GENETIC ANALYSIS OF HEDGEHOG SIGNALLING AND THE REGULATION OF PATCHED GENE EXPRESSION IN VERTEBRATE EMBRYOS

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Genetic Analysis of Hedgehog Signalling


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Cover Photo: Zebrafish Mutations in the Shh Pathway

Results taken from chapter 8. The first two photos show *ptcl* expression in a wild type embryo and a *syu* homozygote respectively; and the second two photos show *shh* and *ptcl* expression in *con* homozygotes respectively. The *con* homozygotes are also stained with a monoclonal antibody for Eng. to identify homozygotes from wt sibs. All the embryos are approximately 18 somites.
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Abstract

This thesis addresses vertebrate Hedgehog (Hh) signalling pathways, and in particular the role played in these pathways by vertebrate homologues of *patched* (*ptc*). Experiments using both zebrafish and chickens embryos are described. *hh* genes encode signalling molecules that have been implicated in a wide variety of developmental patterning events. Ptc has been implicated as the receptor for the Hh signalling pathway and is also a direct target gene of Hh signalling.

I describe the isolation and characterisation of full length cDNA sequence for two zebrafish homologues of *ptc*. The expression of *ptc1* and *ptc2* is described in wild type embryos, in embryos homozygous for a mutation in *shh* and in embryos that are missing expression of all characterised *hh* genes in particular regions of the embryo. Analysis of *ptc1* and *ptc2* expression in embryos in which synthetic RNA from different *hh* genes has been over-expressed is also described. I have also analysed a number of different zebrafish mutations that affect the development of the embryonic midline or somites aswell as the phenotypic consequences of over-expressing *ptc1*.

Experiments that address the regulation of *ptc* gene expression in the chicken and a molecular analysis of the chicken mutation *talpid*\(^3\) are also described. *talpid*\(^3\) is a autosomal, pleiotropic, recessive-lethal mutation in an unknown gene. My analysis has concentrated on the limb, though I have undertaken some analysis of gene expression in the trunk. *talpid*\(^3\) has an interesting limb phenotype which resembles the effects predicted for a uniform ectopic expression of *shh*, or from a partial loss of function of *ptc*. However, *shh* is expressed normally in *talpid*\(^3\). I have analysed the expression of several known Shh pathway genes, and the effect on these gene expression patterns of experimental manipulations such as tissue grafts and exposure to ectopic Shh protein.
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LIST OF ABBREVIATIONS

AER = Apical Ectodermal Ridge
amp = ampicillin
C-terminus = carboxyl terminus
DNA = deoxy-nucleic acid
hpf = hours post fertilisation
hr = hour
min = minutes
MP = muscle pioneer cell
ORF = open reading frame
PCR = Polymerase Chain Reaction
RT-PCR = Reverse Transcriptase PCR
$^{35}$S = radioactive $^{35}$ Sulphur
som = somites
tet = tetracycline
wt = wild type
ZPA = Zone of Polarising Activity

gene and protein names
Ci = cubitus interruptus
hh = hedgehog
N-Hh = amino terminal Hh
shh = sonic hedgehog
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ehh = echidna hedgehog
twhh = tiggywinkle hedgehog
ptc = patched
cptc = chicken patched
mptc = mouse patched
ZFptc = zebrafish patched
HPTC = human patched
ptc1 = patched1
ptc2 = patched2
PKA = Protein Kinase A
dn PKA = dominant negative PKA
RA = Retinoic Acid

mutant names
con = chameleon
cyc = cyclops
dtr = detour
flh = floating head
fss = fused somites
kas = knollase
igu = iguana
mol = monorail
ntl = notail
oepe = one eyed pinhead
smh = schmalhans
sur = schmalspur
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syu = sonic you

ta = talpid

ubo = uboot

wit = white tail

yot = you-too
CHAPTER ONE

INTRODUCTION

This thesis describes the cloning and characterisation of two Zebrafish homologues of the Drosophila segment polarity gene patched (ptc), and a variety of experimental analysis in Zebrafish and chicken embryos that addresses the roles of Ptc and of Hedgehog (Hh) signalling in vertebrate embryonic development. In this chapter I will introduce the roles of the Hh pathway in vertebrate midline signalling (patterning of the neural tube and the somites) and in antero-posterior patterning of the vertebrate limb as these are particularly pertinent to the experimental analysis described later on in this thesis, and then I will introduce the Hh signal transduction pathway and in particular Hh and Ptc proteins. I will also discuss the role of the Hh pathway in human disease, and in particular in cancer, as this has been a particularly exciting recent discovery in this field, and provides further justification for the research described in this thesis being supported by the Imperial Cancer Research Fund. Finally I will discuss why the zebrafish is a valuable model organism for Developmental Genetics. Specific issues will be introduced in more detail at the beginning of the relevant research chapters. For example I will describe the phenotypes of the chicken talpid mutation and the zebrafish "u" mutations in chapter ten and chapter eight respectively.

Hedgehog genes provide crucial signals during embryonic development.

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encode secreted proteins and their structure is highly conserved (Fietz, M. et al. 1994). shh is the most extensively characterised member of the vertebrate hh family as it is expressed in many classically identified signalling centres such as the embryonic shield, prechordal plate and notochord, ventral neural tube, and in the developing limb bud Zone of Polarising Activity (ZPA). In mouse the only overlap in expression of different hh genes is in the gut, where both shh and ihh are expressed (Bitgood, M. J. and McMahon, A. P. 1995) but in zebrafish both ehh and twhh are expressed in a subset of shh expressing cells with twhh expression becoming quickly restricted to the neural tube and ehh being expressed exclusively in the notochord (Ekker, S. C. et al. 1995; Currie, P. D. and Ingham, P. W. 1996). qhh is expressed in the prechordal plate in the early embryo (80% epiboly - tail bud stage) and then from 24 hours post fertilisation (hpf) till about 48 hpf it is expressed in the posterior fore-gut and the anus (Qiao, T. 1997).

In Drosophila, the single hh gene is involved in patterning the embryonic epidermis and the imaginal discs (embryonic structures that give rise to the wings, eyes and legs of the adult fly); for reviews see (Hammerschmidt, M. et al. 1997; Ingham, P. 1995). In vertebrates, Shh has been implicated in the anterior posterior patterning of the limb; patterning of the ventral neural tube, brain and somites; correct spacing of the eyes and development of the central region of the anterior head and face; left-right body asymmetry; and also in the development of teeth, hair follicles, the lung, the urogenital system and the stomach (for review see Hammerschmidt, M. et al. 1997). Ihh has been implicated in chondrocyte differentiation (Vortkamp, A. et al. 1996); Dhh is required for spermatogenesis in the mouse (Bitgood, M. et al. 1996) and Ehh has been implicated in the specification of a specialised class of muscle cells, the muscle pioneer cells in zebrafish (Currie, P. D. and Ingham, P. W. 1996). In addition, the aberrant activation of the Hh signalling pathway, and in particular, mutations in ptc, have been implicated in cancer (for reviews see Ingham, P. W. 1998b; Johnson, R. L. and Scott, M. P. 1997; Ming, J. E. et al. 1998).

The Role of Hh pathways in vertebrate midline signalling

Early in development, mesodermal structures which are important signalling centres, such as the dorsal lip in Xenopus and the embryonic shield in zebrafish, form on the embryonic midline. Signals that induce ectoderm to form neuro-ectoderm and hence the central nervous system originate in these structures (Schier, A. F. and Talbot, W. S. 1998; and references therein). Later in development midline structures such as the notochord and prechordal plate form from these earlier structures and pattern the neural
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tube and the somites, inducing the formation of floor plate and motor neurons in the neural tube, and sclerotome and probably myotome in the somites. There is now considerable evidence that these ventral inducing signals are provided by homologues of hh, most notably by shh. For example, ectopic shh induces ectopic expression of floor plate and / or ventral brain markers in zebrafish (Krauss, S. et al. 1993; Barth, K. A. and Wilson, S. 1995; Macdonald, R. et al. 1995) and in mouse and frog (Ruiz i Altaba, A. et al. 1993; Echelard, Y. et al. 1993); in zebrafish mutants such as cyclops and floating head, loss of midline signalling is closely correlated with loss of shh expression (Krauss, S. et al. 1993; Macdonald, R. et al. 1995); ectopic expression of shh in chick results in ectopic expression of a sclerotal marker, paxl, and a myotomal marker, MyoD, in the developing somites (Johnson, R. L. et al. 1994); purified Shh protein can induce floor plate and motor neuron differentiation in neural plate explants (Marti, B. et al. 1995; Roelink, H. et al. 1995), and sclerotal or myotomal differentiation in pre-somitic mesoderm explants (Fan, C.-M. and Tessier-Lavigne, M. 1994; Munsterberg, A. et al. 1995; Fan, C.-M. et al. 1995); and loss of Shh function in mice produces cyclopia, a lack of floor plate and other ventral neural tube fates such as ventral motoneurons, a severe reduction in sclerotal derivatives and reduced expression of a medial myotomal marker myf-5 (Chiang, C. et al. 1996). In contrast mice which lack Ptc function, and which therefore have an ectopically activated Shh pathway (see discussion of Ptc below), express floorplate markers throughout almost all the neural tube, and lack expression of lateral neural tube markers such as pax6 throughout the embryo, and of dorsal neural tube markers such as pax3 in all but the most caudal region of the embryos (Goodrich, L. et al. 1997).

In mouse and chick, shh is the only hh gene that is expressed in the embryonic midline but in zebrafish ehh is also expressed in the notochord and twhh is also expressed in the floor plate (Ekker, S. C. et al. 1995; Currie, P. D. and Ingham, P. W. 1996). It is probable that these hh genes are also involved in these midline patterning events and consistent with this, and as discussed below, both shh and ehh have been implicated in the patterning of somites and in particular in the induction of a special class of muscle cells, the muscle pioneer cells in zebrafish (Currie, P. D. and Ingham, P. W. 1996). Consistent with this, zebrafish embryos homozygous for a loss of function allele of shh, syu<sup>4</sup> lack muscle pioneer cells and have disturbed somite morphology. However, in contrast to mice lacking Shh function these embryos form a normal medial floorplate, though they lack lateral floorplate cells (Schauerte, H. E. et al. 1998; Chiang, C. et al. 1996). This could be due to redundancy between different hh genes in zebrafish, but an alternative explanation that would also reconcile previous analysis of zebrafish mutations
that do affect the development of the medial floorplate, such as one eye pinhead (oep), cyclops (cyc) and schmalspur (Hatta, K. et al. 1991; Thisse, C. et al. 1994; Brand, M. et al. 1996; Schier, A. F. et al. 1996; Strähle, U. et al. 1997), is that the development of this structure in zebrafish is initially determined by a binary decision between notochord and floorplate fates rather than being induced by Hh signals (Halpem, M. E. et al. 1997; Odenthal, J. et al. 1998) also see (Regbagliati, M. R. et al. 1998; Sampath, K. et al. 1998; Strähle, U. et al. 1997; Zhang, J. et al. 1998). In this scenario Hh signals may be responsible for maintaining the medial floorplate rather than inducing it in zebrafish, though prolonged or later Hh signals might be able to induce medial floor plate cells (for example syu homozygotes start to lose the medial floorplate at late somitogenesis stages, while in contrast cyc and oep embryos start to form a floorplate after 24 hpf) (Strähle, U. et al. 1997; Schauerte, H. E. et al. 1998). However the lateral floorplate cells do appear to be induced and maintained by Hh signals in zebrafish (Schauerte, H. E. et al. 1998; Odenthal, J. et al. 1998).

Zebrafish somites also differ from those in chick and mouse in that they consist mainly of myotome (fig. 1; also see reviews Currie, P. D. and Ingham, P. W. 1998; Tajbakhsh, S. and Cossu, G. 1997). The zebrafish myotome is comprised of three distinct cell types: adaxial cells which form adjacent to the notochord and later migrate through the somite to form a layer of slow muscle fibres at the lateral edge of the somite; muscle pioneer cells which are a subset of adaxial cells that stay located next to the notochord in the medial somite adjacent to where the horizontal myoseptum forms, are the first muscle cells to striate, and are the only muscle cells to express high levels of Engrailed proteins from mid somitogenesis; and fast muscle fibres which form the majority of the myotome and differentiate after the slow muscle fibres have migrated through the somite (fig. 1; Devoto, S. H. et al. 1996; Felsenfeld, A. L. et al. 1991; van Raamsdonk, W. et al. 1978; Hatta, K. et al. 1991; Weinberg, E. S. et al. 1996; Du, S. J. et al. 1997; Blagden, C. S. et al. 1997; Thisse, C. et al. 1993). Previous work has implicated Hh signalling in the induction of both adaxial cells (slow muscle precursors) and muscle pioneer cells in zebrafish (Weinberg, E. S. et al. 1996; Concordet, J.-P. et al. 1996; Hammerschmidt, M. et al. 1996). Moreover, mis-expression of Shh is sufficient to convert the entire myotome to slow-twitch muscle fibres (Blagden, C. S. et al. 1997; Du, S. J. et al. 1997). Conversely, mutations of the bozozok (boz) gene that eliminate the notochord and hence the expression of shh in the midline mesoderm, fail to differentiate slow twitch muscle (Blagden, C. S. et al. 1997). By contrast, notail (ntl) mutant embryos, although lacking a differentiated notochord, retain significant shh expression along their axial midline in cells that ultimately contribute to the neural tube (Krauss, S. et al. 1993;
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Halpern, M. E. et al. 1993; Halpern, M. E. et al. 1997). In these embryos, slow twitch muscle still forms (Blagden, C. S. et al. 1997); but the muscle pioneer (MP) cells fail to differentiate (Halpern, M. E. et al. 1993). This characteristic of ntl mutants led to the suggestion that MP cells may require the activity of an additional notochord derived signal for their induction: a good candidate for such a signal is Echidna hedgehog (Ehh) which is exclusively expressed in the notochord of wild-type fish and is not expressed in ntl mutant embryos (Currie, P. D. and Ingham, P. W. 1996). Consistent with this proposal, it has been previously shown that mis-expression of both Ehh and Shh is necessary for the induction of supernumerary MPs (Currie, P. D. and Ingham, P. W. 1996); however, other authors have reported that high level expression of Shh or of other Hh family members is sufficient to induce MP differentiation (Du, S. J. et al. 1997; Hammerschmidt, M. et al. 1996; Schauerte, H. E. et al. 1998). Therefore there is good evidence that the specification of slow twitch muscle and of muscle pioneer cells in the zebrafish is mediated by Hh signalling, but the precise contribution of the different Hh proteins to these processes remains unclear.

**Figure 1: A comparison of tetrapod and teleost somites**

This figure was adapted from (Currie, P. D. and Ingham, P. W. 1998). A schematic of the formation of the somites in tetrapods is shown in (A-C) and contrasted with teleosts such as the Zebrafish in (D-F). NT = neural tube; NC = notochord; NP = neural plate; MY = myotome. In contrast to tetrapods only a very small fraction of Zebrafish somites forms sclerotome (purple cells) (E&F cf. B&C) with most of the somite forming myotome (red indicates fast twitch, and green indicates slow twitch muscle precursors in E&F; pale blue indicates the myotome in B&C). Note that the somites in teleosts extend much more ventrally, so that the notochord is positioned in the centre of the somites.

In Zebrafish, adaxial cells initially form adjacent to the notochord and acquire a characteristic cuboidal morphology (green cells in (D)). All of these cells except a subset corresponding to the muscle pioneer cells migrate out to the lateral extent of the somite (green in (F) and (G) - the muscle pioneer cells are still adjacent to the notochord). The rest of the myotome starts to express fast twitch markers (red) as the slow muscle precursors (green) migrate past it. The localisation of the dermis in Zebrafish (shown in black in (F)) has not been characterised.

(G & H) Toluidine blue stain of transverse sections of a chick and a zebrafish embryo respectively at comparative stages of development, again showing the predominance of myotome in the Zebrafish somite compared to the chick.
Figure 1
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The Role of Shh in Vertebrate Limb Development

The developing chick limb bud is a well established model system for investigating the cellular and molecular basis of embryonic pattern formation. A wide range of experimental analysis has elucidated aspects of the patterning of all three limb axes: anterior-posterior; proximal-distal; and dorsal-ventral and shown that they are all interdependent to some extent and act co-ordinately to control limb development (for review see Johnson, R. L. and Tabin, C. J. 1997). Shh signalling has been implicated in the anterior posterior patterning of the developing limb bud. Extensive analysis has shown that this axis is established by a group of mesenchymal cells in the posterior of the limb bud known as the Zone of Polarising Activity (ZPA). Grafts of a ZPA to the anterior of another limb bud produce mirror image duplications of the digits of the limb (Saunders, J. W. and Gasseling, M. T. 1968; Tickle, C. et al. 1975; fig. 2). shh is expressed in the ZPA with the correct spatial temporal expression to be the signal mediating the antero-posterior patterning, and ectopic expression of shh in the anterior of the limb mimics grafts of the ZPA; inducing ectopic digits (Riddle, R. et al. 1993; fig. 2). In addition most of the mouse polydactyly mutations investigated so far are associated with ectopic expression of shh in the anterior of the limb bud (Buscher, D. and Ruther, U. 1998; Buscher, D. et al. 1997; Chan, S. C. et al. 1995; Masuya, H. et al. 1995; Masuya, H. et al. 1997; Qu, S. et al. 1997; for review see Niswander, L. 1997). Unfortunately the shh targeted loss of function mice are not very informative for considering anterior posterior pattern in the limb as they lack distal limb structures (Chiang, C. et al. 1996). However, embryos where the ZPA has been carefully excised do have anterior-posterior truncations (Pagan, S. M. et al. 1996).
Figure 2: Shh has Polarising Activity in the Chick Limb Bud

(A) Schematic of a normal chicken wing bud showing the positions of the apical ectodermal ridge (AER) and the Zone of Polarising Activity (ZPA). The chicken wing normally forms three digits: II, III and IV (C); but a graft of a wild type ZPA, or of a bead soaked in Shh protein or of cells expressing shh (B) can all induce full mirror image duplications of the digits (D).

h = Humerus; r = Radius; u = Ulna.

(A & B) were kindly provided by J. Quirk.
(C & D) were taken from (Tickle, C. 1981).
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Several other potential components of anterior posterior patterning in the limb have been identified, partly because of their asymmetrical expression across the anterior posterior axis of the limb bud or their involvement in the Shh signalling pathway. These include members of the *bmp* and the *HOX* gene families. For example *bmp2* and 5' members of the *HoxD* complex (*Hox D-11, D-12 and D-13*) are normally expressed in posterior limb bud mesoderm, overlapping but wider than, the domain of *shh* expression, and they are also ectopically expressed in the anterior of the limb bud after *shh* mis-expression or ZPA grafts (Izpisua-Belmonte, J. C. et al. 1991; Nohno, T. et al. 1991; Riddle, R. et al. 1993; Francis, P. H. et al. 1994; Laufer, E. et al. 1994; Francis-West, P. H. et al. 1995). However, this induction of *bmp* and *Hox-D* gene expression by Shh also requires the AER - a specialised apical ectodermal ridge, that runs along the distal part of the limb bud. This requirement for the AER can be substituted for by *FGF4*, a gene that is normally expressed in the posterior AER and is itself induced in response to *shh* (Laufer, E. et al. 1994). Therefore these *bmp* and *HOX* genes are probably either direct, or indirect targets of Shh signalling that require the convergence of Shh and FGF signalling pathways for their expression. *bmp2* can itself induce expression of posterior *Hox* genes such as *HoxD-13*, but ectopic *shh* induces expression of *Hox D-13* before ectopic *bmp2* does, suggesting that at least the initial induction of *Hox D-13* by *shh* does not go through *bmp2* (Duprez, D. M. et al. 1996). The precise details of how these genes are activated, whether they are direct or indirect targets of Shh, and their individual roles in determining anterior-posterior pattern are still not well understood.

Processing the Hh signal

In order to understand the signalling activities of Hh proteins we need to understand the processing and the transduction of Hh signals. All of the Hh proteins comprise of a highly conserved N-terminal region, preceded by a signal peptide and followed by a more divergent C-terminus (for review see Fietz, M. et al. 1994). During the course of the analysis reported here, biochemical analysis has elucidated several modifications of Hh proteins. These have not been shown for all Hh proteins but most of them have been demonstrated for Shh in more than one vertebrate and for *Drosophila* Hh, suggesting that they are probably conserved throughout the family of Hh proteins. Firstly Hh proteins are auto-proteolytically cleaved, *in vitro* and *in vivo*, to produce a 19kDa N-terminus peptide (N-Hh) and a 26-28kDa C-terminal peptide (Chang, D. T. et al. 1994; Lee, J. J. et al. 1994; Bumcrot, D. A. et al. 1995; Lopez-Martinez, A. et al. 1995). All of the signalling activity of Hh is provided by the N-Hh moiety (Porter, J. A. et al. 1995; Fietz,
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M. J. et al. 1995; Marti, E. et al. 1995; Lopez-Martinez, A. et al. 1995) but the cleavage depends on conserved sequences in the C terminus of the Hh precursor protein. In addition the C-terminus can initiate this cleavage reaction even when attached to sequences unrelated to Hh, and a number of novel C-elegans proteins have been identified that have homology to the C terminus of Hh, at least one of which undergoes a similar auto-cleavage to Hh, suggesting that these related sequences may be an evolutionary conserved general mechanism of protein processing (Burglin, T. R. 1996; Porter, J. A. et al. 1996; Porter, J. et al. 1996). This is consistent with the crystal structure of the C-terminus of Hh, which also has structural similarity to the self splicing regions of inteins (Koonin, E. V. 1995; Hall, T. M. T. et al. 1997; for a review see Perler, F. B. 1998). In addition the crystal structure of N-Shh suggests that this peptide may also have proteolytic activity as it contains a region with structural similarity to zinc hydrolases (Hall, T. M. T. et al. 1995). However, this aspect of the Shh structure does not appear to be conserved with Drosophila Hh.

N-Hh is also lipid modified. During the internal cleavage reaction cholesterol is covalently bound to the C-terminus of N-Hh (Porter, J. et al. 1996; Beachy, P. A. et al. 1997). In addition human N-Shh can also be palmitoylated at its amino terminus (Pepinsky, R. B. et al. 1998), suggesting that at least two forms of N-Shh may exist: cholesterol modified and cholesterol and palmitic acid modified. Both of these modifications are effective at tethering N-Shh to the plasma membrane, and hence both spatially restrict and increase the effective concentration of its signalling activity: both modified forms of the protein are about 30 x more potent than an unmodified soluble form of N-Shh in an in vitro assay (Porter, J. A. et al. 1996; Pepinsky, R. B. et al. 1998).

The significance of these modifications of Hh proteins is emphasised by certain human diseases. For example, mutations in the C-terminal processing domain of human SHH can cause holoprosencephaly (Roessler, E. et al. 1997). The discovery of the cholesterol linkage also provided a plausible explanation for the similarity in phenotype between mammals with defects in cholesterol biosynthesis and those with mutations in the shh gene (Porter, J. et al. 1996). However, more recently it has been shown that inhibitors of cholesterol biosynthesis do not block Shh processing, but instead interfere, in a concentration dependant manner, with the ability of tissues to respond to Shh signalling (Cooper, M. K. et al. 1998; Beachy, P. A. et al. 1997).
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Patched - the putative Hh receptor

*patched* (*ptc*) is the putative receptor for the Hh pathway. Like *hh* it was first discovered as a segment polarity gene in *Drosophila* (Nüsslein-Volhard, C. and Wieschaus, E. 1980) but in contrast to *hh* it encodes a transmembrane protein with twelve putative transmembrane domains (Nakano, Y. et al. 1989; Hooper, J. and Scott, M. P. 1989). In *Drosophila, ptc* is expressed at high levels in cells close to sources of Hh signals (Hidalgo, A. and Ingham, P. 1990), and the protein is located at the plasma membrane (Taylor, A. M. et al. 1993; Capdevila, J. et al. 1994b). Removal of *ptc* causes ectopic activation of Hh target genes, for example *wg* or *dpp* (Martinez Arias, A. et al. 1988; Hidalgo, A. and Ingham, P. 1990; Ingham, P. W. and Hidalgo, A. 1993; Capdevila, J. et al. 1994a), and a *hh ptc* double mutant has the same phenotype as a *ptc* mutant, showing that genetically *ptc* is downstream of *hh* and that in the absence of Hh signals Ptc represses the Hh signal transduction pathway (Ingham, P. W. et al. 1991).

When I started this PhD Lisa Goodrich and Ron Johnson in Matthew Scott’s laboratory had just cloned a homologue of *ptc* in mouse (later published in Goodrich, L. V. et al. 1996); and in collaboration with them we cloned two homologues from zebrafish and Valeria Marigo cloned a homologue from chicken (Marigo, V. et al. 1996; Concordet, J.-P. et al. 1996; chapter three). More recently a second *ptc* gene has been reported in mouse and newt (Takabatake, T. et al. 1997; Motoyama, J. et al. 1998).

As early as 1991 it was suggested that Ptc might be a receptor for Hh (Ingham, P. W. et al. 1991), but at that stage there was no biochemical evidence to support this hypothesis. However, biochemical experiments have now shown that mouse Ptc can bind aminoterminal Shh (N-Shh) and N-Dhh from mouse, and chick Ptc can bind human N-Shh (Stone, D. et al. 1996; Marigo, V. et al. 1996). Genetic experiments in the *Drosophila* wing disc also suggested that Ptc binds Hh and demonstrated a role for Ptc in sequestering Hh and hence localising Hh signalling (Chen, Y. and Struhl, G. 1996). Consistent with this Hh protein diffuses farther in the *Drosophila* embryo in the absence of Ptc activity (Taylor, A. M. et al. 1993). The two functions of Ptc: repressing transcription of Hh target genes and sequestering Hh protein, can also be genetically uncoupled by particular mutations in *ptc* (Chen, Y. and Struhl, G. 1996).

Ptc is an unusual receptor in that it represses transcription of Hh target genes yet it is itself a target of Hh signalling, and both of these things are true for all the vertebrate homologues of *ptc* that have been analysed for either of these two things so far (Gailani, M. R. et al. 1996; Goodrich, L. et al. 1997; Goodrich, L. V. et al. 1996; Marigo, V. et al. 1996; Concordet, J.-P. et al. 1996; Motoyama, J. et al. 1998; chapter six). The predicted structure of Ptc does not resemble other characterised receptors and its twelve
transmembrane domains are more reminiscent of the 6+6 pattern present in some ion channels / bacterial transporters (Nikaido, H. and Saier, H. J. 1992; Nakano, Y. et al. 1989). Until recently the only similar protein that had been remarked upon was Tra-2, which is a C elegans protein involved in sex determination, and this has only gross topological similarity to Ptc (Kuwabara, P. E. et al. 1992; Forbes, A. J. et al. 1993), though intriguingly Tra-2 is also negatively regulated by its ligand Her-1, and the most downstream component of the Tra-2 pathway is a zinc finger protein with significant homology to Ci, a putative transcription factor for the Hh pathway (Zarkower, D. and Hodgkin, J. 1992; Kuwabara, P. E. et al. 1992; Forbes, A. J. et al. 1993). Recently two stretches of homology have been discovered between Ptc and a newly cloned gene involved in cholesterol trafficking disease, Niemann-Pick type C (NPC); one of these regions encompasses a putative sterol sensing domain, based on sequence similarity to sterol regulated proteins HMG CoA reductase and SREBP cleavage-activating protein (fig. 3A; Carstea, E. et al. 1997; Loftus, S. et al. 1997; Johnson, R. L. and Scott, M. P. 1997; chapter three). Even more recently, TRC8, a 664 amino acid gene with a predicted 10 membrane spanning domains, and with sequence similarity to the sterol sensing domain and 2nd major extra-cellular loop of Ptc, was discovered through its disruption by a translocation that is associated with hereditary renal cell carcinoma (Gemmill, R. M. et al. 1998; fig 3B).
Figure 3: Proteins with Sterol Sensing Domains
This figure was taken from (Beachey et al. 1997) (A) & (Gemmill et al. 1998) (B).
(A) Four proteins that contain a sterol sensing domain (dashed box) are schematically depicted. The cylinders denote predicted transmembrane helices. Ptc also has a second region of sequence similarity to NPC in the second half of the protein (see chapter 3). The arrowhead in NPC points to a predicted signal sequence cleavage site. (B) Schematic of the predicted structures of Ptc & TRC8. The thick black lines in Ptc denote regions of similarity to TRC8.
Recent analysis suggests that Ptc forms part of a Hh receptor complex with the product of another *Drosophila* segment polarity gene, *smoothed (smo)* (Nüsslein-Volhard, C. et al. 1984). *smo* genes have also been identified in vertebrates and they encode transmembrane proteins with a structure reminiscent of G protein coupled receptors and homology with frizzled proteins, some of which can act as receptors for Wnt proteins (van den Heuvel, M. and Ingham, P. W. 1996; Alcedo, J. et al. 1996; Bhanot, P. et al. 1996; Quirk, J. et al. 1997; Xie, J. et al. 1998). This suggested that Smo might be the receptor for Hh; however, *in vitro* biochemical experiments were unable to detect Hh binding to Smo, though they were able to detect Ptc binding to Smo (in the presence and absence of Hh) and Hh binding to a Ptc-Smo complex (Stone, D. et al. 1996). In addition genetic analysis in *Drosophila* suggests that *smo* is downstream of *ptc* (Hooper, J. 1994; Alcedo, J. et al. 1996; Chen, Y and Struhl, G. 1996; Quirk, J. et al. 1997). One possible explanation of these results is that Ptc and Smo normally form a receptor complex, with Ptc inhibiting the activity of Smo. This inhibition would then be abrogated by Hh binding Ptc, probably through a conformational change rather than dissociation of the two transmembrane proteins, as Ptc still binds Smo in the presence of Hh. However, as Smo is constitutively active in the absence of Ptc, Smo clearly does not need to interact with either Ptc or Hh for its activity.

Most of the analysis of Hh signalling has implicated Ptc as an essential component of the Hh pathway, and most of the analysis of Ptc has implicated it in Hh signalling. However there are a few examples of Hh activity that seem to be independent of Ptc and vice versa. For example, while the phenotype of *hh ptc* double mutant *Drosophila* embryos is very similar to that of *ptc* embryos it is not identical, suggesting that some Hh signalling can occur in the absence of Ptc (Hidalgo, A. 1991; Bejsovec, A. and Wieschaus, E. 1993); the specification of some cells in the embryonic epidermis depends on Hh signalling but does not seem to involve the repression of Ptc activity, and may instead require the activity of another, as yet un-characterised protein, Lines, to be repressed (Bokor, P. and Dinardo, S. 1996); *ptc* is expressed in places in the *Drosophila* embryo where there is not thought to be any Hh signalling such as the visceral and somatic mesoderm and transiently in the precursors of the CNS (Capdevila, J. et al. 1994b); and *ptc* has been implicated in *hh* independent specification of particular neuroblasts (Bhat, K. M. and Schedl, P. 1997).
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**Other Components of the Hedgehog signal transduction pathway**

When I started this work several components of the Hh pathway had been identified in *Drosophila* (Forbes, A. J. et al. 1993; Hooper, J. 1994). However, besides the *hh* genes themselves and the Gli proteins, which have a zinc finger DNA binding domain that is homologous to a similar domain in cubitus interruptus (Ci) and were cloned before Ci (Kinzler, K. et al. 1987; Kinzler, K. W. et al. 1988; Ruppert, J. M. et al. 1988; Ruppert, J. M. et al. 1990; Orenic, T. V. et al. 1990; Hui, C. C. et al. 1994; Hughes, D. C. et al. 1997), no vertebrate components of Hh pathways had been identified. Since then, as described above, *ptc* and *smo* genes have been cloned in vertebrates and in addition PKA has been identified as a component of the Hh pathway in *Drosophila* (Jiang, J. and Struhl, G. 1995; Lepage, T. et al. 1995; Li, W. et al. 1995; Pan, D. and Rubin, G. M. 1995; Strutt, D. I. et al. 1995) and vertebrates (Fan, C.-M. et al. 1995; Hammerschmidt, M. et al. 1996; Concordet, J.-P. et al. 1996; Ungar, A. and Moon, R. 1996; Epstein, D. J. et al. 1996). However, it is not clear whether PKA acts directly in the Hh pathway or whether it acts in parallel. The initial evidence suggested that PKA is not directly downstream of *ptc* because expression of dominant active PKA at levels which are sufficient to rescue PKA mutants, was unable to rescue clones mutant for *ptc* or repress endogenous Hh signalling (Jiang, J. and Struhl, G. 1995; Li, W. et al. 1995). More recent analysis suggests that PKA may act in different ways at different points of the Hh pathway (Ohlmeyer, J. T. and Kalderon, D. 1997; Chen, Y. et al. 1998), and I will discuss this in more detail in the next section of this chapter.


In *Drosophila*, unlike the vertebrate gli genes, ci is not transcriptionally regulated in response to Hh signalling (transcription of gli is up-regulated and transcription of gli3 is down-regulated in response to Shh signals) (Marigo, V. et al. 1996; Hynes, M. et al. 1997; Lee, J. et al. 1997); but Ci protein is post translationally regulated in response to Hh signalling (Aza-Blanc, P. et al. 1997). In the absence of Hh signalling Ci is cleaved to form a 75 kDa peptide that specifically lacks the C-terminal activation domain of the full length protein (Alexandre, C. et al. 1996), but still includes the zinc finger domain that binds DNA. This form of Ci is detected almost exclusively in the nucleus, and can act as a transcriptional repressor (Aza-Blanc, P. et al. 1997). Recent evidence suggests that this cleavage of full length Ci requires PKA mediated phosphorylation at conserved PKA sites in the C-terminal of the full length Ci protein, and that this may account for the inhibitory effect of PKA on Hh targets (Chen, Y. et al. 1998). Cleavage of Ci is blocked in cells responding to Hh signals, resulting in the accumulation of the full length 155 kDa form of the Ci protein, that, unlike the shorter form, contains sequences that tether it in the cytoplasm in the absence of Hh signals; and a transcriptional activation domain which includes a binding site for CBP, a transcription factor which acts as a co-activator for Ci, and is also required for transcription of Hh target genes in vivo (Aza-Blanc, P. et al. 1997; Alexandre, C. et al. 1996; Akimaru, H. et al. 1997; Hepker, J. et al. 1997). Accumulation of the full length form of Ci correlates with transcriptional activation of Hh target genes, suggesting that this is the transcription factor for these genes, but paradoxically this form of the protein has not yet been observed in the nucleus (Aza-Blanc, P. et al. 1997). The evidence that Ci is a transcriptional activator for at least some Hh target genes (and not just a repressor that is removed by Hh signalling) is, however, considerable: binding sites for Ci have been identified in the promoters of the Hh target genes ptc and wg and these sites are sufficient to mediate Hh-dependant transcription in vitro, and in transgenic flies (Alexandre, C. et al. 1996; von Ohlen, T. and Hooper, J. E. 1997; Von Ohlen, T. et al. 1997); genetic analysis has shown that ci is required in vivo for the Hh-dependant transcription of ptc and wg (Forbes, A. J. et al. 1993); and ectopic expression of ci is sufficient to activate dpp, ptc and wg transcription.
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(Alexandre, C. et al. 1996; Dominguez, M. et al. 1996; Hepker, J. et al. 1997). Similar results have also been obtained for Gli1 and Gli2: Gli binding sites in the HNF3B promoter are required for Shh mediated up-regulation of HNF3B expression (Sasaki, H. et al. 1997); ectopic expression of gli1 can induce transcription of Shh target genes in the mouse, chick, and frog (Hynes, M. et al. 1997; Marigo, V. et al. 1996; Lee, J. et al. 1997); and mice lacking Gli2 function have reduced expression of Shh target genes and fail to develop a floor plate (Ding, Q. et al. 1998; Matise, M. P. et al. 1998). In contrast, Gli3 has been predominantly implicated in repression of Hh signalling, suggesting that the repressor and activator functions of Ci may have been divided between different Gli proteins (Marigo, V. et al. 1996; Sasaki, H. et al. 1997; Buscher, D. et al. 1997; for a review see Ruiz i Altaba, A. 1997); but there is also evidence that Gli2 can repress aspects of Shh signalling and that Gli2 and Gli3 have both distinct and overlapping functions (Ruiz i Altaba, A. 1998; Mo, R. et al. 1997); and it has been suggested, on the basis of the different phenotypes produced by different mutations in human GLI3, that GLI3 may be processed into a repressor and activator form like Ci (Biesecker, L. G. 1997). Thorough investigations into the processing of the different Gli proteins will be necessary to help resolve these issues.

In the last year, the cloning of cos-2 has been reported and this has further illuminated aspects of the transduction of Hh signals, and in particular how the post translational processing of Ci may be controlled. cos-2 encodes a novel protein, but it has an N-terminal domain that has significant sequence similarity to Kinesin super-family proteins (Sisson, J. et al. 1997). Cos-2 forms a complex with Fu and Ci and binds to microtubules in the absence of Hh signals. When the cell receives Hh signals, both Fu and Cos-2 are phosphorylated (though Cos-2 is not phosphorylated by Fu as this phosphorylation occurs even in the absence of Fu) and the complex is released from the microtubules (Sisson, J. et al. 1997; Robbins, D. J. et al. 1997; Aza-Blanc, P. et al. 1997; fig. 4). It is still unclear what phosphorylates Fu and Cos-2 but an unidentified PP2A like serine threonine phosphatase has also been implicated in Gli-dependent and Gli-independent Shh signalling in vitro (Krishnan, V. et al. 1997). Intriguingly, the Drosophila mutation twins, which maps to the gene encoding a regulatory subunit of PP2A, produces symmetrical wing duplications (Uemura, T. et al. 1993), which is reminiscent of perturbations in Hh signalling.

Another protein that has been implicated in this multi-component complex is Suppressor of fused (Su(fu)). Su(fu) was isolated in a screen for mutations that suppress the adult phenotype of fu flies. Loss of the Su(fu) locus completely suppresses loss of function fu phenotypes, whereas additional copies of Su(fu) enhance these fu phenotypes: however,
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homozygous Su(fu) flies are viable and have no obvious phenotype, indicating that Su(fu) function is not essential for Hh signalling (Préat, T. 1992). Su(fu) encodes a novel protein with a PEST sequence, associated with rapidly degraded proteins (Pham, A. et al. 1995). Both Fu and Ci bind to Su(fu) in yeast two hybrid and in vitro biochemical assays, but Fu and Ci only bind to each other in these assays if Su(fu) is also present (Monnier, V. et al. 1998) implying that Su(fu) mediates the physical interaction between Ci and Fu (fig. 4). This is consistent with previous genetic analysis that predicted close interactions between Fu, Su(fu) and Cos-2 (Préat, T. et al. 1993). This genetical analysis also suggests that Cos-2 and Su(fu) may be slightly redundant in function, both acting to repress Ci activation, and both negatively regulated by Fu, as Su(fu) homozygotes have no phenotype, but the loss of Fu activity can be suppressed by the loss of function of either Cos-2 or Su(fu) and loss of Su(fu) activity enhances the Cos-2 phenotype (Préat, T. et al. 1993; Monnier, V. et al. 1998).

More recent genetic screens have also identified other components of the Hh pathway in Drosophila. Tout-velu (ttv) was identified as a new segment polarity gene in a screen for maternal effect mutations and it is required in cells responding to Hh signals for correct diffusion/transport of Hh protein across the cells: in the absence of Ttv activity only the cells adjacent to hh expressing cells can respond to Hh signals (Bellaiche, Y. et al. 1998). Ttv is a putative single span, type II class, integral membrane protein that resembles vertebrate EXT proteins, mutations in which affect bone development (Bellaiche, Y. et al. 1998). It is possible that Ttv could be a plasma membrane protein that interacts directly with Hh, but recent analysis of EXT-1 suggests that Ttv may alternatively play a role in the endoplasmic reticulum in regulating the synthesis and display of cell-surface heparin sulphate glycosaminoglycans (GAGs) (McCormick, C. et al. 1998; also see Ingham, P. W. 1998c).

Slimb (slmb) was identified in two independent screens for recessive mutations that disturb adult development in Drosophila and it has been implicated in both the Hh and the Wg pathways (Jiang, J. and Struhl, G. 1998; Theodosiou, N. A. et al. 1998). slmb encodes a conserved F-box/ WD40 repeat protein which has sequence similarity to a yeast protein, Cdc4p, involved in targeting cell cycle regulators for degradation by the ubiquitin/proteasome pathway. Jiang and Struhl suggest that Slmb negatively regulates Wg signalling by targeting Armadillo (Arm) protein for degradation and may negatively regulate Hh signalling by targeting Ci protein for proteolysis into the Ci 75 repressor form. Their experiments suggest that Slmb acts downstream of Smo in the Hh pathway; and they show that in cells lacking Slmb function, both full length Ci and Arm proteins accumulate at high levels and target genes of the Hh and Wg pathways are ectopically
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activated (Jiang, J. and Struhl, G. 1998). Theodosiou et al. show that Slmb function is also required for correct dorsal ventral patterning in the leg imaginal disc as slmb mutant clones often ectopically express both dpp and wg (the expression of these two genes is normally mutually exclusive in the leg disc). However, their results suggest that Slmb acts upstream of Smo but downstream of Hh. They suggest that their results differ from the other paper because they used null alleles of slmb and smo whereas Jiang and Struhl used hypomorphic alleles (Theodosiou, N. A. et al. 1998). Vertebrate homologues of slmb probably exist: B-TRCP is a Xenopus protein of unknown function that is 80% identical to Slmb at an amino acid level (Jiang, J. and Struhl, G. 1998) and a human homologue of slmb has also been identified (Theodosiou, N. A. et al. 1998). Finally, oroshigane (oro) was identified as a dominant enhancer of the Bar eye phenotype in Drosophila, and genetic interactions implicate it in the Hh pathway (Epps, J. L. et al. 1997). oro homozygotes are recessive lethal and have a segment polarity phenotype, though it is not very penetrant: about 10-15% of homozygotes look like hh homozygotes. ptc is epistatic to oro, but oro homozygotes are rescued from lethality by the loss of one copy of ptc and oro alleles strongly enhance fu and hh phenotypes in a dominant manner. This all suggests that oro is required for correct levels of Hh signalling and the epistasis analysis suggests that oro may act upstream of, or in parallel to, Ptc (Epps, J. L. et al. 1997).

Figure 4. The Hedgehog Signal Transduction Pathway

This figure is based on a figure kindly provided by J. Quirk and published in (Ingham, P. W. 1998a). Negatively acting components are shown in red and positively acting components are shown in green.

In the absence of Hh signalling (A), Ptc inhibits the activity of Smo; the complex of Fu, Cos-2, Su(fu) and Ci binds to microtubules; and Ci is cleaved to form the shorter repressor form of Ci 75, probably through the action of Slmb, PKA and the proteasome. When Hh binds to Ptc (B) inhibition of Smo function is released. There is some evidence that Smo may act through G-proteins (Hammerschmidt, M. and MacMahon, A. P. 1998; Xie, J. et al. 1998). The Cos-2, Fu, Su(fu), Ci complex dissociates from the microtubules and most of the Ci protein in the cell is found in the Ci 155 form. Both Ci and CBP are required for transcription of Hh target genes such as ptc, dpp and wg. PKA may also have a positive action on the pathway independent of Ci protein levels. The molecular nature of oro has not yet been determined but epistasis analysis suggests that it may act upstream of Ptc. Ttv may be located in the plasma membrane or in the ER and is required for correct transport of the Hh signal across responding cells.
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Does the Hh pathway bifurcate?
Recently there has been mounting evidence that the Hh pathway is not a simple linear pathway, but that there are parallel pathways and/or a bifurcation in the pathway. I will discuss this briefly as it is pertinent to some of the results in chapter ten and the discussion in chapter twelve. As discussed above, when PKA was identified as a regulator of Hh signalling some of the data suggested that PKA acts in parallel to the pathway regulated by Ptc (Jiang, J. and Struhl, G. 1995). More recent experiments in *Drosophila* have suggested that the Hh pathway bifurcates downstream of Smo and that in the embryo PKA has opposite effects on the two branches (Ohlmeyer, J. T. and Kalderon, D. 1997). One branch seems to act at least partly by increasing the amount of Ci-155 in a cell, and PKA represses this, probably by acting directly on PKA sites in the C-terminus of Ci and hence targeting Ci for proteolysis into the repressor form as already discussed above (Chen, Y. et al. 1998); and the other branch can induce transcription of Hh target genes without altering the levels of Ci-155, though Ci is still required, and PKA acts positively on this branch (Ohlmeyer, J. T. and Kalderon, D. 1997; Chen, Y. et al. 1998). Paradoxically the repression of Hh signalling by PKA seems to require Smo and Hh activity in the embryo (though it does not require Smo activity in the wing disc (van den Heuvel, M. and Ingham, P. W. 1996)) as inhibition of PKA activity does not activate *ptc* or *wg* transcription in a *smo* mutant background and does not activate *wg* in a *hh* mutant background despite increased levels of Ci-155 (Ohlmeyer, J. T. and Kalderon, D. 1997). In contrast the positive activity of PKA on the Hh pathway requires Smo but not Hh activity: constitutive active PKA induces robust transcription of *wg* and *ptc*, but if anything produces lower levels of Ci-155, and this occurs in a wild type or a *hh* mutant background but does not occur in a *smo* mutant background (Ohlmeyer, J. T. and Kalderon, D. 1997).

Another recent paper has suggested that Ptc mediated repression of *wg* transcription may act through a transcriptional repressor that is not Ci (Lessing, D. and Nusse, R. 1998). The authors identified a sequence that is highly conserved between *Drosophila melanogaster* and *virilus* *wg* promoters that is required for the correct localisation of *wg* expression. A reporter construct with this sequence but without the Ci binding sites that have been previously identified in the promoter and shown to be sufficient for *wg* expression (Von Ohlen, T. et al. 1997; von Ohlen, T. and Hooper, J. E. 1997), has relatively normal expression in wild type embryos but expands in *ptc* mutant embryos and in wild type embryos exposed to ectopic Hh activity. This suggests that normally, in
addition to activating Ci, the Hh pathway has to remove an unidentified repressor of wg transcription, for correct wg transcription to occur (Lessing, D. and Nusse, R. 1998). Finally, in vitro experiments in vertebrate tissue culture cells have identified coup TFII as a direct target of Shh signalling, but they have also shown that its transcription is not mediated by Gli proteins: a Shh responsive region of the coup TFII promoter responds to Shh even in the presence of cycloheximide, and this response can not be titrated out with a Gli response element (Krishnan, V. et al. 1997). In addition, recent analysis of gli1 gli2 double mutant mice suggests that the induction of some ventral neurons by Shh is not mediated by Gli proteins in the mouse (Matise, M. P. et al. 1998). These results suggest that Gli / Ci proteins are not the only end point of Hh signalling pathways, but that other transcription factors (activators and / or repressors) still remain to be identified.

**Do Hh proteins act as morphogens and/or long range signals?**

A moot point, especially with respect to anterior-posterior patterning in the vertebrate limb, is whether Hh proteins act as morphogens and / or act directly on cells at a distance from those expressing hh. For any of the Hh proteins to act as a morphogen, they must be capable of acting both on adjacent cells (to directly induce cell fates that require a high concentration of Hh activity) and as a longer range signal (to directly induce cell fates that require a lower concentration of Hh activity). There is now considerable evidence that Hh can act directly across several cell diameters in *Drosophila*, where for example in the wing disc Hh normally directly induces eng expression over about three cell diameters and dpp and ptc expression over about 8 - 10 cell diameters, whereas an artificially tethered form of Hh only induces expression of these genes in adjacent cells and causes patterning defects (Strigini, M. and Cohen, S. M. 1997). This also suggests that Hh is acting directly as a morphogen in this system, and consistent with this there is also evidence that Hh acts as a morphogen in the *Drosophila* adult abdomen (Struhl, G. et al. 1997b) and the *Drosophila* embryo (Heemskerk, J. and Dinardo, S. 1994). In addition, as already discussed, there is mounting evidence that the spatial regulation of Hh is controlled in many different ways: by modifications to the Hh protein, by binding to Ptc and via Ttv.

In vertebrates it is also now well established that different levels of Hh signals can induce different cell fates in the vertebrate midline, where very high concentrations of Shh are required for floor plate development and lower concentrations induce motor neuron fates (Tanabe 1995; Hynes, M., J. A. et al. 1995; Roelink, H. et al. 1995; Marti, E. et al. 1995; Ericson, J. et al. 1996); and in the limb, where high concentrations of Shh applied
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to the anterior of the limb bud can induce an ectopic digit four and lower concentrations induce progressively a digit three or a digit two (Yang, Y. et al. 1997). There is also mounting evidence that vertebrate Hh proteins do act as direct long range signals. Firstly, vertebrate ptc genes are expressed at high levels a large distance away from cells expressing hh genes in many cases (for example the chicken limb or the mouse neural tube). In Drosophila the induction of high level ptc expression in the wing disc and the adult abdomen is autonomous to cells that directly receive Hh signals (Strigini, M. and Cohen, S. M. 1997; Struhl, G. et al. 1997b), and if this is the case in all systems then high level expression of ptc genes can be interpreted as a marker of cells responding directly to Hh proteins. In addition, the induction of sclerotome in mammalian somites occurs over quite a large distance (about 30 cells) and yet appears to be a direct effect of Shh signalling, as cells adjacent to a source of Shh cannot substitute for Shh in inducing sclerotome (Fan, C.-M. and Tessier-Lavigne, M. 1994). However, recently it was shown that a membrane bound form of Shh can also induce almost full digit duplications in the chicken limb, suggesting that Shh does not need to act directly at a distance to re-specify cell fates in the limb (Yang, Y. et al. 1997). This is in contrast to the similar experiments in the Drosophila wing mentioned above, though it is worth noting that the defects produced by the tethered form of Hh in the Drosophila wing were all in structures close to the source of hh expression, long range Dpp mediated patterning events did not seem to be affected (Strigini, M. and Cohen, S. M. 1997). In addition, it can not be completely ruled out that something in the chicken limb might transport or cleave the altered Shh protein allowing a small amount of protein to escape. It is also worth noting that only two ectopic digits (as opposed to three) were ever induced by this form of Shh protein, though it is not clear if this has any significance (Yang, Y. et al. 1997).

The Hh pathway, human disease and cancer

Gorlin's or NBCC Syndrome

During my PhD, it was discovered that humans heterozygous for mutations in ptc develop Gorlin’s syndrome, otherwise called nevoid basal cell carcinoma syndrome (NBCCS), which is characterised by a wide range of variable developmental defects and by a high predisposition to basal cell carcinomas (BCCs) of the skin (Hahn, H. et al. 1996; Johnson, R. L. et al. 1996; Gorlin, R. J. 1995). Sporadic BCCs are also often caused by somatic mutations in ptc in individual skin cells (Gailani, M. R. et al. 1996) and in addition ptc mutations have also been found in other cancers, particularly in medulloblastomas but also for example in breast carcinomas (Xie, J. et al. 1997; Pietsch,
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T. et al. 1997; Vorechovsky, I. et al. 1997a; Raffel, C. et al. 1997; Wolter, M. et al. 1997; Vorechovsky, I. et al. 1997b; Maesawa, C. et al. 1998). More than 70 different ptc mutations from sporadic BCCs or individuals with NBCCS have now been reported and these map throughout the ptc gene with no obvious genotype - phenotype correlations (Johnson, R. L. and Scott, M. P. 1997; Wicking, C. et al. 1997; Chidambaram, A. et al. 1996; Unden, A. B. et al. 1996). Most of the mutations produce truncations of the protein, but of the mis sense mutations about half map to the two putative extra cellular loops of PTC. However, only 9/23 mis-sense mutations alter highly conserved amino acids and a mutation has been detected both in the variable region of the cytoplasmic loop in the middle of the protein and in the variable region of the C-terminus of the protein (Johnson, R. L. and Scott, M. P. 1997).

Mutations in other components of the Hh pathway have also now been discovered in BCCs, consistent with the fact that overexpression of ptc, gli and smo is seen in BCCs even when no mutation in ptc can be detected (Gailani, M. R. et al. 1996; Unden, A. B. et al. 1997; Dahmane, N. et al. 1997; Reifenberger, J. et al. 1998; Kallasy, M. et al. 1997). For example constitutive active mutations in smo (Xie, J. et al. 1998; Reifenberger, J. et al. 1998) and a putative gain of function mutation in shh (Oro, A. et al. 1997) have been found in sporadic BCCs and other tumours. In addition transgenic mice that over-express shh in their skin, or grafted human skin or frogs that express glil in their epidermis also develop multiple BCCs (Oro, A. et al. 1997; Dahmane, N. et al. 1997; Fan, H. et al. 1997); and an up-regulation of shh expression has also been found in lung squamous carcinomas (Fujita, E. et al. 1997).

Other Human syndromes associated with the Hh pathway

A number of human developmental syndromes have also been linked to mutations in particular components of the Hh pathway (for a recent review see (Ming, J. E. et al. 1998). For example one of the loci responsible for holoproencephaly (HPE3) was recently found to be shh (Roessler, E. et al. 1996; Belloni, E. et al. 1996); truncations of GLI3 just before the C termi

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these syndromes are, like Gorlin's / NBCC syndrome, dominant syndromes, caused by haplo-insufficiency of the gene in question. This is in contrast to mice heterozygous for mutations in shh, which have no phenotype (Chiang, C. et al. 1996), but is consistent with mice heterozygous for mutations in ptc or gli3, which do show defects reminiscent of their respective human syndromes (Goodrich, L. et al. 1997; Lyon, M. F. et al. 1967; Hui, C. C. and Joyner, A. L. 1993).

Why study the Hh pathway in zebrafish?
As a vertebrate model organism, Zebrafish has several advantages for developmental biology and genetics. The embryos develop outside of the mother and are transparent, enabling the development of even interior structures of the embryo to be observed easily under a microscope; the adult fish are small which makes it possible to keep a large number of different mutant strains on site; the fish are cheap to maintain compared to other vertebrate systems; an individual female can lay up to 1000 eggs at one time; it is easy to inject RNA and DNA into embryos; and it is possible to perform heterotypic and homotypic cell transplantations. Many of these characteristics combine to make zebrafish a particularly attractive vertebrate system for large scale mutagenesis and for combining genetical and embryological methods of analysis. A number of zebrafish mutations had already been identified when I started this PhD, including mutations that result in the loss of particular midline structures, for example cyclops (Hatta, K. et al. 1991; Thisse, C. et al. 1994) and floating head (Halpern, M. et al. 1995; Talbot, W. S. et al. 1995) that are very useful for examining the role of different signals in the midline. In addition, during my PhD, a large number of mutations were generated in two large scale mutant screens in Boston and Tübingen and in particular a class of these mutations were identified as potential candidates for components of Hh signalling pathways (van Eeden, F. et al. 1996). Subsequent to this one of these mutations has been identified as a loss of function mutation in shh (Schauerte, H. E. et al. 1998). I will discuss the phenotypes of this class of mutations in more detail in the introduction to chapter eight, before I describe my analysis of ptc expression in these, and other mutants from the Tübingen screen.

Outline of the Thesis
The main body of this thesis is divided into two major parts: my analysis using the zebrafish as a model organism is described and discussed in chapters 3-9; and my analysis of the chicken talpid3 mutation, and of the regulation of ptc expression in the chicken limb, is described and discussed in chapters 10-12. In general, the main
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discussion of the results and of the conclusions that they suggest is to be found in chapters 9 and 12, though there is a very brief summary of some of the main findings and/or conclusions at the end of each of the results chapters. Some of the analysis described in this thesis has been undertaken in collaboration with other people; where this is the case it has been clearly indicated in the relevant chapter.
CHAPTER TWO

MATERIALS AND METHODS

Molecular Biology Techniques

DNA Preparation
Plasmid DNA was obtained using either a lysis by boiling mini prep protocol (Sambrook, J. et al. 1989) or for higher quality or higher yields of DNA, Qiagen Maxi prep Kits.
Cosmid DNA was obtained using Qiagen Maxi prep Kits (cosmid protocol), but starting from an initial culture of 200 - 400ml and heating the elution buffer to 50°C before use.
Lambda DNA was obtained using liquid cultures or plate lysate methods. Standard protocols for liquid cultures (using LB as a culture medium and PEG 6000 precipitation) did not give a very high yield so after trying a variety of conditions the following protocol was used. An aliquot from the library was used or a single plaque was cut out from a plate and eluted for 2-3 hours at room temp or overnight at +4°C in 110 µl of λ dil. 100µl of λ in λ dil. was then added to 100µl Y1090 cells and 100µl 10mM MgCl₂/10mM CaCl₂ and placed on ice for 20 minutes. This was then added to 50 mls NZC medium; 250µl 20% casamino acids and 20µl of 100µg / µl ampicillin and cultured with shaking for 4 hours + (a variety of times were used), until lysis started to occur. Then 50 µl of chloroform was added and the culture was shaken to lyse the cells. The culture was then spun at 4000rpm for 30 mins and this supernatant transferred to a new tube and spun at 8000 rpm for 15 mins. The supernatant was then filtered through gauze into a new tube. 30 mls of this supernatant was then processed using a Qiagen lambda DNA kit and a Qiagen-tip 100 following the kit protocol but using 60µl L1; 6ml L2; and 1.8ml of L3, L4 and L5. Before precipitating the DNA 0.5µl of tRNA (10mg/ml)was added to aid precipitation. To check the resulting DNA a 20 µl aliquot was added to 5µl EDTA 0.5M and 2.5µl 20% SDS and heated to 70°C for 10 minutes before loading on a gel.

λ dil. = 20mM Tris Cl (pH8); 20mM MgCl₂; or 10mM Tris Cl (pH 7.5); 10mM Mg SO₄.
NZC = 10g NZ Amine A; 5gm NaCl; 2gm MgCl₂.6H₂O - made up to 1 litre with water and autoclaved for 30 minutes. Casamino acids were only added after the solution had cooled to less than 50°C.

Y1090 bugs were grown on tet amp plates and cultured in 49 ml LB broth + 0.5 ml 20% maltose + 0.5 ml 1M Mg SO₄ + 50 µl/ ml ampicillin
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Purifying DNA fragments from gels
Large DNA fragments (such as cosmid fragments) were purified from agarose gels using the Qiagen Qiaex II gel extraction kit. Smaller fragments were purified from agarose gels using either this kit or gene clean.

Transforming bacteria
Bacteria were transformed using a heat shock method. Competent bacteria were stored at -70°C. Between 50 and 200 µl were defrosted on ice and incubated with a 1/3 of a ligation reaction or a minimum amount of plasmid for 30 min - 1 hour on ice; placed in a water bath at 42°C for 90 seconds; and then added to 600 µl LB and incubated at 37°C for between 30 and 60 minutes. The reaction was then centrifuged at 4,000 revs/min for 5 minutes. All except 200 µl supernatant was removed and the pellet of bacteria was re-suspended by pipetting up and down in the remaining supernatant. This was then plated on amp+ plates, previously dried at 37°C for 30 - 60 minutes, using small glass beads to evenly spread the bacteria.

Screening cDNA and genomic DNA libraries
Radioactive probes were prepared by random priming using the Amersham life sciences rediprime kit and ^32P dCTP

Hybridisation of filters lifted from bacteriophage plates
Phage were plated on LB-agar plates using a LB/agarose top layer. Filters of the plates were prepared using Hybond™-N nylon membranes and the protocol provided with them. The DNA was fixed to the filters by baking at 80°C for 2 hours. Filters were pre-hybridised for 6 hours for primary screens and for at least an hour for subsequent re-screens and then hybridised overnight at 42°C. They were then washed with 2X SSC, 0.1% SDS at room temperature for an hour and then if still highly radioactive, at 65°C for 10 minutes. Filters were then placed in 5X SSC at room temperature and laid down with autoradiographic film in cassettes. The hybridisation buffer used was 5X SSC; 5X Denhardts; 200µg/ml salmon sperm DNA; 50mM NaPO₄ pH 7; 0.1% SDS; 43% Formamide.

Hybridisation of gridded library filters
Initially, when screening the gridded cDNA library with ptcI, filters were hybridised using the same hybridisation buffer as the other library screens. When I screened either
the cDNA gridded library with *ptc2* or the genomic DNA gridded libraries I used the following hybridisation buffer: 4X SSC; 10X Denhardts; 50 μg/ml salmon sperm DNA; 0.1% SDS; 0.1% SPP and hybridised at 68°C. Filters were pre-hybridised for 2-4 hours and then hybridised in fresh hybridisation buffer + probe overnight. They were then washed in 2X SSC 0.1X SDS for 2 X 30 minutes and 0.1X SSC 0.1X SDS for 2 x 30 minutes - all at 68°C

**Southern blots of cosmid DNA**
Cosmid DNA was digested with EcoR1 in a volume of 100μl and then precipitated and re suspended in a small volume. This was heated to 70°C for 7 minutes and then loaded on a 0.8% TAE gel and run at 15 volts overnight. The gel was photographed and a southern blot was prepared using a nitro-cellulose filter as described in Maniatis - protocol 9.41(Sambrook, J. et al. 1989). The Southern Blot was then probed with randomly primed 32P dCTP labelled DNA and the hybridisation buffer and protocol described above under the gridded library section (for *ptc2* 3’ sequences the 3-7 fragment was used as a template for the probe).

**PCR**

**RNA prep - for RT PCR.**
To prepare RNA for RT PCR I used the following protocol:
1.) Collect embryos at a particular stage, clean and dechorionate. From this stage on use sterile tubes, tips etc. and DEPC- treated aqueous solutions (except those containing Tris) [DEPC water: add 0.01% DEPC, leave for at least 12 hours and then autoclave].
2.) Add 100 μl solution D to < 10 mg tissue in a micro centrifuge tube and pipette up and down through a syringe till the tissue disintegrates. (We added 2ml solution D for 150 mg embryos or 1 ml for 60 mg embryos).
3.) Add 10 μl 2 M sodium acetate pH 4 and mix
4.) Add 100 μl *unbuffered* water saturated phenol and mix
5.) Add 20 μl chloroform/isoamyl alcohol (49:1) and mix very well
6.) Chill on ice for 15 min centrifuge at 15000g for 15 min at 4 °C
7.) Transfer aqueous (top) phase to new tube. If started with less than 1mg tissue add 1 μl 10 mg/ml RNAase free glycogen
8.) Add 240 μl ethanol, mix and put at -20°C overnight
9.) Thaw and centrifuge at 15 000 g for 15 min at 4 °C
10.) Remove supernatant and re suspend the pellet in 300 μl solution D.
11.) Transfer to 1.5 ml ependorf; add 600 μl ethanol; put on solid CO2 for at least 1 hr.
**Materials and Methods**

12.) Thaw and centrifuge at 13 000 rev/min for 30 min at 4 °C
13.) Pipette off supernatant and wash the pellet with 150 μl 80% ethanol, drain and dry
11.) Re dissolve in 100 μl DEPC water. Heat to 65 °C for 10 min and snap chill on ice
12.) Store at -70 °C

Solution D = 25 gm guanidinium thiocyanate; 29.3 ml DEPC water; 1.76 ml 0.75 sodium citrate pH 7; 2.64 ml 10% sarkosyl (sodium N-laurylsarcosinate); 38 μl 2-mercaptoethanol. Store aliquots at -70 °C or keep them in the dark at room temp for up to one month.

**Making 1st strand cDNA**

I took 5 μl of RNA and heated to 65°C for 10 minutes, followed by snap chilling on ice and then adding the reaction mixture (see below). The reaction was placed at 37°C for 1 and a half hours; heated to 95°C for 5 minutes, cooled on ice and then stored at -20°C. I then used 5 μl of this as template for each PCR reaction.

Reaction Mix = 5 μl RNA; 10 μl 5X RT (1st strand cDNA) buffer; 0.5 μl dNTP mix (a mix of 25mM of each (formed by mixing equal amounts of 100mM stocks of all four dNTPs)); 1 μl RNase Inhibitor (40 units); 1 μl specific primer (0.5 - 1μg); 2 μl Reverse Transcriptase (Boehringer M-MuLV - 40 units); 30.5 μl depc water.

5 X RT buffer = 250mM Tris HCl (pH 8.3); 30mM MgCl₂; 200 mM DTT; 200 mM KCl

**PCR protocol**

Generally Boehringer Taq was used for RT-PCRs using degenerate primers; an ICRF in house Pic Taq enzyme was used for diagnostic PCRs and for PCRs using plasmids as templates; and Boehringer Taq + Taq extender was used for the bridging fragment PCRs. All reactions were 50 μl. Between 250 ng - 1 μg of each primer was used per reaction. (The higher concentrations of primer were used with the initial genie primers, otherwise 250 - 500 ng of each primer was used per reaction). Standard PCR protocols were used, with extension times as recommended for the enzyme and expected length of product. Each reaction was usually 35 cycles long, used 200 μM dNTP and was hot started. For example:

**Rev and Genie PCRs**

94 °C 5 minutes
72 °C - add enzyme
Then X 35
94 °C 30 sec
50 °C 30 sec
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72 °C 1 min 30 sec
Then
72 °C 10 minutes

Inverse PCR
I developed this protocol based on (Huang, S. et al. 1990); Promega protocols and applications guide 3rd edition page 184; and Maniatis Molecular Cloning 2: 8.11 - 8.17 (Sambrook, J. et al. 1989)

Step One - make 1st strand cDNA
As protocol above but omit the final 95°C step

Step Two - making the second strand cDNA
(This step does not need a primer because the RNase H causes nicks in the RNA which act as primers for the DNA Polymerase. The protocol uses the 1st strand reaction as a template but dilutes it 5X so you end up with a final reaction volume of 250µl.)
1.) Take the 50 µl 1st strand cDNA reaction and add 100 µl 2nd strand buffer X2.5 (see below); 8 µl DNA Pol I (60 units); 2 µl RNase H (2 units); 2 µl dNTP mix; 88 µl depc water.
2.) Put at 16°C for 2-4 hours (should be 14°C. I did 16°C for 2 hours 50 minutes)
3.) Heat 70°C 10 minutes
4.) Centrifuge quickly and place on ice
5.) Add 10 units T4 DNA Polymerase and incubate at 37°C for 10 minutes (should be 2 units T4 DNA Polymerase / µg input RNA - I used 10 units)
6.) Dilute with 200µl of depc water
7.) Concentrate with Centricon 100, 1000g 20 minutes

2nd strand buffer x2.5: 100 mM Tris HCl pH 7.2; 225mM KCl; 7.5mM MgCl₂; 7.5mM DTT; 0.125 mg/ml BSA.
(I made 10 mls using 1 ml 1 molar Tris (actually about pH 7.3); 2.25 ml 1 molar KCl; 75 µl 1 molar MgCl₂; 75µl 1 molar DTT; 1.25 ml of 10 mg/ml BSA; and made up to 10 ml with depc water)

Step Three - Circularisation
1.) Take the concentrated cDNA - 100 µl and add 12 µl ligation buffer X10; 6 µl T4 DNA ligase (1U/µl) - (I added 24 units); and 2 µl RNA ligase (40µg / µl).
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2.) Put at 16°C over night
3.) Add distilled water to dilute to 1 ml
4.) Use Centricon 100 to concentrate by spinning for 30 minutes at 1000g
5.) Collect the result (was about 50 µl)
6.) Freeze to store

Step Four - PCR
1.) Use 5µl of the final 50µl mixture as a template and PCR as usual using the two primers you have designed (Inverse1 and Inverse2 - design these with different restriction sites and then you can simply directionally clone in to KS)
   Assemble reaction mix: 5 µl template cDNA; 5µl PCR buffer X10; 0.4µl dNTP mix; 0.5µl primer 1 (1 µg / µl); 0.5µl primer 2 (1 µg / µl); 38µl distilled water.
2.) Heat to 94°C for five minutes
3.) Hold at 72°C
4.) Add 0.5µl of Boehringer Taq (about 2.5 units)
5.) Set cycling X 35
   94°C 30 seconds
   50°C 30 seconds
   72°C (I did 2 minutes the 1st time and 1 minute the second time - both worked)
6.) 72°C 10 minutes

Genotyping individual zebrafish embryos with PCR

Preparation of genomic DNA from individual zebrafish embryos

Individual embryos were placed in an epindorf with 50 µl of DNA extraction buffer and incubated at 98°C for 5 minutes. Then 5 µl of 10mg/ml Proteinase K was added to each epindorf and they were incubated overnight at 37°C. The proteinase K was then heat inactivated by heating the reactions to 98°C for 5-10 minutes and 10µl of each preparation was used for a 50µl PCR reaction.

DNA extraction buffer: 20µl 1 M Tris (pH 8); 100 µl 1 M KCl; 6µl 1 M MgCl2; 60 µl 10% Tween 20; 60 µl NP 40; 1.754ml dH2O

Genotyping for the shh locus
**Materials and Methods**

Two primers that amplify a CA repeat in the 3' UTR of the zebrafish *shh* gene were used to identify zebrafish embryos that had a deletion of the *shh* locus. These primers were kindly provided by Heike Schauerte (Schauerte, H. E. et al. 1998). Primers for a region of the forkhead gene, *fkd3*, were kindly provided by Jörg Odenthal as a positive control (Odenthal, J. and Nusslein-Volhard, C. 1998).

The Shh primers were: forward: ctcgcgtgaactcaggctgg; reverse: ggagcccacggttgcttaccc

**Sequencing**

Sequencing was done either manually using $^{35}$S and standard protocols or using Pharmacia or Applied Biosystems automated sequencers. Sequence analysis was performed using Geneworks (Intelligenetics) GCG (Devereux, J. et al. 1984) and DNA STAR (Applied Biosystems) software packages.

**In Situ Hybridisation on Zebrafish embryos.**

Embryos were fixed in 4% PFA in PBS, overnight at 4°C or for 4 hours at room temperature. They were then washed several times with PBS; at least 3 X 10 minutes with 100% MeOH; and stored in 100% MeOH at -20°C.

Embryos were rehydrated through a MeOH/PBS series: 10 minutes each in 75:25 50:50 25:75 and 100% PBS. Then embryos that were fixed at 24 hpf or older stages were treated with proteinase K at 10μg/ml for 20 minutes; re-fixed in 4% PFA for 20 minutes; and washed 3X 10 minutes in PBT. These steps were missed out for embryos that were fixed at stages younger than 24 hpf.

Embryos were then equilibrated in 50% fish cheap hyb : 50% PBT, and then in 100% fish cheap hyb. This was then replaced with Fish Hybridisation Buffer and embryos were pre-hybridised at 70°C for 4 hours (2 - 8 hours, preferable at least 4 hours) and then hybridised with RNA probes in Fish Hybridisation Buffer overnight. All the steps at 70°C were done without shaking. Probes were prepared at a final concentration of 1:20000 in Fish Hybridisation Buffer (a 1/40 dilution of the stock) and placed at 70°C for at least an hour before use. Washes were also prepared and placed at 70°C over night to equilibrate to the temperature.

Embryos were then washed at 70°C with fish cheap hyb 3X 5 minutes; 2X SSC 0.1% tween 3X 20 minutes; 0.2 X SSC 0.1% tween 3X 20 minutes; PBT 3X 5 minutes.

Then embryos were washed in PBT 3X 5 minutes at room temp; put to gently shake in blocking solution for at least an hour at room temp; gently shaken in 1/2000 pre-absorbed
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antibody in blocking solution for 2 hours; washed at least 8X 15 minutes with PBT and then washed overnight at 4 °C in PBT.

If dig probes were used embryos were then washed for at least 3X10 minutes in NTMT buffer and then stained with NBT/BCIP at a concentration of 4.5 μl of 75 mg/ml NBT in 70% dimethylformamide and 3.5μl of 50 mg/ml BCIP in dimethylformamide per ml of NTMT.

If a mixture of dig and fluorescein probes were used then these were visualised sequentially. The alkaline phosphatase was inactivated between the two staining reactions by rinsing twice and then incubating for 30 minutes with glycine 0.1M/pH2.2, tween 0.1%; followed by thorough washing in PBT. Embryos were blocked for 1 hour before each antibody incubation, and both anti flu alkaline phospatase antibody and anti dig alkaline phosphatase antibody were used at a concentration of 1:2000. One antibody was visualised using NBT/BCIP as above and the other was visualised using fast red(Boehringer). One fast red tablet was dissolved in 2ml 100mM Tris pH 8.2 and used within 30 minutes. Embryos were equilibrated with 3 washes of 5 minutes in 0.1M Tris-HCl pH 8.2 and then placed in stain solution

NTMT buffer = 0.1M NaCl, 0.05M MgCl, 0.1M Tris pH 9.5, 0.1% tween
Fish Cheap Hyb = 50% formamide; 5X SSC; 0.1% tween
Fish Hybridisation Buffer = Cheap Hyb + 500 μg/ml yeast RNA + 50 μg/ml heparin and pH'd to pH 6.0 with 1 M citric acid
NTMT buffer = 0.1M NaCl, 0.05M MgCl, 0.1M Tris pH 9.5, 1% tween
PBT = PBS + 0.1% tween

Probes were prepared by linearising plasmid equivalent to 1μg of insert DNA with the appropriate enzyme, treating with proteinase K (0.05μg/μl) for 30 minutes at 37°C, and then extracting the DNA with phenol chloroform followed by ethanol precipitation. This was then added to 2μl of 10X nucleotide mix (with digoxigenin-UTP or fluorescein-UTP); 2 μl of transcription buffer; 20 units of RNase inhibitor; 40 units of the appropriate polymerase; and water to give a final reaction volume of 20 μl. The reaction mixture was incubated for 2 hours or overnight at 37°C, then 40 units of DNaseI were added and the reaction was incubated for a further 15 minutes at 37°C and then the reaction was stopped with 2μl of 200 mM EDTA pH 8.0. The RNA was then precipitated with 2.5μl 4M LiCl and 75 μl pre-chilled ethanol and re-suspended in 100 μl RNase free water containing 40 units of RNase inhibitor. 2.5μl of the probe was usually run on a gel to check it. Probes were normally then diluted with 400 μl of Fish Hybridisation buffer.
and stored at -20°C. This stock was then diluted 1/40 in the appropriate Hybridisation buffer for the final concentration and placed at 70°C for at least an hour before use.

Probes were synthesised using the following templates:

- **shh** (Krauss, S. et al. 1993);
- **MyoD** (Weinberg, E. S. et al. 1996);
- **ptc1** (Concordet, J.-P. et al. 1996);
- **ptc2** - 1.8kb bridging fragment (b.f. 10, 14 or 16) was linearised with EcoR1 and transcribed with T7;
- **twhh** (Ekker, S. C. et al. 1995);
- **ehh** (Currie, P. D. and Ingham, P. W. 1996);
- **paxb** (Krauss, S. et al. 1991a);
- **krox-20** (Oxtoby, E. and Jowett, T. 1993);
- **nucleolin C23** (Qiao, T. 1997);
- **fkd-4** (Odenthal, J. and Nusslein-Volhard, C. 1998).

**In Situ Hybridisation on Chicken embryos.**

Embryos were fixed in 4 % PFA in PBS or PBT, over night at 4°C. They were then washed with shaking for at least one lot of 10 minutes with PBT; at least once for 10 minutes with 50% MeOH; at least once for 10 minutes with 100% MeOH and then stored in fresh 100% MeOH at -20°C.

Embryos were rehydrated through a MeOH/PBS series: 10 minutes each in 75:25 50:50 25:75 and 100% PBS. They were then treated with proteinase K at 20μg/ml for 20 -25 minutes depending on the stage of the embryos; re-fixed in 4% Formaldehde, 0.2% gluteraldehyde for 20 minutes; and washed 2X 10 minutes in PBT. Embryos were then equilibrated in 50% chick cheap hyb: 50% PBT, and then in 100% chick cheap hyb. This was then replaced with Chick Hybridisation Buffer and embryos were pre-hybridised at 70°C for a minimum of 4 hours and then hybridised with digoxigenin labelled RNA probes at a concentration of 1: 20000 in Chick Hybridisation Buffer overnight, preferably with mild shaking. Probes were heated at 70°C for at least an hour before use and washes were also prepared and placed at 70°C over night to equilibrate to the temperature.

Embryos were then washed at 70°C with chicken cheap hyb 3X 30 minutes; 50 % formamide 2X SSC 0.1% tween 3X 30 minutes. Then embryos were washed in KTBT for at least 3X 10 minutes at room temp; put to shake in blocking solution for at least 4 hours at room temp; put to shake in 1/2000 pre-absorbed antibody in blocking solution overnight at +4°C; and washed at least 6 X 1hr at room temperature and overnight at +4°C with KTBT.

Embryos were then washed for at least 3X10 minutes in NTMT buffer with 1% tween and then stained with NBT/BCIP at a concentration of 0.263 mg/ml NBT, 0.187 mg/ml...
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BCIP in NTMT with 1% tween. Reactions were stopped by rinsing with NTMT with 1% tween and embryos were stored in 4% PFA in PBS.

Chick Cheap Hyb = 50% formamide, 5X SSC, 1% SDS, 0.1% tween

Chick Hybridisation Buffer = Chick Cheap Hyb + + 50μg/ml heparin and 50μg/ml tRNA

KTBT = 0.05M Tris/HCL ph 7.5, 0.15M NaCl, 0.01M KCl, 1% Tween

Blocking solution = 10-20% sheep's serum in KTBT

Chick NTMT buffer = NTMT but with 1% tween

Templates for Chick probes

\[ ptc \quad \text{(Marigo, V. et al. 1996); gli} \quad \text{(Marigo, V. et al. 1996); gli3} \quad \text{(Marigo, V. et al. 1996); coupTFII} \quad \text{(Lutz B et al. 1994); BMP-2} \quad \text{(Francis, P. H. et al. 1994); shh} \quad \text{(Cohn, M. J. et al. 1995).} \]

Antibody staining on fish embryos

Antibody staining was as in (Westerfield, M. 1995) but without step 12 and stopping after step 14. When embryos had already been through an \textit{in situ} hybridisation procedure they were fixed for 20 minutes in 4% PFA, washed in PBT a few times and then put straight into the blocking step of the protocol. The antibody incubations were done in PBS/BSA/DMSO. For BAD5 staining, embryos were fixed with a MeOH/PBS series (5 minutes in each of 25:75; 50:50; 75:25; 100:0; 75:25; 50:50; 25:75; 0:100 MeOH:PBS. They were then stored in PBS at 4°C.

Antibody stainings for Engrailed used 4D9 monoclonal antibody (Patel, N. H. et al. 1989) obtained from the Developmental Studies Hybridoma Bank at the University of Iowa, Department of Biological Studies, Iowa city, IA 52242

Slow muscle fibres were visualised using BAD5 antibody (mouse monoclonal) (Blagden, C. S. et al. 1997)

Antibody staining on chicken embryos

I attempted Shh whole mount antibody (Marti, E. et al. 1995) staining using the following protocol. I tried a variety of different fixation times for the embryos, and also tried dehydrating the embryos into EtOH before the antibody procedure (and storing at -20°C) and not dehydrating the embryos.

Incubate embryos in 1% H₂O₂ in PBS for 15 minutes at room temperature; wash in PBS + 0.5% triton; block in PBS + 0.5% triton + 10% Goat serum + 1% BSA for 2 hour at room temperature; and then incubate in the 1° antibody (1:500) in PBS + 0.5% triton + 1% goat serum + 0.1% BSA overnight at +4°C. Wash 3 x 1 hour in PBS + 0.5% triton.
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+ 1% goat serum and then incubate in goat anti-rabbit 2° antibody (1:250 Jackson labs antibody) overnight at +4°C. Then wash 3 x 1 hour in PBS and pre-incubate in 0.5mg/ml DAB in PBS for about an hour. Then replace with 0.5mg/ml DAB in PBS + 2μl 30% H₂O₂ in 10ml. Stop the reaction with PBS.

Embryonic Sections
 Sections were obtained by embedding embryos in wax after the in situ hybridisation procedure. Embryos were taken through an Ethanol/Butanol series at room temperature with each step being for half an hour: 80% EtOH; 80% EtOH/BuOH 3:1; 90% EtOH/BuOH 1:1; 100% EtOH/BuOH 1:3. Then embryos were placed in 100% BuOH for at least an hour (or stored at +4°C). The embryos were taken through a BuOH/wax series at 60°C: BuOH/wax 1:1 20 minutes; 100% wax 15 minutes; fresh wax 15 minutes and then embedded in wax at 60°C and left to cool.
 Sections were cut and placed on microscope slides using water to spread them out on the slide. Slides were allowed to dry. To mount slides and remove the wax the slides were heated to 50 - 55 O°C for 30 minutes and then taken through at least 3 changes of Histoclear for at least 20 minutes in total. Excess Histoclear was then removed and coverslips were mounted over sections using Permount. Air bubbles were removed and the slides left to dry before examining under a compound microscope.

Over-Expression Studies in Zebrafish
 The following expression constructs were used: p64Tshh (Krauss, S. et al. 1993); p64Tehh (Currie, P. D. and Ingham, P. W. 1996); pT7TStwhh (Ekker, S. C. et al. 1995); pCS2dnReg (Strähle, U. et al. 1997); ptc1A (described in chapter 3) - linearised with sall and transcribed with sp6 polymerase.
 RNA was synthesised using Ambicon Message Machine kits and injected at empirically determined concentrations. Phenol red was used to make it easier to visualise the RNA as it was injected. Large amounts of phenol red were injected as a negative control. This had no effect on embryo morphology or expression of either ptc gene. Injections were performed on 2-4 cell stage embryos using back-filled capillaries (Clarks Micro-electrical Instruments, Reading) and a pressure pulsed Narishige micro injector. Once injected, embryos were cultured in Hanks solution with added penicillin/streptomycin, in petri dishes coated with 1.5% agarose made with Hanks solution With all of the hh injection experiments, individual experimental replicates used a constant concentration of RNA, but there was a slight variation in the effects on different embryos, probably due to a
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slight variation in the volume of RNA injected and/or the distribution of the synthetic RNA between different cells in the embryo. I controlled for these differences by dividing the injected embryos from each individual experiment into two pools, making sure to divide embryos of similar phenotype equally between the two pools. One pool was then examined for ptc1 expression and the other pool for ptc2 expression. I could then directly compare the effects on the two different ptc genes.

Hanks solution was made up fresh from stock solutions as described in The Zebrafish book (Westerfield, M. 1995), with the exception that stock solution 6 (NaHCO3) was always added as a solid as a last step.

Photography
Specimens were analysed using a Zeiss Axioplan microscope, or a Zeiss Stemi SV6 dissecting microscope and photographed with Kodak Ektachrome 64T film. Images were scanned on either a Sprintscan 35 slide scanner or a Nikon LS-1000 35mm film scanner and processed using Adobe Photoshop software.

Zebrafish stocks
Wild-type Danio rerio were bred from a founder population obtained from the Goldfish Bowl, Oxford, or from the AB strain, initially from the University of Oregon. The flh^nl strain was obtained from T. Jowell (Newcastle University, UK). The cyc and ntl strains were obtained from C. Kimmel (University of Oregon, USA) and the syu, ubo, con, igu, you, yot and dtr strains were obtained from P. Haafter (Max Planck Institute for Developmental Biology, Tübingen, Germany). I also collected embryos from these and other mutant strains during two visits that I paid to Dr. Pascal Haffter's lab. Fish were maintained on a constantly recirculating system at 28°C on a 14 hours light / 10 hours dark cycle.

Alleles used
Except where noted to the contrary the following alleles were used in the analysis in this thesis: yot = yotty119; ubo = ubopl39; you = youty97 (van Eeden, F. et al. 1996); igu = igu(ts294e) (Brand, M. et al. 1996); syu = syu4(Schauerte, H. E. et al. 1998).

In most cases this is the strongest allele. The syu4 allele is a stable inherited deletion that covers the shh gene (Schauerte, H. E. et al. 1998) and has a similar phenotype to other alleles of syu. The syu(ts294e) allele that was used to make some of the double mutants
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with *igu* is a regulatory allele of *syu* that has reduced expression of *shh* but still makes reduced levels of functional protein at some stages (Schauerte, H. E. et al. 1998; chapter 8)

Mutant embryos were identified either on the basis of somite morphology or expression of *Eng.* proteins. In the latter case the *Eng.* antibody staining was the last step in the analysis, which allowed mutant and wild type embryos to be processed in the same tube and hence treated identically throughout the collection, fixation and *in situ* hybridisation procedures.

Chicken stocks

Fertilised wild type White Leghorn chicken embryos were purchased from Needle Farm. Fertilised *talpid^3* (*ta^3*) embryos were obtained from the stock maintained by Dave Burt at Roslin Institute, Roslin, UK. Eggs were incubated at 38°C: normal embryos were staged according to (Hamburger, V. and Hamilton, H. 1951); and *ta^3* embryos were staged according to (Hinchliffe, J. R. and Ede, D. A. 1967). Homozygous *ta^3* embryos were identified by the characteristic limb morphology (broader - mushroom shaped) and/or by the reduced spacing of the eyes. If there was any ambiguity then identification was confirmed by either grafting a wing onto the wing stump of a wild type host to allow it to develop further (in which case the *ta^3* shape would become more obvious) or by *in situ* hybridisation on one limb with a gene that has an altered pattern of expression in *ta^3* limbs (e.g. initially *bmp-2*, and after we had established the pattern of expression in *ta^3* limbs *ptc, gli* or *gli3*).
CHAPTER 3

CLONING AND ANALYSIS OF ZEBRAFISH PTC SEQUENCES

A. Cloning of ptcl and ptc2

Background

When I first arrived in Philip Ingham's laboratory Lisa Goodrich, Ron Johnson and Matthew Scott at Stanford University had just cloned a homologue of ptc in mouse using a degenerate PCR approach (later published in (Goodrich, L. V. et al. 1996)). This was the first example of a vertebrate homologue of ptc. They provided us with their successful degenerate PCR primers, modified in light of the mouse Ptc sequence and I started working on this project in collaboration with a post doctoral fellow in the lab, Jean-Paul Concordet.

The isolation and cloning of the initial PCR fragments of both ptcl and ptc2 and the subsequent cloning of ptcl from cDNA libraries was done in collaboration with Jean-Paul Concordet: I made the major contribution to the PCR amplification and cloning of the ptc related fragments; some of the sequencing of clones described here was performed by a technician in the lab, John Moore, using an automatic sequencer; the rest of the sequencing, and the analysis of all the sequence, was performed by Jean-Paul Concordet and/or myself. The determination of the ptc2 sequence outside the initial 1.8kb PCR bridging fragment was all my own work, as was the analysis of the Ptc protein sequences. John Postlethwait's laboratory mapped both ptcl and ptc2 and Heike Schauerte in Pascal Haffter's laboratory also mapped ptcl.

PCR amplification of ptc-related sequences

We were initially provided with four degenerate PCR primers which we used to amplify two short fragments (about 300bp and 320bp) of putative zebrafish patched genes. The degenerate PCR primers were based on amino acid sequence comparisons of Drosophila melanogaster (fruit fly), Anopheles gambiae (mosquito), Precis coenia (butterfly), Tribolium casteneum (beetle), and Mus Musculus (mouse) Ptc proteins (Goodrich, L. V.
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et al. 1996). The Rev2 and Rev4 primers were used to isolate Rev fragments; and the Genie1 and Genie2 primers were used to isolate Genie fragments (fig. 5).

Rev2: ggacgaattcSYTCNRGCCARTGCAT
Rev4: ggacgaattcYTNGANTGYTTYTGGA
Genie1: ggacgaattccGAYGGNATNATNAAYC
Genie2: ggacgaattcRTAYTGYTCCCARAANA

(large lettering corresponds to sequences conserved between mouse and different insect Ptc sequences, small lettering corresponds to an EcoRI site and arbitrary flanking sequence introduced to facilitate subsequent cloning of PCR products).

Jean-Paul Concordet and I both prepared total RNA from bud stage, 5 somite stage, 14 somite stage and 24 hpf zebrafish embryos in case the ptc gene(s) were temporally regulated. We then synthesised 1st strand cDNA using either a random primer, the Genie2 or the Rev2 primer and attempted RT PCR with the different cDNAs, varying Mg^2+ and primer concentration, annealing temperature and extension time. We obtained and sub-cloned 5 positive PCR fragments: three separate Rev products, all using a Mg^2+ concentration of 1.5mM, a primer concentration of about 250ng/50μl reaction, and either 14 somite random primed cDNA, 14 somite Rev2 specific primed cDNA or bud stage random primed cDNA (fig. 6); and two separate Genie products, using a primer concentration of about 1μg/50μl, a Mg^2+ concentration of 2.5mM and 14 somite random primed cDNA or 14 somite Genie 2 specific primed cDNA. The latter reaction yielded two bands of a very similar size (fig. 6). We isolated a number of subclones from each of these reactions and sequenced them manually. We identified two Rev fragments that had homology to ptc and one Genie fragment. We sequenced several subclones for each fragment, and then sequenced our remaining subclones using just one termination reaction to see if we could identify any different sequences. Any sequences that looked different were then sequenced in full. In total we analysed about 75 Rev fragments and about 50 Genie Fragments but we found no more fragments with homology to ptc.
Cloning and analysis of zebrafish ptc sequences

Figure 5: PCR primers used in the initial identification and cloning of fragments of ptc1 and ptc2.
The coloured rectangles represent PCR products and the arrows represent PCR primers. The black arrows are the degenerate PCR primers used to amplify the initial Rev and Genie fragments; the red arrows are specific PCR primers, designed to amplify the middle section; the blue arrows are specific PCR primers designed to test whether other PCR products and/or library clones contained these sequences.
* indicates primers that were used to make 1st strand cDNA pools.
Row A and Row D show the initial PCR fragments obtained (two 5' Rev fragments and one 3' Genie fragment).
Row B, C, E and F show attempts to amplify the middle section of both genes. Note that the particular combination of primers represented in row E did not work - which was not un-expected as we had only managed to PCR one 3' fragment and therefore had only one specific 3' PCR primer. This was the reason for also using the 3' degenerate primer in PCRs with the 5' specific primers (rows C & F).
Rows A, B and C correspond to fragments of what became known as ptc2 and Rows D and F correspond to fragments of what became known as ptc1.
Figure 5
Cloning and analysis of zebrafish ptc sequences

**Figure 6: Cloning the First Fragments of Zebrafish ptc Genes.**

A mixture of *Drosophila* and mouse ptc was used as a positive control and reactions were assembled without template as negative controls. The MgCl₂ concentration was varied in order to maximise the efficiency of the reaction. Positive reactions that were sub-cloned and analysed are highlighted in bold in the description below.

**Gel 1. 27/10/94**

Lanes 1 - 7 show Rev fragment PCRs; lane 8 is a 1 Kb ladder and lanes 9-11 show positive control Genie fragment PCRs with different magnesium concentrations.

**Rev Fragment PCRs**

1.) 14 somite stage specifically primed cDNA; [MgCl₂ ] = 1.5mM
2.) 14 somite stage randomly primed cDNA; [MgCl₂ ] = 1.5mM
3.) 14 somite stage specifically primed cDNA; [MgCl₂ ] = 3.5mM
4.) 5 somite stage specifically primed cDNA; [MgCl₂ ] = 1.5mM
5.) tail - bud stage specifically primed cDNA; [MgCl₂ ] = 1.5mM
6.) negative control; [MgCl₂ ] = 1.5mM
7.) positive control; [MgCl₂ ] = 1.5mM

**Gel 2. 1/11/94**

Lanes 12 - 16 show Rev fragment PCRs; lane 17 is a 1 Kb ladder and lanes 19 - 23 show Genie fragment PCRs

**Rev Fragment PCRs all with [MgCl₂ ] = 1.5mM**

12.) positive control
13.) tail - bud stage randomly primed cDNA
14.) 24 hpf randomly primed cDNA
15.) 24 hpf specifically primed cDNA
16.) negative control

**Genie Fragment PCRs all with [MgCl₂ ] = 2.5mM**

18.) negative control
19.) 24 hpf randomly primed cDNA
20.) 14 somite specifically primed cDNA
21.) 14 somite stage randomly primed cDNA
22.) tail-bud stage specifically primed cDNA
23.) positive control
The first Zebrafish Rev fragments cloned

The first Zebrafish Genie fragments cloned (and another Rev reaction)

Figure 6
Cloning and analysis of zebrafish ptc sequences

In an effort to obtain a larger fragment for library screening and *in situ* hybridisation, we designed specific primers from each of our three fragments.

RevR3/id = ggacgaattcaTGACTGGTGGCTGCTATGGGC
RevR1/id = ggacgaattcctTGAACTTCAGGGTGGTTGCCA
GenieR3/cp = gcacgaggatccTCATTGCTCACCCAGGCAGAC

We set up PCR reactions with each of the Rev specific primers and the Genie specific primer (fig. 5 B & E). To allow for the possibility that one of the Rev fragments derived from a second *ptc* gene, we also performed PCR reactions with each of the Rev specific primers and the degenerate Genie2 primer (fig. 5 C & F). We obtained bands of the appropriate size (1.8 - 2.0 kb), not present in control reactions, with both RevR3/id and Genie2 (fig. 5C) and with RevR1/id and Genie2 (fig 5F), and we obtained a band with RevR3/id and GenieR3/cp (R3G3) (fig. 5B) but did not obtain a band with RevR1/id and GenieR3/cp (fig. 5E). Sequence analysis of these fragments revealed that the Genie region in the RevR1/id Genie2 (R1G2) fragment was more divergent in sequence than the other *ptc* genes and that the sequence in the Genie1 primer region was not conserved. This explained why we had not initially isolated a genie fragment for this gene and probably explains why a chick and mouse homologue of this *ptc* gene were not initially identified. We then used these bridging fragments as probes for *in situ* hybridisation analysis and cDNA library screens in an effort to obtain full length clones. Because we were initially successful with the R1G2 fragment this was designated *ptcl* and the R3G3 fragment was designated *ptc2*.

Restriction analysis of the R1G2 PCR product revealed the existence of internal EcoR1 sites, so this bridging fragment was cloned as three sub-fragments L, S and VS. Several isolates of each of these were restriction mapped. Subclones of a particular sub-fragment were all the same length and had the same restriction sites, so a number were sequenced and a consensus established.

In contrast, we isolated 5 separate subclones of the bridging fragment R3G3: three were the same size (b.f. 10, b.f. 14, b.f. 16); one was slightly shorter (b.f. 17) and one behaved strangely (b.f. 11). We abandoned b.f. 11 and restriction mapped the other four fragments. This confirmed that b.f. 14 = b.f. 16; b.f. 10 is very similar but has an extra Bam H1 site; and b.f. 17 is about 150 bp shorter than the other three and also has an extra Bam H1 site. All four of these fragments were sequenced on both strands and a consensus established. There are no BamH1 sites in this final consensus sequence, suggesting that the sites in b.f. 10 and b.f. 17 are either star sites or are due to PCR

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Cloning and analysis of zebrafish ptc sequences

errors. Sequencing determined that b.f. 17 has a 114 nucleotide, or 38 amino acid, deletion compared to the other three fragments, at what I eventually identified as amino acid 566 of the final ORF of the Ptc2 consensus sequence. We thought that this might be the result of alternative splicing and designed PCR primers either side of this site to see if we could amplify two different sized bands. These primers were:

R3G3 gap1/id: ggacgaattcGTGGACGATGTCTTTCTCCT
R3G3 gap2/cp: ggacgaattcTACTGCACGTGCTGCTGCTGTT

These primers amplified the expected sized bands when the bridging fragments were used as templates, so I used them to PCR amplify 1st strand cDNA. Using one sample of cDNA I only obtained one band, which was at the larger size. With another type of cDNA I obtained two bands of roughly the right sizes, but when compared to the bands amplified from the bridging fragments the smaller of the cDNA products was slightly smaller than the positive control. This region of ptc2 contains an Nco site so I also investigated whether the PCR products contained this site. The larger band did but the smaller band did not. Also, when I tried to re amplify these products from a dilution of the initial PCR reaction only the larger band re amplified. This all suggested that the smaller band was an artefact. When I compared the ptc2 sequence to the sequence of other ptc genes I found that the deletion in b.f. 17 was in a highly conserved region and contained 2 conserved cysteines, suggesting that it was unlikely to be real. Therefore I assumed that it was probably a PCR artefact and abandoned b.f. 17. However, when a full length construct of ptc2 is isolated it might be worth while checking this region.

Screening cDNA libraries

We screened oligo dT primed cDNA libraries using the R3G3 bridging fragments or the L fragment from R1G2 as templates for randomly primed 32P labelled DNA probes (for more details see chapter two). The first five cDNA libraries that we screened were unsuccessful: none of them gave final round positives with homology to ptc. In parallel I also tried RACE PCR to try to obtain more 5' and more 3' sequence for both of the genes. I tried this a number of different times with different primers and PCR conditions - but also with no success. The cDNA libraries that we initially screened were made by David Grunwald (9-16 hours, 20-28 hours, adult) and by Domingos Henrique (9-16 hours). No positive clones were obtained with any homology to ptc. (Positive clones did come through three rounds with some of the Grunwald libraries but when extracted with helper phage and sequenced, or analysed by PCR, did not correspond to ptc. For
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eexample, two separate positive clones came through three rounds of the R1G2 screen of the Grunwald 9-16 hour library, but when these were sequenced neither had any significant homology to ptc or to anything else in the database. One of these also behaved strangely, with different mini preps after the helper phage protocol showing different restriction patterns. No positive clones came through all three rounds of re-screening, with the R3G3 screen of this library).

A gridded cDNA library from 26 somite embryos made by Matt Clarke (Lehrach laboratory, ICRF) was also screened (filters ZFCS1 and ZFCS2, 20,000 clones per filter). We obtained a large number of positives with the ptc1 probe. A number (eventually about 100) were picked and analysed with restriction enzymes to see if they fell into particular classes. This analysis gave unexpected results that suggested that the inserts contained repeated sequence (depending on the restriction enzyme used the insert appeared to be radically different in size - suggesting that some enzymes were excising a number of similarly sized fragments from the insert). The clones were divided into groups based on their restriction digest patterns and one was sequenced. The consensus sequence had homology to ptc, though it was not very high, but when I searched databases with the sequence I discovered that it was 100% identical to yeast 2µ plasmid. It turned out that the gridded library was contaminated with yeast 2µ plasmid and that our ptc1 probe was hybridising to this. Just in case some of our positive clones also contained ptc1, Matt Clarke hybridised the filters with yeast 2µ plasmid, and we compared the clones that he identified with those that we had identified with our ptc1 probe. A number were positive for ptc1 but not for yeast 2µ plasmid, so we isolated these and analysed them with restriction enzymes that we knew had sites in the L2 fragment. We also analysed all the clones in groups of 10 with PCR to see if any contained either the Genie or Rev fragments, using specific primers designed against these sequences (see fig. 5A), as we reasoned that even if the clones were contaminated with yeast 2µ plasmid they might also contain ptc1. No positive clones were obtained.

At a later date this library was also screened using the ptc2 probe and a different hybridisation buffer and higher temperature to try and avoid hybridising to the yeast 2µ plasmid (see chapter two). No positives were obtained.

Our next attempt was more successful. We screened a 33-36 hour λgt11 phage library from Kai Zinn. 2×10^6 plaques were screened for each gene at low stringency according to standard procedures. We did not obtain any positive clones with the ptc2 bridging fragment probe, that contained ptc2 sequence as assayed by PCR. However, 20 positive
Cloning and analysis of zebrafish ptc sequences

plaques from the screen using the ptc1 fragment were purified and several of these contained ptc1 sequence as assayed by PCR. The longest clone that contained both Rev and Genie sequence (as assayed by PCR using oligonucleotides against these sequences and against the lambda vector), λ105, was sequenced on both strands using either Pharmacia or Applied Biosystems automated sequencers with primers from the cloning vector and internal primers. This clone turned out to contain the complete ORF of ptc1. The ORF was significantly shorter than in other ptc genes characterised at that time so I also checked the sequence of the cDNA in the region of the stop codon, and the position of the stop codon, by amplifying this region from 1st strand cDNA with a proof reading enzyme Pvu. We sub cloned fragments from three separate reactions and sequenced these on both strands to confirm the sequence in this region.

The full length open reading frame sequence of ptc1 was deposited in the EMBL database under the accession number X98883 (fig. 7).

A full length expression construct for ptc1, ptc1A, was created by first of all cloning the ptc1 ORF plus about 900 bp of λgt11 sequence downstream of the ORF, from λ105 into the litmus 29 vector. This was then excised and inserted into the psp64TXB vector. The excess λgt11 sequence and part of the 3' end of the gene were then excised and replaced with a PCR fragment of the 3' of the ptc1 ORF, produced using a proof reading enzyme, Vent, and sequenced to check that no errors had been incorporated.
Figure 7 - The DNA sequence of ptc1

This figure contains all the sequence of ptc1 that has been determined so far. The small boxes show the start and stop codons of the Open Reading Frame. This sequence was all determined from cDNA, either by RT-PCR or by sequencing the cDNA library clone λ105.
Further degenerate PCR to amplify more ptc2 sequence

As described above, the ptc2 bridging fragment was used to screen a number of cDNA libraries but no clones with homology to ptc were isolated. Therefore, I designed specific primers from the known sequence and used them in combination with a number of more 5' and 3' degenerate primers, which I designed by comparing the sequences of zebrafish Ptc1, mouse Ptc, chicken Ptc and Drosophila Ptc. Four more PCR fragments were isolated, each using one specific primer and one degenerate primer; two included progressively more 5' sequence and two included more 3' sequence (fig. 8). All the PCR reactions used random primed 1st strand cDNA synthesised from RNA isolated from 24 hpf zebrafish embryos. In order to minimise errors due to the PCR procedure, the same reaction was usually repeated 2-3 times, the product from each separate reaction was subcloned, a number of subclones from each reaction were sequenced on both strands and a consensus was established. Sequencing was mainly performed using primers either from the cloning vector or specific sequencing primers that I designed from known ptc2 sequence. With all of the PCR primers described here, the lower case letters refer to a linker sequence, used to incorporate restriction enzyme sites (usually EcoR1 and BamH1) into the ends of the PCR product for directional cloning. The upper case letters are sequence based on the known nucleotide sequence of ptc2 (specific primers) or a comparison of different Ptc proteins (degenerate primers).

Figure 8: Establishing the full length open reading frame of ptc2

Schematic representation of the different fragments from which the sequence encoding the full length open reading frame of ptc2 was established. Rev2, Genie3 and the bridging fragment were the initial PCR fragments isolated (Concordet, J.-P. et al. 1996). IQR, 5-1, EVL and 3-7 are four PCR fragments that were generated using one specific primer and one degenerate primer. inverse refers to sequence obtained from a modified inverse PCR procedure and genomic refers to sequence gained from genomic DNA.
**Figure 8**

Diagram showing the genomic relationships between different ORFs and regions:

- **5'** End

- **ptc2 ORF**

- **3'** End

- **Rev2** to **Genie3** bridging

- **IQR**

- **EVL**

- **5-1**

- **3-7**

- **Inverse genomic**
Cloning and analysis of zebrafish ptc sequences

Five Prime fragments

IQR :- 530 nucleotide fragment
specific primer from initial Rev2 fragment:
ptcfin2/cp - ggacgaattcATCTTCAACTCAGCAATGAAGC
degenerate primer:
ptc2IQRdeg/id - atacgaggatccATICARARRMAYTGYGGIAA

5-1 :- 320 nucleotide fragment
specific primer from IQR fragment:
P2-5P-AMFS/cp - atacgaggatccACTAAACATGGCCTCCTCTCCGATC
degenerate primer:
P2MOCHFl/id - ggaatgaattcAGTATCTGCAGCGGCCGAGCTA

Three prime fragments

EVL :- 440 nucleotide fragment
specific primer from Genie3 fragment:
R3G3THREEOUT - ggacgaattcGCCATCTGCAACAACTA
degenerate primer:
Ptc2/PEVL/cp - atacgaggatccSYISCIARCATIARIACISC

3-7 :- 350 nucleotide fragment
specific primer from EVL fragment:
P2-3P-RNKR/id - ggacgaattcATCGGTGACAGGAACAAGCGAG
degenerate primer:
P2-3P-SDSEY/cp - aggcgaggatccTAYTCISWRTCISWISWITC

Genomic library screening

The rest of the 3' ORF sequence was obtained using the 3-7 fragment as a template for a
$^{32}$P dCTP labelled randomly primed probe to screen a gridded genomic cosmid library.
Three filters of ZFTu (ICRF c71) (Burgtorf, C. et al. 1998) were screened and 2 strong
positives were obtained: cosmids E19.108 and J01.94. These were digested with EcoR1;
Southern Blotted and then probed with $^{32}$P dCTP labelled 3-7. Both cosmids had a
positive band on the Southern - with each cosmid it was the largest fragment (roughly
Cloning and analysis of zebrafish ptc sequences

15kb) - though the fragment from cosmid J was larger than the fragment from cosmid E. These fragments were cloned into Bluescript KS and sequenced. A sequencing primer designed against the 3' end of the known sequence was used to determine further 3' sequence (3Psequen1/id). The genomic subclones were also sequenced using a primer designed against the opposite strand of the 3' end of the previously identified sequence to confirm that any sequence obtained was really ptc sequence (3Psequen2/cp).

3Psequen1/id - TTGTCCTGAGGTATCTCCAGCGGATG
3Psequen2/cp - TGTGACTTTGGAGCATGGTGAAAG

Sequence was obtained that extended the known sequence and which uncovered a stop codon, just a little way into the new sequence. Sequencing the opposite strand confirmed known ptc2 sequence and then gave what I presume is intron sequence (see * in fig. 9). To check that the new 3' sequence was coding sequence, the sequence in the region of the stop was checked by RT PCR. Two specific primers were used, one from either side of the stop codon. A proof reading enzyme - vent DNA polymerase- was used to minimise PCR errors. A PCR product of the right size was obtained (about 350 base pairs). This PCR product was sub cloned and two separate subclones were sequenced. These confirmed the sequence obtained from the cosmids and confirmed that the stop codon was real and in coding sequence.

ptc2end1/cp - atacgaggatccCTGGAGGTTGTACTGCTGAAG
ptc2end3/id - ggacgaattcCTCTGCTAGGAGTCTTGATG

This genomic library (ZF Tü) was also screened using 32P labelled 5-1 as a probe, but no positive cosmids were obtained that provided any further 5' sequence. The two cosmids that hybridised to the 3-1 probe did not hybridise to the 5-1 probe, suggesting that these cosmids only contain part of the ptc2 gene. Two new cosmids were identified by the 5-1 screen: J20, 111 and N24, 002 and both of these were also positive in a southern blot of an EcoR1 restriction digest. The positive band was about 9kb long in the EcoR1 digest, but there were two bands of this size, very close together on the gel. Both bands were sub cloned, but because there was likely to have been contamination from one band to the other because they were so close in size, I identified the correct subclones by restriction analysis and by probing with the 5-1 probe. Promising subclones were sequenced using different sequencing primers designed from 5-1 sequence (5Psequ1, 5Psequ2, 5Psequ3, 5Psequ4).

5Psequ1/cp - CCGCAGTTCTTTTGTATGTAACAGCC
5Psequ2/CP - CCACCTTCTACCCAGAGTTTCTC
5Psequ3/cp - CGTGTGTACTTGAGTTCCTGATTC
Cloning and analysis of zebrafish ptc sequences

5Psequ4/cp - CGCTTTCTCCAGTCGCATTC

Only one reaction worked, the most 3' sequencing primer (5Psequ3) used on a 10kb fragment from the J cosmid (Js10), and this gave unknown sequence. This sequence had lots of stop codons in all three frames and did not identify anything in sequence databases suggesting that it was not ptc2 coding sequence; however, it could have been intron sequence. I tried cutting the 10kb fragments with restriction enzymes for which there were sites in the 5' sequence that I had already identified - EcoRV and PstI. The cosmid fragments I analysed had three EcoRV sites and one PstI site. I therefore cloned the two fragments generated from the PstI digest (roughly 3kb and 7kb) and sequenced from the PstI end of these clones to see if the sequence around the PstI site in the fragment corresponded to the sequence around the PstI site in fragment 5-1. T3 and T7 primers from the Bluescript KS plasmid sequence were used for sequencing. The sequence obtained bore no resemblance to ptc2 5' sequence or to anything in the database (FASTA search). Therefore I concluded that if these cosmids did contain ptc2 sequence they did not extend as far 5' as the PstI site in the sequence that I had already obtained, probably due to a large intron within the 5-1 cDNA sequence, close to 5Psequ3.

Consequently, I made a shorter 5' probe of about 250 base pairs of the most 5' known sequence. This was made by PCR using 5Psequ2/cp and P2MOCHF1/id primers. This PCR product was used as a template to make a ^32^P dCTP labelled probe, which was used to re-screen the genomic library filters. No really strong positives were identified though some weaker ones were. N24, 002 did not light up at all and J20, 111 only lit up very weakly. None of the weak positives were pursued since at this point the inverse PCR strategy began working (April 1997).

Inverse PCR

The rest of the 5' sequence was obtained using an inverse PCR technique on specifically primed cDNA, (see chapter two). First strand cDNA was made as for all of the other PCRs but with a specific primer close to the 5' end of the known sequence. Two different types of cDNA were made using different specific primers. The rest of the protocol was then followed in duplicate. The two primers used were:

5Psequ1/cp (which is about 150 nucleotides downstream from the 5' end of the sequence that we had isolated at that point) and 5Psequ3/cp (which is about a further 140 nucleotides downstream).
Cloning and analysis of zebrafish ptc sequences

5Pseq1/cp - CCGCAGTTCTTTTGATGTAACAGCC
5Pseq3/cp - CGTGTGTACTTGAGTTCCTGATTC

The inverse PCR reaction was performed using two specific primers, directed away from each other:
Inverse1/cp - ggacgaattcTGATATCTGCTCCAGAAAG
Inverse2/id - atacgaggatccGAATGCGACTGGGAGAAGCG

Both of the circularised cDNAs produced PCR fragments, roughly 0.52kb and 0.67kb, consistent with the difference between the positions of the two initial specific primers for the 1st strand cDNA. These PCR products were sub-cloned. Three different sub-clones of each product were sequenced, the sequence re-linearised and a consensus established.

This completed the sequence of the ORF of ptc2 (fig. 9). The full length ORF sequence of ptc2 was deposited in the EMBL database under the accession number AJ007742.

I also attempted to create a full length construct of ptc2 for overexpression studies but I was not successful. I designed a number of different PCR primers in an effort to amplify the ORF of ptc2 in 2 sections. I attempted to amplify these fragments using a proof reading enzyme Pfu (Stratagene) and a variety of different PCR primer combinations and conditions but with no success.

Figure 9: ptc2 DNA sequence.

This figure contains all the sequence of the ptc2 gene that has been determined so far. The small dark boxes show the start and stop codons of the Open Reading Frame. All of the sequence is from 1st strand cDNA, or has been checked with 1st strand cDNA (RT PCR) apart from the boxed sequence at the 3' end of the gene, which has been determined solely from genomic DNA. The sequence boxed in the light shading was determined by me and the more 3' sequence that is boxed in darker shading was determined by Heike Schauerte and is based on only 1 or 2 sequencing runs so it may not be completely accurate. None of the genomic sequence after the stop codon has been checked as thoroughly as all the sequence 5' to the stop codon. The asterix (*) indicates the position of the nearest intron to the stop codon: this is the first point at which the sequence of the antisense strand of the genomic DNA clone differs from the sequence determined by RT PCR.
Cloning and analysis of zebrafish ptc sequences

B. Genetic Mapping of ptc1 and ptc2

ptc1 and ptc2 map to different linkage groups on the zebrafish genetic map. ptc1 maps to linkage group 2, which is genetically very close to twhh (Postlethwaite, J. H. et al. 1998). ptc2, however, maps to linkage group 8 which is not near any currently known hh gene (Postlethwait personal information).

C. Analysis of Ptc protein sequences and structures

Hydropathy analyses of both the ZF-Ptc1 and ZF-Ptc2 amino acid sequences predict that they both include 12 transmembrane domains, like all the other characterised Ptc proteins (fig. 10). Both of the zebrafish Ptc proteins have a significantly shorter carboxyl terminal domain than the Ptc proteins characterised in other species, with the exception of mouse Ptc2 which is similar in length (fig. 13). ZF-Ptc2 is slightly longer than ZF-Ptc1 due to a small insertion into the middle of ZF-Ptc2 in the region between the putative 6th and 7th transmembrane regions. All of the Ptc proteins are quite divergent in this region, including the different Drosophila species, and the vertebrate Ptc proteins also all have an insertion relative to Drosophila Ptc (Concordet, J.-P. et al. 1996; Forbes, A. J. 1992). The second small insertion seems however to be specific to ZF-Ptc2.

The amino acid sequence of zebrafish Ptc2 is more closely related to the Ptc proteins initially isolated from other vertebrates than to zebrafish Ptc1. For example ZF-Ptc2 shows 82% identity with mPtc1 and chick Ptc1 but ZF-Ptc1 is only 64% identical to mPtc1 and chicken Ptc1 and ZF-Ptc2 is only 64% identical to ZF-Ptc1 (fig. 11). However, ZF-Ptc2 is more similar to ZF-Ptc1 than it is to mouse Ptc2 (it is only 58% identical to mPtc2). Both zebrafish proteins are roughly equally conserved with Drosophila Ptc (ZF-Ptc1 is 40% identical and ZF-Ptc2 is 43% identical). Unlike ZF-Ptc1, ZF-Ptc2 has the same potential glycosylation sites that are conserved between Drosophila mouse, human and chick Ptc1. Drosophila Ptc has seven putative glycosylation sites, of which the same three are conserved (though the precise amino acid sequence of the sites differs) in mPtc1 chick Ptc1, human Ptc1 and ZF-Ptc2 but only one is conserved in ZF-Ptc1 or mPtc2 (fig. 12). In addition to these sites ZF-Ptc2 has an additional seven putative glycosylation sites, two of which are in regions of the protein predicted to be extra-cellular. One of these is conserved in all the vertebrate Ptc proteins including ZF-Ptc1 but not in Drosophila Ptc (though there is a putative glycosylation site close by in the
Cloning and analysis of zebrafish ptc sequences

*Drosophila* sequence) and one is conserved in mPtc1, human Ptc1 and chick Ptcl (but not mouse Ptc2 or ZF-Ptc1) (fig. 13 & 12). Eight cysteine residues are conserved in all the Ptc proteins (fig. 13). All of this evidence suggests that ZF-Ptc2 is most closely related to chick, mouse and human Ptc1 (it is 82% identical to all of them) and that there is a family of vertebrate Ptc proteins that consists of at least two members. ZF-Ptcl is more similar to mPtc2, but not by much (ZF-Ptc1 is 68% identical to mPtc2 and 65% identical to mPtcl) so these two genes might also belong to different classes (fig. 14).

Ptc proteins also have homology in their hydrophobic regions to a protein involved in cholesterol trafficking disease, Niemann-pick type C (NPC). This homology can be divided into two main regions: the first spans the 3rd to the 5th transmembrane regions of Ptc and includes a putative sterol sensing domain, based on sequence homology with other sterol-regulated proteins SREBP cleavage -activating protein (SCAP) and HMG CoA; and the second spans the 8th to 12th transmembrane regions (Loftus, S. et al. 1997; Carstea, E. et al. 1997; Johnson, R. L. and Scott, M. P. 1997). The homology between mouse NPC and the two different classes of Ptc proteins in fish and in mouse is almost identical (34-35% identical, 44-47% similar in the first region; 35-38% identical, 48-49% similar in the second region; fig. 15 & 16) suggesting that any functional significance of this homology is common to all Ptc proteins.

**D. Brief Summary and Conclusions**

This chapter demonstrates that at least two *ptc* genes are expressed in zebrafish and that these genes are located on different chromosomes. A comparison of the amino acid sequences of all the vertebrate Ptc proteins isolated so far demonstrates that ZF-Ptc2 is more closely related to mPtc1, human Ptc1 and chick Ptcl than ZF-Ptc1 is, and suggests that the vertebrate Ptc proteins can be divided into at least two classes on the basis of their amino acid sequences. However, all the Ptc proteins are predicted to have a similar structure with 12 putative transmembrane domains and 8 conserved cysteine residues, and they all have similar homology to NPC. These results and conclusions will be discussed in more detail in chapter nine.
Cloning and analysis of zebrafish ptc sequences

Figure 10: Hydropathy plots of the Drosophila and Zebrafish Ptc proteins

Hydropathy plots were calculated for the predicted amino acid sequences of the open reading frames of all three proteins using Geneworks. All three proteins have a very similar hydropathy profile, suggesting that they have a very similar structure, and all form proteins with 12, similarly spaced, transmembrane domains.

Figure 11: Comparison of the sequences of different Ptc proteins

A comparison of the amino acid sequence of the open reading frames of Ptc proteins from different organisms. The first number gives the percentage identity between the two proteins in question and the number in brackets gives the percentage similarity. These were all calculated using the GCG best fit programme using the default options of a gap creation penalty of 12 and a gap extension penalty of 4.
Figure 10

K-D Drosophila Ptc

K-D zebrafish Ptc1

K-D zebrafish Ptc2
<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Mouse Ptc2</th>
<th>Mouse Ptc1</th>
<th>Chick</th>
<th>ZF ptc2</th>
<th>ZF ptc1</th>
<th>Drosophila</th>
</tr>
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<td>40 (51)</td>
<td>100 (100)</td>
</tr>
</tbody>
</table>

**Figure 11: Comparison of the sequences of different Ptc proteins**
Cloning and analysis of zebrafish ptc sequences

Figure 12: Sequence alignment of the Drosophila, mouse & zebrafish Ptc1 proteins.

Putative transmembrane domains are indicated by black lines above the sequence. Orange shading indicates identities between all three species. Blue shading indicates identities between the two vertebrate proteins. Putative N-glycosylation sites in Drosophila are indicated by pink lines and conserved cysteine residues by asterisks. Zebrafish Ptc1 is significantly shorter at the C-terminus than the other Ptc-1 proteins. We checked the sequence of the cDNA in the region of the stop codon, and the position of the stop codon, by amplifying this region from cDNA prepared from zebrafish embryos at somitogenesis stages.

Figure 13: Sequence Alignment of Zebrafish and Mouse Ptc Proteins.

The amino acid sequences of the open reading frames of zebrafish Ptc2 (zfptc2), zebrafish Ptc1 (zfptcl), mouse Ptc1 (mptcl) and mouse Ptc2 (mptc2) were aligned using DNA STAR. Conserved amino acids are boxed in orange. Putative transmembrane domains are indicated by purple lines above the sequence. Red and black asterisks above the sequences identify cysteine residues that are conserved between all Ptc proteins. Square brackets indicate potential N-glycosylation sites that are conserved between zf-Ptc2, m-Ptc1, chick Ptc1 and human Ptc1; green brackets indicate sites that are also conserved with Drosophila Ptc and yellow brackets indicate sites that are not conserved with Drosophila Ptc. Note that only the most 5' yellow site and the most 3' green site are conserved with ZF-Ptc1 and mPtc2.

Figure 14: Phylogeny Tree for the Different Ptc Proteins

A phylogeny tree based on the amino acid sequences of the open reading frames of different Ptc proteins. This was aligned by the clustal method within the programme DNA STAR.
Figure 12
Figure 14: Phylogeny Tree for the Different Ptc Proteins
**Cloning and analysis of zebrafish ptc sequences**

**Figure 15: Comparisons between NPC and Ptc protein sequences**

A comparison of the amino acid sequences of zebrafish and mouse Ptc1 and Ptc2 with Niemann-pick type C (NPC). Two regions of homology are compared: the 454th to the 502nd amino acid of zebrafish Ptc2, which covers the 3rd-6th transmembrane regions and the putative sterol sensing domain; and the 1023rd to the 1194th amino acid of zebrafish Ptc2, which includes the 8th to the 12th transmembrane domains. The corresponding regions of homology of the other proteins were identified using the GCG Best fit programme. The percentage identity and similarity between the different Ptc proteins in these regions are also given for comparison. The charts give the percentage identity between the specific fragment of the two proteins in question and the number in brackets gives the percentage similarity. These figures were all calculated using the GCG Best fit programme using the default options of a gap creation penalty of 12 and a gap extension penalty of 4.

**Figure 16: Alignments of NPC and Ptc proteins**

These alignments were created using Geneworks and are for the same regions of the proteins as in figure 15. Grey boxes show amino acids that are conserved between all the proteins. Black lines and numbers above the sequences show the relevant transmembrane domains of the Ptc proteins.
**Comparison of 1st region of similarity between Ptc and NPC.**

This comparison is for the region of zebrafish Ptc2 that runs from amino acid 454 to 602, which covers the 3rd - 6th transmembrane regions of Ptc including the putative sterol sensing domain.

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<tr>
<th></th>
<th>zebrafish Ptc1</th>
<th>zebrafish Ptc2</th>
<th>mouse Ptc1</th>
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**Comparison of 2nd region of similarity between Ptc and NPC.**

This comparison is for the region of zebrafish Ptc2 that runs from amino acid 1023 to 1194 and includes the 8th - 12th transmembrane regions of Ptc.

<table>
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**Figure 15: Comparisons between NPC and Ptc protein sequences**
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<th>Fish Ptc1</th>
<th>MPTC2</th>
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</thead>
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<td>-K01LAVL -G111LISV  -AG1LCS1L  -CT1LTLV -V1FL1VAG</td>
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</table>

Figure 16: Alignments of NPC and Ptc Proteins
CHAPTER FOUR

WILD TYPE EXPRESSION OF \textit{ptc1} AND \textit{ptc2}

Background

The expression of \textit{ptc1} and \textit{ptc2} during zebrafish embryogenesis was analysed by \textit{in situ} hybridisation and compared to the expression of the three zebrafish \textit{hh} genes characterised to date: \textit{shh}, \textit{ehh}, and \textit{twhh} (Krauss, S. et al. 1993; Ekker, S. C. et al. 1995; Currie, P. D. and Ingham, P. W. 1996). \textit{ehh} and \textit{twhh} are both expressed in subsets of the \textit{shh} expression domain (e.g. cf. fig. 17S-U & C-E) so I concentrated principally on comparing the expression of \textit{ptc1} and \textit{ptc2} with \textit{shh}. The characterisation of \textit{ptc1} expression was done in collaboration with Jean-Paul Concordet but the analysis of \textit{ptc2} expression was solely my own work. With the exception of (fig. 18 R & S), the figures in this chapter show \textit{in situ} hybridisations that were performed, analysed and photographed by myself.

Results

Expression of \textit{ptc1} is first detected at 70\% epiboly in the presumptive mesodermal cell layer in two stripes of cells flanking the axial mesoderm, which at this stage already shows robust expression of \textit{shh}. In contrast \textit{ptc2} expression is first detected about an hour later (85\% epiboly), in the neurectoderm above the axial mesoderm where it persists until early somitogenesis. At the end of gastrulation a longitudinal stripe of \textit{ptc2} expression, 8-9 cells wide, can be seen extending along the rostro-caudal axis of the neurectoderm (fig. 17A). At this stage \textit{ptc1} is strongly expressed in the adaxial cells of the pre-somatic mesoderm as well as more weakly in the pre-somatic mesoderm and in the neurectoderm above the axial mesoderm (fig. 17B). At these early stages of development all three \textit{hh} genes are expressed in the midline: \textit{shh} and \textit{ehh} are expressed in the notochord and \textit{twhh} is expressed in the ventral neurectoderm (fig. 17C-E).

By the early stages of somitogenesis, 3-4 somites, a prominent stripe of expression of \textit{ptc2} is apparent in the presumptive hindbrain and this persists throughout early somitogenesis. No equivalent \textit{ptc1} expression is observed in this region (fig. 17F & G). A comparison with \textit{krox-20} expression at about 5-6 somites (Oxtoby, E. and Jowett, T. 1993), and with \textit{pax-b} expression at about 8 somites (Krauss, S. et al. 1991a), suggests that this stripe of \textit{ptc2} expressing cells is located in presumptive rhombomere 3 (fig. 17H)
Wild Type Expression

& I). A weaker stripe of ptc2 expression is also visible in presumptive rhombomere 5 by about 8 somites (fig. 17I). Throughout somitogenesis, ptc2 and ptc1 are both expressed in the ventral midline of the presumptive brain.

The early expression of ptc2 in the trunk is quite dynamic, contrasting with the relatively static expression of ptc1 in the ventral neur ectoderm and the adaxial cells. Initially, as gastrulation ends and the first few somites form, ptc2 is only expressed weakly in the neur ectoderm overlying the midline as described above, but by about 5 somites expression is apparent throughout the medial lateral extent of the somites, in the adaxial cells of the pre-somitic mesoderm and around the tail-bud (fig. 17J & L). At about 10 somites ptc2 is still expressed throughout the medio-lateral extents of the somite except that it is now down regulated in a triangular patch of cells adjacent to the notochord, and expressed at elevated levels just lateral to these cells (fig. 17M & O). In this respect the highest levels of ptc2 expression in the somite appear complementary to ptc1 expression (fig. 17N & P). In contrast to ptc1, which is evenly expressed along the rostro-caudal axis, expression of ptc2 in the trunk becomes weaker more caudally: there is only very weak expression throughout the pre-somitic mesoderm but slightly stronger levels of expression persist in the most caudal adaxial cells and around the tail bud.

By 18 somites, the expression patterns of ptc1 and ptc2 are more clearly resolved and distinct though both genes continue to be expressed in similar parts of the embryo (cf. fig. 17Q to 17R). Both genes are still highly expressed in regions around sites of shh, twhh, and ehh expression, for example in the somites and in the neural tube, but in both instances the ptc2 expression domain is broader than that of ptc1. For example, ptc2 is expressed throughout the dorso-ventral extent of the somites whereas ptc1 expression is concentrated around the notochord in the middle of the somites.
Wild Type Expression

Figure 17: Wild type expression of ptc and hh genes during early development

Comparison of ptc and hh gene expression during the first 18 hours of wild type zebrafish embryogenesis. Transcripts were revealed by in situ hybridisation with antisense RNA probes to ptc1 (B, G, K, N, P & R); ptc2 (A, F, J, L, M, O & Q); shh (C & S) ehh (E & U); twhh (D & T); krox20 (H) and pax-b + ptc2 (I).

(A-E) show dorsal views of embryos at the end of gastrulation (tail - bud stage). ehh is only expressed in the notochord (E); twhh is expressed in the tailbud, the neurectoderm above the notochord, and the presumptive ventral brain (D) and shh is expressed in all of these places (C). ptc1 and ptc2 have different expression patterns at this stage: ptc2 is just expressed in the neurectoderm above the midline (A) whereas ptc1 is also expressed in the adaxial cells and the pre-somitic mesoderm (B).

(F-H) show lateral views and (I-P) show dorsal flat preps (with the yolk removed) of embryos at 6-12 somites of development: (F) shows ptc2 expression at 5-6 somites and the arrow points to the stripe of high level ptc2 expression in presumptive rhombomere 3; (G) shows ptc1 expression slightly later in development at 8-9 somites; (H) shows krox-20 expression at about 5-6 somites in presumptive rhombомeres 3 and 5; (I) shows ptc2 and pax-b expression at about 8 somites. pax-b is expressed in the midbrain hindbrain boundary (shown with an *), and the presumptive otic vesicle which is centred on presumptive rhombomere 5 but extends into presumptive rhombomeres 4 and 6 (indicated with a square bracket). A stripe of ptc2 expression is visible in presumptive rhombomere 3 and a much weaker stripe of expression is present in rhombomere 5 (indicated with yellow double arrowhead lines). (J & K) show ptc2 and ptc1 expression respectively at 5-6 somites and (L) shows a close up of ptc2 expression in the caudal trunk at this stage. ptc2 is expressed throughout the medial lateral extent of the somites at this stage but ptc1 expression is still confined to the adaxial cells. ptc2 expression in the somites is still broader than ptc1 expression at 10-12 somites (N & M) and close up views (O & P).

(Q-U) show lateral views of embryos at 18 somites. ehh is still only expressed in the notochord (U) and twhh is only expressed in the floor plate and ventral brain (T) whereas shh is still expressed in both of these domains (S). ptc2 expression (Q) is now clearly broader than ptc1 expression (R) in the brain, neural tube and somites.
<table>
<thead>
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<th>6 - 12 somites</th>
<th>18 somites</th>
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<td><img src="6-12-somites.png" alt="Image" /></td>
<td><img src="18-somites.png" alt="Image" /></td>
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Figure 17
**Wild Type Expression**

By 24 hours post fertilisation (hpf) both *ptc* genes are still widely expressed: at this stage *ehh* expression has faded from most of the trunk and is only weakly expressed in the caudal notochord and tailbud while both *twhh* and *shh* are expressed in the floor plate and the ventral neur ectoderm in the head and *shh* is also weakly expressed in caudal regions of the notochord (fig. 18C). The domain of *ptc2* expression in the somites and neural tube remains broader than that of *ptc1*: expression of *ptc2* extends throughout the full dorso-ventral extent of the somites and the neural tube except for the floor plate in the rostral trunk (fig. 18 A, F, G, O & P), whereas *ptc1* expression is much more tightly localised to the medial somites and ventral neural tube, though it is also not expressed in the rostral floor plate (fig. 18 B, M, N &Q). Neither *ptc* gene is expressed in the notochord (fig. 18 F, G & M-Q). A dorsal view reveals the dynamic nature of *ptc2* expression within the somites at this stage (fig. 18H & K). The more rostral somites express *ptc2* at their most lateral extents but expression becomes progressively more medial in more caudal somites, and the expression in the pre-somitic mesoderm is in the adaxial cells and around the tail bud. Intriguingly this pattern resembles the migration through the somites of the slow twitch myoblasts that derive from the adaxial cells, as visualised by an antibody BA-D5 specific for slow myosin HC (cf. fig. 18K to 18L) (Devoto, S. H. et al. 1996; Blagden, C. S. et al. 1997). This dynamic expression of *ptc2* contrasts with that of *ptc1* - which remains restricted to the somitic cells immediately adjacent to the notochord: by this stage these comprise a sub population of the slow muscle cells, the muscle pioneer cells (fig. 18I & J).

The localisation of *ptc* expression within the somites also varies along the rostro-caudal axis of the embryo. At this, and later stages, both *ptc1* and *ptc2* are expressed in the anterior half of the most caudal somites (fig. 19D & G) but in the posterior half of the more rostral somites (fig. 19B & F). This transition from anterior to posterior occurs gradually along the embryo such that some somites have expression in the middle of the somite (fig. 19C). Examination of *ptc1* expression at earlier stages suggests that *ptc* expression is initially restricted to the anterior portion of newly formed somites, shifting posteriorly as the somites continue to develop (fig. 19A & E). This contrasts with *myoD* expression which is initially restricted to the posterior of newly formed somites ((Weinberg, E. S. et al. 1996; fig. 19E).

In the brain at 24 hpf, *ptc1* and *ptc2* are both expressed in the ventral midline and in a peak in the diencephalon which follows the peak of *shh* expression that extends up toward the epiphysis at this stage (fig. 18A-C). In the hindbrain there are six vertical
Wild Type Expression

stripes of ptc2 expression which mark the boundaries of rhombomeres 2-6. This rhombomeric expression of ptc2 is similar, but not identical to the expression of ptc1 - which is elevated in rhombomere 2, 4, and the edges of rhombomere 6 (fig. 18 D & E). Both ptc genes are also just beginning to be expressed in the developing gut.

ptc1 and ptc2 continue to be expressed in the brain - at 32 hpf they are expressed in a similar manner to each other in the hindbrain, diencephalon, and forebrain (fig 20 E & F). Both genes are still expressed in the neural tube and weakly in the somites, with the expression differing between them in the same ways as at 24 hpf. Both ptc1 and ptc2 are now expressed in the fin buds and the branchial arches, which express shh but not ehh or twhh (Krauss, S. et al. 1993; Ekker, S. C. et al. 1995; Currie, P. D. and Ingham, P. W. 1996; fig. 20C & D). The expression of ptc2 in the fin buds is broader and weaker than that of ptc1 (fig. 20A & B).

At 3 days ptc2 expression extends throughout the fins, whereas ptc1 expression is still much more tightly localised (fig. 20 K&L). At this stage of development ptc1 and ptc2 are also expressed in the mouth parts, branchial arches and hindbrain in very similar patterns (fig. 20 I - N). In the mouth, shh is expressed in the epithelial layer and the ptc genes are expressed mainly in the mesenchymal cells adjacent to cells expressing shh. Both ptc genes are also expressed in the developing gut but this is the only notable trunk expression at this stage.

Brief Summary and Conclusions

In summary, the expression patterns of ptc1 and ptc2 differ: ptc1 is expressed at high levels in cells adjacent to sources of hh expression, whereas ptc2 is generally expressed more widely. However, both genes are expressed in embryonic regions that respond to Hh signals. This suggests that the expression of both of these ptc genes might be regulated by Hh signalling and I address this issue in the next chapter.
Wild Type Expression

Figure 18: Wild type expression at 24 hours post fertilisation.

Transcripts were revealed by in situ hybridisation with antisense RNA probes to ptc1 (B, D, I, J, M, N, & Q); ptc2 (A, E- H, K, O & P) and shh (C, R, S) and slow muscle fibre types were detected by immunolocalisation with the BA-D5 antibody (L). All embryos were 24 hpf. (F, G, Q - S) were obtained by embedding embryos in wax and sectioning them after the in situ hybridisation procedure.

(A-C) Lateral whole mount views of ptc1, ptc2 and shh. ptc2 is still expressed more broadly than ptc1, most notably in the neural tube and somites. The double headed arrows indicate the rhombomere expression which is shown in close up in (D & E). At this stage ptc1 is expressed in rhombomere 2, 4 and the edges of rhombomere 6 (D) and ptc2 is expressed at the edges of rhombomeres 2 - 6 (E).

(H - K) Dorsal flat preps of ptc expression. Both ptc1 and ptc2 are expressed similarly in the heads at this stage but the expression of the two genes differs considerably in the somites. In particular ptc1 is only expressed in the pre-somatic and somitic mesodermal cells that are adjacent to the notochord, whereas ptc2 has a dynamic expression along the rostro-caudal axis which is very similar to that of BA-D5 (L) which marks slow muscle fibre types.

(M-P) Close ups of lateral views of ptc expression in the rostral trunk where neither gene is expressed in the floor plate (M & O) and the tail where both genes are still expressed in the floor plate (N & P).

(F, G, Q, R & S) all show transverse sections through the trunk. All except R are from rostral regions of the embryo. The one cell wide medial floor plate is indicated with a red arrow and the ventral neural tube is outlined with yellow dashes. (F & G) show ptc2 expression. (F) which is more weakly stained than (G) shows expression throughout the neural tube except for the floor plate and (G) shows expression in the neural tube and the somites. This demonstrates that the neural tube expression of ptc2 is slightly stronger than the somite expression. (Q) shows ptc1 expression to be much more tightly localised than ptc2: it is expressed only in the ventral neural tube (apart from the floor plate) and the medial somite. Neither ptc gene is expressed in the notochord. (R) shows a rostral section where shh is only expressed in the floor plate and (S) shows a more caudal section where shh is also still expressed in the notochord.
Figure 18
Wild Type Expression

Figure 19: Somite Expression of \textit{ptcl}, \textit{ptc2} and \textit{MyoD}

Transcripts were revealed by \textit{in situ} hybridisation with antisense RNA probes to \textit{ptcl} (A - E); \textit{ptc2} (F & G) and \textit{MyoD} (A & E). \textit{ptc} expression is visualised in blue and \textit{MyoD} expression is visualised in red.

(A & E) show \textit{ptcl} expression in blue and \textit{MyoD} expression in red at 12 somites. (A) shows a lateral view of rostral somites and (E) shows a dorsal view of caudal somites. In both cases \textit{ptcl} is expressed in the anterior of the somites. \textit{MyoD} is expressed in the posterior of the caudal somites but in more rostral somites its expression expands into the anterior somite.

At later stages (24 - 33 hpf) \textit{ptc} genes are still expressed in the anterior of caudal somites but they are now expressed in the posterior of rostral somites: (B & F) shows \textit{ptcl} and \textit{ptc2} expression respectively in the posterior of rostral somites at 32 hpf; (C) shows \textit{ptcl} expression in the middle of the somites at the end of the yolk extension at 24 hpf; (D) shows \textit{ptcl} expression in the anterior of caudal somites at 24 hpf; and (G) shows \textit{ptc2} expression in the anterior of caudal somites at 32 hpf.
Figure 19

caudal 24 hpf  |  middle 24 hpf  |  rostral 32 hpf

12 somites
**Wild Type Expression**

**Figure 20: Wild type expression of ptc and hh genes at 32 and 72 hpf**

Transcripts were revealed by *in situ* hybridisation with antisense RNA probes to *ptcl* (A & E); *ptc2* (B & F) *shh* (C & G); *twhh* (D & H); *ptcl + shh* (I, K, M) and *ptc2 + shh* (J, L, N). Where *ptc* and *shh* expression is visualised simultaneously *ptc* expression is visualised in blue and *shh* expression is visualised in red. The *shh* staining in these embryos is weak and does not show up very clearly in these photographs.

(A-D) show fin bud expression at 32 hpf: *shh* (C) but not *twhh* (D) is expressed in the fin buds and *ptc2* (B) is expressed more broadly than *ptcl* (A). In each case the fin buds are indicated with red arrows.

(E - H) show lateral views of embryos at 32 hpf: *ptc2* is still expressed more broadly than *ptcl* in the trunk.

(I-N) show different rostral views at 72 hpf. Both *ptc* genes are expressed in the hindbrain, branchial arches, fins, mouth and gut. *ptc2* is expressed throughout the fins but *ptcl* is more tightly localised to the posterior of the fin.
Figure 20
Background

As discussed in chapter one, in *Drosophila* high level *ptc* expression is induced in response to Hh signals. The wild type expression patterns of *ptc1* and *ptc2*, described in the previous chapter, suggest that this relationship between *ptc* expression and Hh signalling might be conserved in vertebrates. Consequently we investigated whether Hh signals were sufficient to increase transcription of either *ptc* gene in zebrafish. In collaboration with Jean Paul Concordet I showed that ectopic expression of either *shh* or of a dominant negative regulatory subunit of Drosophila PKA is sufficient to induce ectopic high level *ptc1* expression, suggesting that the normal expression of *ptc1* is regulated by Shh signals (Concordet, J.-P. et al. 1996). I will not discuss these experiments here, but I will discuss more recent experiments that were my sole work, and that confirm and extend this earlier analysis. As the *ptc2* expression pattern differs from that of *ptc1*, and in particular is not as closely associated with *hh* expressing cells, I was interested in investigating whether *ptc2* transcription was also inducible by Hh signals, and whether there was any specificity between the activity of particular *hh* genes and transcription of the different *ptc* genes. The experiments described in this chapter also address these questions.

Inhibition of PKA activity

Jean Paul Concordet and I previously showed that reducing the activity of PKA in zebrafish embryos resulted in ectopic expression of *ptc1*. These experiments used a dominant mutation of the regulatory subunit of *Drosophila* PKA. I repeated these experiments using a dominant mutation of the mouse PKA regulatory subunit (dnPKA) and examined the effect on the expression of *ptc1* and of *ptc2*. Over-expression of mouse dnPKA results in ectopic expression of both *ptc* genes but the pattern of the ectopic expression at 24 hpf differs between *ptc1* and *ptc2*. Both *ptc* genes are expressed ectopically in the brain in a similar manner, with the most severely affected embryos having ectopic expression virtually throughout the brain, except in the telencephalon (fig
Ectopic Activation of Hh Pathways

21B, C, J, M & N. Less severely affected embryos often have a punctate ectopic expression of the ptc genes (e.g. fig 21B & C). In the trunk, however, the expression of the ptc genes differs quite considerably. In severely affected embryos, ptc1 is expressed throughout the dorsal-ventral and medio-lateral extents of the somites and the dorsal-ventral extent of the neural tube (fig 21 B & E). This pattern is reminiscent of, but much stronger than, ptc2 expression in the trunk of normal embryos. By contrast, ptc2 expression is suppressed in the ventral trunk of severely affected embryos, leaving only a narrow domain of strong expression in the most dorsal part of the neural tube (fig. 21M). However, the trunk expression of both of the ptc genes varies between individual injected embryos. This is most clearly seen in the case of ptc1: in less affected embryos just a few cells in the trunk, usually in the neural tube, express ptc1 ectopically (fig 21C & F). Less severely affected embryos also either express ptc2 normally in the trunk or have very slightly weaker ventral trunk expression (fig. 21N).

Figure 21: ptc1 and ptc2 expression after ectopic expression of dnPKA

Transcripts were revealed by in situ hybridisation with antisense RNA probes to ptc1 (A - F) or ptc2 (G - N). (B, C, E, F, H, J, M & N) show embryos that were injected with dnPKA at the 1-4 cell stage and then fixed at 24 hpf. (A, D, G, I & L) show control uninjected embryos that were processed in parallel. ptc1 and ptc2 are ectopically expressed in the brains of injected embryos (B, C, J, M & N); ptc1 is often also ectopically expressed in the trunks of injected embryos (B, E and in a few cells in C &F), but in severely affected injected embryos ptc2 expression is lost from the ventral trunk (e.g. see H & M).
Figure 21
Ectopic Activation of Hh Pathways

Overexpression of shh

I next investigated whether shh was sufficient for ptc2 expression by injecting synthetic shh mRNA into 2-4 cell stage embryos and examining these embryos at either 10 hpf or 24 hpf for ptc1 and ptc2 expression. I found that over-expressing shh at high levels is also sufficient to induce high level ectopic expression of both ptc genes (fig. 22 A-I & K-S). However the pattern of the altered expression at 24 hpf differs between ptc1 and ptc2 in the same manner as with the dnPKA injections described above: ptc1 is ectopically expressed in both the head and the trunk of strongly affected embryos but ptc2 is ectopically expressed in the head and suppressed in the ventral trunk.

Investigating whether there is any specificity between the over-expression of different hh genes and the induction of ptc1 and ptc2

I was concerned that my injections of shh RNA might be at such a high concentration that I was artificially activating different Hh pathways. Therefore to try to determine if different Hh proteins specifically activate transcription of different ptc genes, I over-expressed all three hh genes individually, and reduced the concentration of injected synthetic RNA until I observed greater effects on the expression of one ptc gene than on the other. I reasoned that if there was a specificity between particular hh genes and the expression of the two different ptc genes, I should either only observe ectopic expression of one of the ptc genes as a result of over-expressing a particular hh (which I had already shown not to be the case for shh) or I should reach a concentration with each of the hh genes where more embryos showed ectopic expression of one of the ptc genes than of the other.

I found that ectopic expression of high concentrations of all of the hh genes induces ectopic expression of both of the ptc genes and suppression of ptc2 expression in the ventral trunk: strikingly no obvious differences were observed between the effects of the different hh RNAs (shown for shh in fig. 22 B, C, E, G-I, L, M, O & Q-S and for ehh in fig. 22 J & T). As described above for shh and dnPKA injections, both ptc genes are ectopically induced in the heads of injected embryos, and this is the area of the embryo most often affected by hh over-expression; and in the trunks of more severely affected embryos ptc1 is ectopically expressed and ptc2 expression is suppressed ventrally. When I injected lower concentrations of RNA, I found that I reached a concentration with all of the different hh genes where ptc2 expression is affected more often than ptc1 expression.
**Ectopic Activation of Hh Pathways**

(fig. 23). At these concentrations the effects on both ptc genes are weaker than when hh genes are over-expressed at higher concentrations.

**Brief Summary and Conclusions**

In summary, in this chapter I demonstrate that mis-expression of shh, ehh or twhh, and inhibition of PKA activity, can all induce ectopic expression of both ptc1 and ptc2. However, there is a difference in the response of the two ptc genes to high levels of ectopic Hh signalling in the trunk: ptc1 expression becomes more widespread but ptc2 expression decreases, particularly in the ventral trunk. These results suggest that Hh signals are sufficient for high level expression of both of the ptc genes and that there is no specificity between the activity of particular hh genes and the transcription of the different ptc genes. I will address the question of whether Hh signals are required for high level expression of either ptc gene in chapters seven and eight, and I will discuss the results presented in this chapter in more detail in chapter nine.

**Figure 22: ptc1 and ptc2 expression after ectopic expression of hh RNA**

Transcripts were revealed by in situ hybridisation with antisense RNA probes to ptc1 (A-J) or ptc2 (K-T). (B, C, E, G - I, L, M, O, Q - S) were injected with shh RNA and (J & T) were injected with ehh at the 1-4 cell stage and then fixed at 24 hpf or 10 hpf (E & O). (A, D, F, N, K & P) show control uninjected embryos that were processed in parallel. Both ptc1 and ptc2 are ectopically expressed 10 hours after hh injection (E & O). 24 hours after hh injection both ptc genes are ectopically expressed in the brains of injected embryos (B, C, G-J, L, M, & Q-T); ptc1 is also ectopically expressed in the trunks of some embryos (B shows an example of strong ectopic expression and C shows an example of weaker ectopic expression); but in contrast ptc2 expression is lost from the ventral trunks of severely affected embryos (L).
Ectopic Activation of Hh Pathways

Figure 23: Comparing the effects on ptc expression of over-expressing different hh genes.

Percentage of embryos showing ectopic expression of ptc1 or ptc2 at 24 hpf, after injection of synthetic hh RNA at bud - 4 cell stage.

Injections were performed at a variety of concentrations but each individual experimental replicate used a constant concentration. However, the severity of the resulting phenotype varied between embryos in each replicate, probably due to slight differences in the volume of RNA injected and/or in the cells which inherited the RNA. Therefore in each experimental replicate embryos were examined at 24 hpf and divided into two equal pools: embryos with similar phenotypes were divided equally between the pools. in situ hybridisation was done on one pool using ptc1 as a probe and on the other pool with ptc2. Two main categories of result were observed: injections where most embryos showed ectopic expression of ptc genes; and injections where lower numbers of embryos showed ectopic expression, and more embryos showed ectopic expression of ptc2 than of ptc1. Total results for each of these categories are given. All of the total results for the lower concentration category show a significant difference between ptc1 and ptc2 (p<0.001 for the hypothesis that there is no difference between ptc1 and ptc2, using the X² test). The last column gives the results from individual experimental replicates as ratios of (embryos with ectopic expression) / (total number of injected embryos). On each line the first ratio shows the number of embryos that showed ectopic ptc1, and the second ratio gives the results for ptc2.
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<th>total percentage of embryos with ectopic ptc2</th>
<th>individual experimental results</th>
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<td>42.6% (n=108)</td>
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<td>55% (n=161)</td>
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Figure 23
CHAPTER SIX

ECTOPIC EXPRESSION OF ptc1
REPRESSIONS HH SIGNALLING

Background

As well as being transcriptionally regulated by Hh signalling activity, Ptc activity normally represses the Hh pathway in the absence of Hh signals and overexpression of very high levels of ptc RNA in Drosophila can suppress the response of cells to Hh signalling (Johnson, R. L. et al. 1995; Schuske, K. et al. 1994). I was interested in establishing if zebrafish ptc1 could also repress Hh signalling so I cloned the full length ptc1 cDNA clone that we had obtained from the Kai Zinn library into an expression vector and removed excess lambda sequence as described in chapter two. I then injected very high concentrations of ptc1 RNA into zebrafish embryos at the 1-4 cell stage and examined the embryos for resulting phenotypes. Unfortunately I was unable to obtain a full length construct for ptc1 and therefore I was unable to extend this over-expression analysis to ptc2.

Results

At 24 hpf embryos injected with ptc1 showed a variety of morphological phenotypes. Some embryos had u shaped somites and patches of floor plate that were less distinct than normal (fig. 24B, H &F), some had smaller eyes and a few had eyes that were closer together or even fused (fig. 24D). in situ hybridisations with pax-b (pax-2.1) on embryos with morphologically fused eyes confirmed that the normally separate domains of high level pax-b expression in the optic stalks had also fused, in a similar manner to that reported for cyclops embryos (Krauss, S. et al. 1991a; Krauss, S. et al. 1991b; Hatta, K. et al. 1994; fig. 24 I-N). in situ hybridisations with fkd-4, a gene that is normally expressed in both medial and lateral floor plate cells (Odenthal, J. and Nusslein-Volhard, C. 1998), showed that expression was reduced in injected embryos (fig. 24 O & P).

As several embryos had u shaped somites I examined the somite phenotype of injected embryos in more detail. At early somitogenesis stages (3-5 somites) myoD is normally expressed at high levels in adaxial cells (Weinberg, E. S. et al. 1996) but myoD expression was variably reduced in the adaxial cells of ptc1 injected embryos. Out of 69 injected embryos that had all undergone gastrulation properly and had wild type krox20
expression in the hindbrain (Oxtoby, E. and Jowett, T. 1993), 16 had normal myoD expression and 53 had reduced myoD expression in the adaxial cells. This ranged from a very slight reduction or patchy expression on one side of the embryo to complete loss of adaxial expression on both sides, with only tail bud expression remaining (cf. fig. 25 E-H, J, L & M). Several embryos were only, or predominantly, affected on one side (e.g. see fig. 25 G, H & L). In addition, at 24 hpf 11/11 embryos had reduced numbers of muscle pioneers. Again the severity of the effect varied from an embryo that was just missing muscle pioneers in 3 somites on one side, to an embryo that had no muscle pioneers on one side of the embryo and just three short stretches of about 3 somites each that had muscle pioneers on the other side (fig. 25 O & Q).

**Brief summary and conclusions**

In summary, the analysis presented in this chapter suggests that high levels of Ptc1 activity can suppress the response of cells to Hh signals, in at least the ventral neural tube, somites and brain; suggesting that this function of Ptc has been conserved from *Drosophila* to vertebrates. In addition, the severity of some of the phenotypes suggests that Ptc1 can inhibit all Hh signals, and I will discuss this in greater detail in chapter nine.

**Figure 24: Phenotypic effects of over-expressing ptc1**

(A-H) show live embryos at 24 hpf: (B, D, F & H) were injected with *ptc1* RNA at the 1-4 cell stage and (A, C, E & G) are uninjected siblings. (B & H) show u shaped somites, the embryo injected with *ptc1* (H) has u shaped somites, reminiscent of the u mutants (see chapter eight), whereas the uninjected sibling (G) has chevron shaped somites; the arrow in (F) points to a region where the floor plate is indistinct; and (D) shows an embryo with fused/cyclopic eyes.

(I - N) show expression of *pax-b* at 24 hpf, revealed by *in situ* hybridisation with an antisense probe. (J, K, M & N) were injected with *ptc1* RNA at the 1-4 cell stage and (I & L) are uninjected siblings.

(O & P) show expression of *fkd-4* at 24 hpf, revealed by *in situ* hybridisation with an antisense probe. (P) was injected with *ptc1* RNA at the 1-4 cell stage and (O) is an uninjected sibling.
*ptc1* overexpression

**Figure 25: Over expressing *ptc1* reduces slow muscle cell types**

Embryos were subjected to *in situ* hybridisation with antisense RNA probes to *MyoD* (A - M) or immunolocalisation with 4D9 antibody to visualise Eng. expressing muscle pioneer cells (N - Q).

(B, E - H, J, L, M, O & Q) show embryos that were injected with *ptc1* RNA and (A, C, D, I, K, N & P) show uninjected siblings that were processed in parallel. (B) shows a number of different injected embryos to demonstrate the range of phenotypes produced, but also compare the individual whole mounts (E - H) and the different dorsal flat preps (J, L, & M). These show examples of the different extents of reduction of adaxial *MyoD* expression that are produced by over-expressing *ptc1*. These vary from almost total loss of adaxial *MyoD* expression (F & M) to just partial loss in some adaxial cells on one side of the injected embryo (J). (G, H J & L) are affected on just one side of the embryo, presumably because the *ptc1* RNA was only inherited by cells that eventually formed that side of the embryo.

(N & O) show lateral views and (P & Q) show dorsal views of muscle pioneers. (O) shows a lateral view of an embryo that has lost all of its muscle pioneers on one side, though very weak out of focus staining indicates that there are a few muscle pioneer cells on the other side of the embryo. (Q) shows a dorsal view of an embryo that has normal muscle pioneers on one side and complete lack of muscle pioneers on the other side. In both of these cases uninjected siblings are shown for comparison (N & P respectively).
Chapter Seven

Analysis of ptc Expression in cyclops and floating head

Background

As discussed in chapter one, experimental analysis in Drosophila has established that ptc expression is directly regulated by Hh signalling. When we first cloned ptc1 and ptc2 in Zebrafish we wanted to establish whether vertebrate ptc expression is also regulated by Hh signalling. As already described in chapter 4, ptc genes are expressed in embryonic regions that are known to respond to Hh signals and as I showed in chapter 5, overexpression of hh genes is sufficient to induce ectopic transcription of ptc genes. We also wanted to establish whether high level expression of ptc1 and ptc2 required Hh signalling. When we started these investigations there was no available mutation in any of the hh genes. Since then a mutation has been isolated in shh, syu, and I will discuss this mutation in the next chapter. However, there is still no known mutation in either twhh or ehh, and as the expression patterns of twhh and ehh overlap that of shh, this means that even in the syu mutation there is still some Hh signalling in most regions of the embryo. Therefore, I examined the expression of ptc1 and ptc2 in mutations that are missing all Hh activity in particular regions of the embryo. Embryos homozygous for cyclops (cyc) or floating head (flh) lack expression of all of the characterised zebrafish hh genes, and/or express certain subsets of hh genes, at different positions along the rostro-caudal axis of the embryo, at particular times of development. I examined the expression of ptc1 and ptc2 in these mutants to establish if the high level expression of either of these genes requires Hh activity; and to investigate the spatial relationship between discrete sources of Hh activity and ptc1 and ptc2 expression. The initial analysis of ptc1 expression in cyc and flh homozygotes was conducted by both myself and Jean Paul Concordet (Concordet, J.-P. et al. 1996) but the analysis of ptc2 expression in these mutants is all my own work, and all of the in situ and photographs shown here, except for (fig. 27 D, F, K & M), are my sole work.

cyclops

Mutation of the cyc gene disrupts the specification of the prechordal plate mesoderm and concomitantly the induction of the overlying neurectoderm in the brain, and this produces
**cyclops and floating head**

A cyclopic phenotype. *cyc* mutants also have delayed development of the floor plate in the trunk (Hatta, K. et al. 1991; Hatta, K. et al. 1994; Thisse, C. et al. 1994; Strähle U. et al. 1997). The *cyc* locus has recently been cloned and is a *nodal* related gene (Sampath, K. et al. 1998; Regbagliati, M. R. et al. 1998).

The disruption of prechordal plate formation in homozygous *cyc* embryos results in a complete loss of *shh* and of *twhh* expression in the brain, with the exception of a small stripe of *shh* expression that extends dorso-ventrally in the diencephalon at about 24 hpf and a very small dot of *shh* expression in the very anterior of the head (fig. 26 E). At this stage a ventral patch of *twhh* and *shh* expression also forms posterior and ventral to this stripe in the presumptive gut (fig. 26 E & F). However, in the hindbrain and the trunk the expression of *shh* and *ehh* in the notochord is normal but there is no expression of *shh* or *twhh* in the ventral neural tube, until the floor plate finally forms. At 24 hpf most of the wild type expression of *ptc2* and *ptcl* is missing from the forebrain and midbrain of homozygous *cyc* embryos (fig. 26 cf. G to C & H to D). The remaining expression of the *ptc* genes correlates closely with the remaining *hh* expression: *ptc2* and *ptcl* are only expressed around the dorsal ventral stripe of *shh* expression and the region of the developing gut, where *twhh* and *shh* are expressed (fig. 26 G & H). The hindbrain expression of both *ptc* genes is also reduced, though it is still present. However *ptc2* and *ptcl* are both still expressed at normal levels in the trunk.

**Figure 26: ptc expression in cyclops homozygotes**

Transcripts were revealed by in situ hybridisation with antisense RNA probes to *shh* (A & E); *twhh* (B & F); *ptc2* (C & G) and *ptcl* (D & H).

(E - H) show expression in *cyclops* homozygotes and (A - D) show expression in wild type siblings processed in parallel. All embryos were fixed at 24 hpf. Expression of both *ptc* genes is lost from all the areas of the anterior brain except for regions around the cells now expressing *shh* and/or *twhh*.
Figure 26
**cyclops and floating head**

**floating head**

*flh* is a mutation in the zebrafish homologue of *Xnot*, a homeobox gene which is expressed in the embryonic shield and the notochord, and the main phenotype of *flh* homozygotes is a disruption of notochord specification (Talbot, W. S. et al. 1995). Cells that would normally form notochord transiently express both notochord and muscle markers and then are mis-specified as muscle (Halpern, M. et al. 1995). This leads to a premature loss of *shh* expression from the axial mesoderm posterior to the midbrain-hindbrain boundary. In contrast to *shh*, which is lost gradually from the midline but is initially expressed, *ehh* is never expressed in *flh* (Currie, P. D. and Ingham, P. W. 1996). The expression of *hh* genes in the ventral neural tube in the trunk is also affected. In the anterior trunk a continuous floor plate is still induced and expression of *shh* and *twhh* is normal. However, in the posterior trunk the development of the floor plate is disturbed and instead of a continuous line of floor plate cells, small discontinuous groups of floor plate-like cells develop. These cells express both *shh* and *twhh*. In contrast to homozygous *cyc* embryos, expression of *shh* and *twhh* is normal in the midbrain and forebrain of homozygous *flh* embryos.

I analysed *ptc* expression in *flh* homozygotes to establish whether *ptc1* or *ptc2* expression in the trunk requires *ehh* and to compare how the expression of *ptc2* and *ptc1* relates to discrete sources of *twhh* and *shh* activity. As in *cyc* homozygotes, the expression of *ptc* genes correlates with the expression of *hh* genes in *flh* homozygotes. *ptc1* and *ptc2* are expressed normally in the brain (fig. 27 B & F), but at 24 hpf, expression of both genes is substantially reduced in the trunk (fig. 27 B, F, H & M). In the caudal region of the trunk, *ptc1* and *ptc2* are mainly expressed in small groups of neural cells dorsal to those expressing *shh* and *twhh*, with the *ptc2* expression extending slightly more dorsally from the *hh* expressing cells than the *ptc1* expression, mirroring the more dorsal expression of *ptc2* in the wild type neural tube. In the rostral trunk where there is a continuous stretch of floor plate, *ptc2* and *ptc1* are expressed in the ventral neural tube and *ptc2* is also expressed more dorsally in the neural tube. However, there is no expression of either *ptc* gene in the anterior somites, apart from maybe a few mesodermal cells that are in contact with the *shh* and *twhh* expressing cells in the neural tube. Weak expression of *ptc2* is however sometimes seen in posterior / tail somites of the embryo.
Brief summary and Conclusions

In summary, the analysis presented in this chapter suggests that Hh signals are required for high level expression of both \textit{p\textit{tcl}} and \textit{p\textit{tc2}}. In addition the expression of \textit{p\textit{tc2}} in the trunks of \textit{flh} homozygotes suggests that Hh signals can regulate the transcription of \textit{p\textit{tc2}} over a longer distance than \textit{p\textit{tc1}}, which is also consistent with the wild type expression pattern of the two genes described in chapter four. I will discuss all of these issues in more detail in chapter nine.

\textbf{Figure 27: \textit{p\textit{tc}} expression in \textit{flh} homozygotes}

Transcripts were revealed by \textit{in situ} hybridisation with antisense RNA probes to \textit{shh} (C, D, J & K); \textit{shh + p\textit{tc1}} (N); \textit{shh + p\textit{tc2}} (I); \textit{p\textit{tc2}} (A, B, G & H) and \textit{p\textit{tc1}} (E, F, L & M). Where \textit{shh} and \textit{p\textit{tc}} expression are visualised simultaneously, \textit{shh} expression is visualised in red and \textit{p\textit{tc}} expression in blue.

(B, D, F, H, I, K, M & N) show expression in \textit{flh} homozygotes and (A, C, E, G, J and L) show expression in wild type siblings processed in parallel. (A-F) show whole mount lateral views, and the trunk above the yolk extension, at 24 hpf. Expression of both \textit{p\textit{tc}} genes is lost from the somites and from most of the caudal trunk apart from around the groups of floor plate like cells that still express \textit{shh} and \textit{t\textit{whh}}. In addition \textit{p\textit{tc2}} expression extends more dorsally in the neural tube from these groups of cells than \textit{p\textit{tc1}} expression does (cf. I & N).
C H A P T E R  E I G H T


Background

As mentioned in the previous chapter, when I started this PhD there were no identified mutations in any of the hh genes. However large scale screens for mutations affecting zebrafish embryonic patterning were underway in Tübingen and in Boston (Driever, W. et al. 1996; Haffter, P. et al. 1996). As hh genes had been implicated in the patterning of the somites and neural tube in zebrafish as well as in other vertebrates (Chiang, C. et al. 1996; Krauss, S. et al. 1993; Ruiz i Altaba, A. et al. 1993; Echelard, Y. et al. 1993; Johnson, R. L. et al. 1994; Fan, C.-M. and Tessier-Lavigne, M. 1994; Fan, C.-M. et al. 1995; Barth, K. A. and Wilson, S. 1995; Macdonald, R. et al. 1995; Munsterberg, A. et al. 1995; Roelink, H. et al. 1995; Marti, E. et al. 1995), mutations that affected the development of either of these two embryonic structures were possible candidates for mutations in components of Hh pathways. I investigated the expression of ptc1 in a number of different mutations that had been isolated in the Tübingen screen to try to identify potential Hh pathway mutations. This was a collaboration with Pascal Haffter's laboratory at the Max Planck Institute in Tübingen and involved me visiting Tübingen twice to collect embryos from a variety of different mutant lines, some of which I also established in our own facility. In general, I examined mutations that affected the development of either the floor plate or the somites, and in particular I investigated the class of mutations initially called the u mutants, that all affected the morphology of the myotome; one of which was eventually identified as a mutation in shh (van Eeden, F. et al. 1996; Schauerte, H. E. et al. 1998). In this chapter I will first of all introduce the u mutants and two floor plate mutants which share some phenotypic characteristics with the u mutants and which I found affected ptc1 expression: iguana (igu) and detour (dtr). Then I will describe my analysis of ptc1 and hh expression, and of the myotome phenotype in these mutants, and a comparative analysis of ptc1 and shh expression in two different alleles of syu. This will be followed by my analysis of ptc2 expression in these mutants and of ptc1 and ptc2 expression in the fin buds of these mutants. Finally I will describe my analysis of other floor plate mutants that did not alter ptc1 expression, and of other mutants that I investigated because I thought that they might affect Hh
Analysis of zebrafish mutations

signalling. Where I indicate numbers in square brackets these refer to the number of identified mutants over the total progeny of a cross between heterozygous parents. Occasionally I indicate the total numbers of identified mutants and identified wild type sibs, where known numbers of each were examined. Apart from where indicated to the contrary the results and analysis described in this chapter are my sole work.

A. Analysing syu, con, you, yot, ubo, igu, dtr

Introduction

Mutant screens in zebrafish have now identified a number of genes that are required for correct patterning of the somites, and in particular the shape of the myotome, which constitutes the majority of the somite in zebrafish. Instead of forming wild type chevron shaped (v on its side) somites, embryos homozygous for these mutations have somites which are more curved or u shaped and do not form a normal horizontal myoseptum (for example see fig. 24 G & H in chapter 6; for a more subtle example see fig. 28E). These mutations fall into two major classes, those that affect correct notochord formation, some of which had been identified in earlier screens (momo, notail, floating head, doc, bozozok and dino)(Stemple, D. et al. 1996; Odenthal, J. et al. 1996; Halpern, M. E. et al. 1993; Talbot, W. S. et al. 1995; Hammerschmidt, M. et al. 1996; Blagden, C. S. et al. 1997) and those that have apparently normal notochords suggesting that they may be components of signalling pathways from the notochord that pattern the somites (the "u" mutants: sonic you (syu), chameleon (con), you, you-too (yot) and uboot (ubo))(van Eeden, F. et al. 1996). In agreement with this, mosaic analysis established that the products of two of these genes, yot and ubo, are required in the somites (van Eeden, F. et al. 1996), suggesting that they act downstream of notochord derived signals, and more recently one of the other mutations in this class, syu, was identified as a mutation in shh (Schauerte, H. E. et al. 1998). The similarity of the somite phenotypes (most of the mutations have defects in muscle pioneer cell (MP) formation and in adaxial expression of the myogenic gene MyoD) suggested that the other u mutants might be part of the same signalling pathway as syu. However, other aspects of the phenotypes of these mutants differ suggesting that the genes might act in different pathways. For example yot and con differ from syu, you and ubo in that they have a rectinotectal phenotype and a variably reduced spacing of the eyes and they were also recovered independently in a separate rectinotectal path finding screen; and syu and con homozygotes have specifically reduced pectoral fins whereas in ubo all the fins are irregularly indented and yot and you by
Analysis of zebrafish mutations

contrast show no obvious fin phenotype (van Eeden, F. et al. 1996; van Eeden, F. et al. 1996b; Karlstrom, R. O. et al. 1996). I therefore examined the expression of ptc1 in these mutants to identify which ones, if any, were potential components of the Hh signalling pathway. We also analysed the myotome phenotype of these mutants in more depth to see if it correlated with the expression of ptc1 and was consistent with Hh signals patterning the myotome. As previously discussed in chapter one Hh signalling has been implicated in the induction of both adaxial cells (slow muscle precursors) and MPs in zebrafish (Weinberg, E. S. et al. 1996; Concordet, J.-P. et al. 1996; Currie, P. D. and Ingham, P. W. 1996; Blagden, C. S. et al. 1997; Du, S. J. et al. 1997).

However, Zebrafish express two hh genes in the notochord: ehh and shh, and while previous analysis of mutations with reduced Hh signalling in the midline had suggested that ehh is not required for slow muscle formation, and that shh is primarily responsible for the induction of this cell fate, we were unable to conclusively test this hypothesis without a mutation that specifically removes Shh activity (Currie, P. D. and Ingham, P. W. 1996; Halpern, M. E. et al. 1993; Weinberg, E. S. et al. 1996; Concordet, J.-P. et al. 1996; Blagden, C. S. et al. 1997; Du, S. J. et al. 1997).

I also identified two mutations that had been characterised as having late floor plate, curly tail and ipsilaterial rectinotectal path finding defects, as affecting ptc1 expression: iguana and detour (Brand, M. et al. 1996; Karlstrom, R. O. et al. 1996). I therefore also examined these mutations in more detail and investigated whether they had any myotome defects. These two mutations will also be described in this section. iguana and yot also share an additional phenotypic characteristic in that embryos homozygous for either mutation sometimes form an ectopic lens in the place of the mouth (fig. 28F).

Figure 28: The phenotype of iguana homozygotes

This figure was kindly provided by Pascal Haafter. It illustrates the phenotype of iguana homozygotes, aspects of which are also shared with many of the u mutations. For example igu homozygotes sometimes form an ectopic lens in the position of the mouth - shown with an arrow in (F), and this is also the case with yot homozygotes; igu homozygotes have a curved "curly tail down" body shape (B) as do most of the u mutants; and igu homozygotes have a slightly u shaped somites (E) which resembles a weak u type phenotype. igu homozygotes also have an ipsilateral pathfinding defect as do dtr, con and yot homozygotes and the eyes are turned inward posteriorly, again like in embryos homozygous for dtr, con and yot.

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RESULTS

ptc1 expression at late somitogenesis stages

Initially, I examined the expression of ptc1 towards the end of somitogenesis, generally at 18-20 somites or at 24 hpf, to determine whether any of these mutations altered Hh signalling. Consistent with ptc1 being a primary target of Shh activity embryos homozygous for syu have a dramatic reduction in ptc1 expression: at 18-20 somites transcript is eliminated from the somites and only very weak expression in the ventral neural tube and ventral brain remains [16/54] (fig. 29B).

ptc1 expression is also dramatically reduced in embryos homozygous for yot. At the 18-20 somite stage the reduction is most severe in the trunk where only very weak ventral neural tube expression remains [9/40] (fig. 29D). However, ptc1 is still expressed at higher, though still reduced, levels in the heads of homozygous yot embryos. Amongst the siblings of yot homozygotes, approximately two thirds exhibit a less pronounced reduction in ptc1 expression [24/40] (fig. 29C), consistent with the previous finding that this yot mutation is semi-dominant (van Eeden, F. et al. 1996).

Embryos homozygous for con [29/113] also show a significant effect on ptc1 expression with very weak, if any, expression detectable in the somites and greatly reduced levels of transcript in the ventral neural tube (fig. 29F & J cf. E & G). However, the head expression of ptc1 is, like in yot homozygotes, more substantial than the trunk expression though the levels of transcript are still lower than in wild type sibling embryos.

The effects of you on ptc1 expression are more subtle. ptc1 expression is reduced but to much less of an extent than in con, yot, or syu and the reduction of transcript levels is most obvious in the somites, though the ventral neural tube expression is also slightly reduced (fig. 29 cf. H to K & I to L) [12/50; and 25/25 mutant vs. 24 wt]. This is consistent with the previous finding that embryos homozygous for you<sup>y97a</sup> have a relatively weak phenotype (van Eeden, F. et al. 1996).

In contrast to the other mutations ptc1 is ectopically expressed in igu homozygotes and in the trunk the ectopic expression is of a similar level to the wild type expression [39/138] (fig. 29P). In igu homozygotes ptc1 is expressed throughout the dorsal ventral and the medial-lateral extents of the somites, and strong neural tube expression extends more dorsally than in wild type embryos (fig. 29X-a). ptc1 is also ectopically expressed in the
Analysis of zebrafish mutations

heads, most noticeable in the optic stalks and lenses (fig. 29V & W). However, this head expression of ptc1 is weaker than wild type expression at this stage.

Homozygous detour embryos have slightly weaker expression of ptc1 in the somites, the neural tube and the head. In addition, the domain of expression in the neural tube is expanded dorsally in a manner similar to that observed in igu homozygotes. This phenotype was observed in all three alleles of dtr examined. \([dtr^{ts269} - 17/56; dtr^{te370a} - 14/53; dtr^{tm276b} - 12/61]\) (fig. 29Q-U).

Unlike the other mutations discussed above the expression of ptc1 was unaffected in embryos homozygous for ubo (fig. 29N).
**Analysis of zebrafish mutations**

**Figure 29: Expression of \( ptc1 \) in wild-type and mutant embryogenesis at late somitogenesis stages**

Transcripts were revealed by *in situ* hybridisation with antisense RNA probes to \( ptc1 \) and in addition immunolocalisation for Engrailed proteins with the antibody 4D9 (brown staining) was used in (E, F, G & J) to confirm the genotype of embryos. (A-G & J) show embryos at 18-20 somites; (H, I & K - a) show embryos at 22-24 hpf. The arrows in E and G point to MPs stained with 4D9 and the square brackets in E and F show the midbrain / hindbrain region that also expresses Engrailed proteins.

(A & E) wild-type expression of \( ptc1 \); (E) is a sib of the \( con \) homozygote in (F)

(B) syu (D) yot and (F) \( con \) homozygotes: note strong reduction in \( ptc1 \) expression. (C) yot heterozygote: note weak reduction in \( ptc1 \) expression

(G & J) detail of (E & F) note the correlation between loss of \( ptc1 \) expression and loss of MPs.

Lateral and dorsal views of wild-type (H & I) and \( you \) homozygous embryos (K, M & L) showing reduction in expression of \( ptc1 \) in somites and neural tube.

(N) \( ubo \) homozygote showing normal expression of \( ptc1 \).

(P) \( igu \) homozygote showing expansion of \( ptc1 \) expression in the somites compared to wild-type (O). The dorsal and ventral expansion is shown at higher magnification in a lateral view of the trunk in (Y) - compare with wild type in (X); note also the dorsal expansion of expression in the neural tube (Y); and the ectopic expression of \( ptc1 \) in the optic stalks and lenses (W) - compare with wild type in (V);

(a) dorsal view of an \( igu \) homozygote showing lateral expansion of \( ptc1 \) expression in each somite; compare with restriction to medial somite in wild type embryos (Z);

(Q) homozygous \( dtr \) embryo showing slightly reduced expression of \( ptc1 \) throughout the somites and neural tube, seen more clearly in (S) cf. (R) and (U) cf. (T). Note the dorsal expansion of expression in the neural tube in (U) cf. (T).
Figure 29
Analysis of zebrafish mutations

The expression of hh genes in con and you and igu

These results implicated all of these mutations except ubo in Hh signalling. However, it was possible that one or more of these mutations could, like syu, be a mutation in a hh gene or could be a mutation in an upstream regulator of hh gene expression. yot and ubo are unlikely to be either of these things as they are both required in the somites (van Eeden, F. et al. 1996). However the phenotypes of con, you or igu could be due to a reduction of hh transcripts. Therefore I investigated the expression of hh genes in con, you and igu. twhh, ehh and shh are expressed normally in you and con homozygotes (fig. 30D-G - data shown for shh in con) and there is no reduction or increase of hh expression in igu. However, there is a slight but consistent difference in the shape of shh expression in the brain of igu homozygotes at 24 hours of development, presumably due to differences in the brain morphology of mutant embryos [27/92]. The peak of expression of shh that normally rises from the ventral floor of the brain towards the epiphysis is more perpendicular in mutant embryos (fig. 30 H-K).

Genetic mapping of these mutations relative to the different hh genes

Heike Schauerte and Pascal Haffter also investigated whether any of these mutations were genetically linked to any of the characterised hh genes. They found that con, dtr and you do not map close to ehh, twhh or shh but igu maps very close to ehh. However they cloned and sequenced the coding sequence for ehh from igu homozygotes and did not find any mutations. They have also more recently separated igu and ehh on a radiation hybrid map (personal information Schauerte and Haffter).
**Analysis of zebrafish mutations**

**Figure 30: hh expression in wt and mutant embryos**

Transcripts were revealed by *in situ* hybridisation with antisense RNA probes to *shh* (A, D-K); *ehh* (B) and *twhh* (C). In addition (D-G) were subjected to immunolocalisation for Engrailed proteins using the antibody 4D9 (visualised in brown). (A-G) show embryos at 18-20 somites and (H-K) show embryos at 22-26 hpf.

(A, B & C) show the wild type expression of *shh*, *ehh* and *twhh* respectively. *ehh* and *twhh* are both expressed in different subsets of the cells that express *shh*: *ehh* is just expressed in the notochord and *twhh* is expressed in the floor plate and ventral brain.

(D-G) show that *shh* is expressed normally in embryos homozygous for *con*. Embryos were processed in the same tube and then identified as mutant or wild type using immunolocalisation for Engrailed proteins to identify wild type embryos that had MPs (D and F - the arrows point to the MPs and the square bracket in (D) shows the midbrain hindbrain region that also expresses Engrailed proteins) and mutant embryos that lacked MPs (C and G). *shh* expression was identical in both classes of embryos.

(H-K) show the slight difference in the shape of *shh* expression that is seen in *igu* homozygotes (I and K): the peak of *shh* expression that normally extends dorsally to the epithesis (indicated with a * in (K)) is slightly more perpendicular, presumably due to changes in the morphology of the brain.
Figure 30
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**ptcl expression in these mutants is dynamic**

I also examined *ptcl* expression in these mutants at other stages and it quickly became apparent that in most of these mutations *ptcl* expression was dynamic, so I investigated this in more detail.

*ptcl* expression is not reduced in *ubo*, *you*, *con*, or *suy* homozygotes at the end of gastrulation (tail-bud stage) (fig. 31A, C, E, G). However by about 10 somites the adaxial cell expression of *ptcl* expression is reduced in homozygous *con*, *you* and *suy* embryos (fig. 32D, F, J), though there is still hardly any difference in the head expression (fig. 32 O, Q & N) [*you* 10-12 som 18/78; *con* 7-8 som 17/77, 12-13 som 11/54; *suy* 9-10 som 8/32]

In contrast to *you*, *con* and *suy* *ptcl* expression is already reduced in *yot* homozygotes [63/259] at the end of gastrulation. *ptcl* is still expressed in the adaxial cells but the expression is much weaker than wild type (fig. 31L). This reduction persists throughout somitogenesis. By 10 somites the adaxial expression of *ptcl* in *yot* homozygotes has been eliminated [27/115, fig. 32L] though there is still weak ventral neural tube expression and more substantial expression in the ventral midline of the presumptive brain (fig. 32 R).

In *igu* homozygotes, *ptcl* expression is also already reduced at the end of gastrulation but it is also expanded into the pre-somatic mesoderm [13/49] (fig. 31 J). This expanded expression persists and increases in level throughout somitogenesis: At 10-12 somites *ptcl* expression is expanded throughout the somites but it is still expressed at higher levels in the adaxial cells than in the more lateral somitic cells [6-7 somites 14/67; 9-12 somites 17/80] (fig. 31 H & P); and by 24 hours, as described in the previous section, the ectopic *ptcl* expression in the trunk is as strong as the wild type *ptcl* expression.

I also examined *ptcl* expression at later stages and found that at 32 hpf, while the levels of *ptcl* transcript were still severely reduced in the trunks of mutant embryos, there was still substantial expression of *ptcl* in the heads of embryos homozygous for *con*, *yot* or *suy*. In *you* homozygotes the head expression of *ptcl* is not obviously reduced at this or earlier stages (fig. 33D). However, at 32 hpf the expression of *ptcl* in the heads of *yot* homozygotes also appears normal (fig. 33 E & L). In *suy* and *con* homozygotes *ptcl* expression is reduced, but this reduction is more obvious in particular subsections of the brain: in *suy* homozygotes *ptcl* expression is reduced in the forebrain but is still quite strong (though weaker than in wild type embryos) in the mid and hindbrain (fig. 33 B &
Analysis of zebrafish mutations

G) and in con homozygotes ptc1 expression is reduced in the hindbrain (fig. 33 C & J). I will discuss the expression of ptc1 in the fin buds at this stage later in this chapter.

Characterising the muscle phenotypes

As many of these mutations had already been shown to affect the development of the muscle pioneer (MP) cells and Hh signals had been implicated in the patterning of the zebrafish myotome, I examined the expression of myoD and the MP phenotype in all of these mutations and P. Currie analysed the distribution of slow and fast myosin isoforms (discussed in chapter nine).

Muscle Pioneer Cells

yot and syutd homozygotes have the most severe MP phenotype of the u mutants as they both completely lack MPs (van Eeden, F. et al. 1996; Schauerte, H. E. et al. 1998; my results shown here: fig. 34 H & I). con and you also have a severe reduction in MPs but they sometimes have a small number of MPs in some anterior somites (usually just one MP per somite in a few somites, sometimes just on one side of the embryo) (van Eeden, F. et al. 1996; my results shown here: fig. 34 D-G)

In ubo homozygotes I found that the MPs that strongly express Engrailed proteins are absent but unlike in yot, syu, con or you there are still a group of cells in each somite that express weaker levels of Engrailed proteins [12/12 mutant cf. 21 wild type embryos]. This group / cloud of weaker expressing cells is also seen in wild type embryos around the MPs, especially in anterior somites (fig. 34 M cf. 1A & B). (A similar description of the ubo MP phenotype can be found in van Eeden, F. 1997).

Embryos homozygous for igu also initially have slightly u shaped somites but later a myoseptum forms. In contrast to the other mutations there is an expansion of MPs in the anterior somites, mainly in the ventral half of the somite, which Engrailed positive cells now extend throughout [20/74] (fig. 34 J). These Engrailed positive cells are also more widely distributed throughout the medial lateral extent of the somites (fig. 34 L). However, in the most posterior and tail somites there is a normal number of MPs (fig. 34 K).

I also investigated the MP phenotype of two different detour alleles - dtrfe370a and dtrm276b but did not see any differences between mutant and wild type embryos.
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**MyoD**

I also examined the expression of myoD in these mutants at various stages and compared it to the expression of ptc1. Previous investigations had already established that myoD is reduced in the adaxial cells of embryos homozygous for yot, con, syu, and yot at late somitogenesis stages but not reduced in ubo homozygotes. The reduction in myoD expression in yot homozygotes is more severe than in the other mutations and can be seen as early as the end of gastrulation (van Eeden, F. et al. 1996; Schauerte, H. E. et al. 1998). In addition I also found no change in myoD expression in ubo homozygotes [18 som - 0/144] or in detour homozygotes [12 somites - 0/47].

However, as with ptc1, when I examined myoD expression in younger embryos I found that there is no reduction of myoD expression in embryos homozygous for con, you, ubo, igu or syu at the end of gastrulation / tail-bud stage [you - 0/82; ubo - 0/86; syu - 0/54; con - 0/33; igu - 0/153] (fig. 31 B, D, F, H, K). (I investigated myoD expression in ubo homozygotes at this stage just incase ubo was only required for early Hh signalling). However, by 10-12 somites the adaxial expression of myoD is reduced in con, you, igu and syu homozygotes [con - 7/41; you - 15/82; syu - 11/45; igu - 6-7 somites 8/38, 9-10 somites 23/93, 13 somites 26/109] (fig. 32 C, E, G, I). The reduction in syu homozygotes is more severe than the reduction in the other mutants and resembles, but is still not quite as severe as, the reduction in yot homozygotes at this stage (fig. 32 K, discussed below).

Unlike the other mutants, yot homozygotes already have reduced expression of myoD by the end of gastrulation [16/82]. There is still strong expression of myoD around the tail-bud / in the most posterior adaxial cells at this stage, but transcript is lost from the rest of the adaxial cells (fig. 31 M). This reduced expression continues throughout somitogenesis. At 10 somites yot homozygotes still have no adaxial expression of myoD, and only very weak expression of myoD remains around the tail bud [12/50] (fig. 32 K).

To check whether the adaxial cells in yot homozygotes ever expressed myoD I also examined myoD expression at 80% epiboly. At this stage all of the embryos expressed myoD in the same two triangular domains either side of the midline [82/82]. There were very slight differences in expression levels due to slight differences in age between the embryos but there was no evidence of a smaller domain of expression in homozygotes.

With all of the mutants at 10 somites there is slightly more expression of myoD just around the tailbud than in the adaxial cells in the pre-somitotic mesoderm (fig. 32). Also, in
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contrast to the adaxial expression, the medio lateral somite expression of myoD is normal in most of the somites, though it is slightly reduced in the most anterior somites (fig. 32 - also described for yot in (van Eeden, F. et al. 1996)).

Establishing when con, you and syu are first required for myoD and ptc1 expression.

To attempt to establish when con, you, and syu are first required for Hh signalling I examined myoD and ptc1 in these 3 mutants at 5-6 somites. At this stage there is a very slight but consistent reduction in ptc1 expression in some embryos (fig. 35 A, C, E, H-J). The numbers were roughly Mendelian for syu and con [syu - 7/36; con 11/52] but were less than Mendelian for you [6/67]. However there is a more noticeable difference in myoD expression at this stage (fig. 35 B, D, F, L - N). In all three mutants myoD expression in the adaxial cells is reduced and slightly patchy and the numbers were roughly Mendelian for all three of the mutants [syu - 16/80; you 15/64; con 14/56].

Figure 31: Expression of ptc1 and MyoD at the end of gastrulation

Transcripts were revealed by in situ hybridisation with antisense RNA probes to ptc1 (A, C, E, G, I, J & L) or MyoD + krox-20 (B, D, H, K, M) or just MyoD (F). Expression of krox-20 provides an accurate independent indication of developmental stage and staining levels in individual embryos.

(A - H and K) show a random selection of embryos from an in situ hybridisation to demonstrate that none of the progeny from syu, you or con heterozygous parents have an altered expression of MyoD or ptc1 and none of the progeny from igu heterozygous parents have an altered expression of MyoD at this stage: (A & B) are from a syu cross; (C & D) are from a con cross; (E & F) are from a you cross (G & H) are from a ubo cross and (K) is from an igu cross.

However, the expression of ptc1 is reduced in igu homozygotes at this stage ((J) cf. wild type expression in (I)); and yot homozygotes have reduced expression of both ptc1 and MyoD (L & M respectively, wild type embryo on the left in (L) and on the right in (M)), but ptc1 is still expressed weakly throughout the adaxial cells, whereas MyoD is only expressed in the tail bud and most posterior adaxial cells.
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**Analysis of zebrafish mutations**

**Figure 32: ptcl and MyoD expression at 10-15 somites.**

Transcripts were revealed by *in situ* hybridisation with antisense RNA probes to *ptcl* (A, C, E, G, I, K & M-R) or *MyoD* (B, D, F, H, J & L). (A, B, M) show expression in wild type embryos at 10-12 somites (B & M) and 15 somites (A).

The expression of *MyoD* is reduced in the adaxial cells of embryos homozygous for *syu*, *con*, *igu*, *you* and *yot* (C, E, G, I, K respectively) at 14-15 somites (*syu* and *con*) or 11-13 somites (*you*, *yot* and *igu*). *yot* and *syu* have the most substantial reduction in *MyoD* expression, there is less of a reduction in *con*, *igu* and especially *you* embryos. In all cases, even *syu* and *yot*, there is persistent expression of *MyoD* in the tailbud / posterior adaxial cells. The lateral somite expression of *MyoD* is also slightly reduced in the most rostral somites, most notably at this stage in *syu*, *con* and *yot*.

At 10-12 somites *ptcl* expression is also reduced in the adaxial cells of embryos homozygous for *syu*, *con*, *you* and *yot* (D, F, J, L respectively) but in contrast *ptcl* expression is expanded in the somites and pre-somatic mesoderm of *igu* homozygotes (H). The most dramatic loss of *ptcl* expression is in embryos homozygous for *yot* which have almost no adaxial expression of *ptcl* at this stage, though there is still weak neural tube expression (see R).

(O - R) show whole embryo dorsal flat prep views of *ptcl* expression in *con*, *you*, *syu*, *igu*, and *yot* homozygotes respectively, at the same stages as the trunk views shown above. These show that in *con*, *you*, *syu* and *yot* there is more considerable expression of *ptcl* in the heads of mutant embryos than in the trunks. This is particularly striking in *syu* homozygotes (N) and *yot* homozygotes (R).
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**Figure 33: ptc1 expression in syu, con, you, and yot at 32-34 hpf**

Transcripts were revealed by *in situ* hybridisation with antisense RNA probes to *ptc1* on embryos at 32-34 hpf. (A-E) show flat preps of *ptc1* expression in the head, whereas (F & G) show *ptc1* expression in the whole embryo and (H-M) compare expression in the head (H, J, L) with expression in the trunk (I, K, M). (A, F, H and I) show *ptc1* expression in wild type embryos for comparison with the expression in homozygous mutant embryos.

(B & G) show that *ptc1* expression is strongly reduced in *syu* homozygotes at this stage, most noticeably in the trunk where it is only expressed in the ventral neural tube. The head expression of *ptc1* is more considerable than the trunk expression, but is still reduced, especially in the very anterior of the brain (behind the eye in (G) where there is normally a peak of *ptc1* expression that extends up towards the epithesis, also see (B) where the anterior of the diencephalon is hardly expressing any *ptc1*.

(C, J, And K) show that in *con* homozygotes *ptc1* expression is also reduced more considerably in the trunk than in the brain. However, in *con* homozygotes the anterior expression of *ptc1* in the brain is still strong and it is the hindbrain expression that is more obviously reduced (C & J). In *you* homozygotes (D) and *yot* (E, L) the expression of *ptc1* is normal in the brain, though in *yot* homozygotes *ptc1* expression is still considerably reduced in the trunk in a similar way to *con* and *syu* homozygotes (M).
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Figure 34: Muscle Pioneers in wild type and mutant embryos

(A) lateral view of the trunk region of a wild type embryo at 24 hpf stained with mAb 4D9 to reveal Engrailed expressing MPs. Each somite has a small number (1-5) of cells that express high levels of Engrailed proteins surrounded by a cluster of cells expressing Engrailed at lower levels. These are more clearly seen in a lateral (B) or dorsal (C) view at higher magnification. Note that the MPs are located in the middle of the somite adjacent to the notochord.

(D) lateral view of a you embryo showing the sporadic differentiation of a single MP in some rostral somites.

(E) another example of a you homozygote that completely lacks MPs on one side (though some can be seen out of the plane of focus on the other side of the embryo).

(F) con homozygote that has one MP in each of its rostral somites.

(G) dorsal view of a con homozygote that is devoid of MPs.

No MPs are seen in syu or yot homozygotes (H & I respectively).

(J and L) igu homozygotes have ectopic MPs in rostral somites but a normal number of MPs in caudal somites (K).

Ectopic MPs are mainly located in the ventral half of the somite (J) but extend throughout the medial lateral extent of the somite (L).

(M) ubo homozygotes do not form MPs but have clusters of cells expressing low levels of Engrailed similar to those that surround MPs in wild type embryos: note that this embryo was stained for longer than those shown in (B & C) to reveal the weaker Engrailed staining.

Figure 35: Expression of ptc1 and MyoD in syu, con and you at 5-6 somites

Transcripts were revealed by in situ hybridisation with antisense RNA probes to ptc1 (A, C, E & G - J) or MyoD (B, D, F & K - N). (A - F) show whole mount views of one wild type embryo (on the left) and one mutant embryo (on the right). (G & K) show dorsal flat preps of wild type embryos and (H - J & L - N) show dorsal flat preps of mutant embryos. (A, B, H &L) show syu; (C, D, I & M) show con and (E, F, J & N) show you. All three mutations result in slightly reduced expression of ptc1 and reduced and discontinuous / patchy expression of MyoD in the adaxial cells.
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**Analysing syu/igu double mutant embryos**

Because *igu* and *syu* have different effects on *ptc1* expression I was able to use *ptc1* expression to investigate the epistasis of these two genes, and to determine whether *shh* was required for *ptc1* expression in the absence of *igu*. Heike Schauerte generated double mutants between *syu<sup>q252</sup>* and *igu<sup>s43e</sup>* and between *syu<sup>4</sup>* and *igu<sup>s43e</sup>*. She was unable to identify embryos homozygous for both mutations by morphological criteria, so she fixed embryos at 24 hpf for me to examine *ptc1* expression.

I observed three different expression patterns in the progeny of fish heterozygous for both *igu<sup>s43e</sup>* and *syu<sup>q252</sup>*: wild type; *syu*-like (dramatic reduction of *ptc1* expression especially in the trunk); and *igu*-like (ectopic *ptc1* expression especially in the somites) [148 : 53 : 64] (fig. 36 A, C, E). This suggests that the double mutants have the same expression of *ptc1* as *igu* single mutants (The predicted numbers for this hypothesis would be 149 : 50 : 66 and the $X^2$ test gives $0.8 < p < 0.9$).

I also examined progeny from parents heterozygous for both *igu<sup>s43e</sup>* and *syu<sup>4</sup>*. Again I only identified the same three classes of *ptc1* expression. [61:16: 23 - so for the hypothesis that the double mutants are in the *iguana*-like class 0.5 <p< 0.7] (fig. 36 B, D, E). In this latter case I was able to test my hypothesis that the double mutant embryos were in the *iguana* like class using PCR. Heike Schauerte provided me with primers that amplify a short (about 300 bp) fragment in the 3' UTR of the *shh* gene and Jörg Odenthal provided me with primers that amplify a 570 bp fragment from an unrelated gene (*fkd3*) as a positive control (Odenthal, J. and Nusslein-Volhard, C. 1998; Schauerte, H. E. et al. 1998). I made DNA from all 23 individual embryos in the *iguana* like class and 3 embryos from the *syu* like class as controls. PCR with a mixture of primers amplified the *fkd3* fragment from all of the embryos but the *syu* fragment did not amplify from any of the *syu* like embryos, and only amplified in 17/23 of the *igu* like embryos (fig. 37). This confirmed that the *iguana* like class of embryos contained 6 *syu* homozygotes, which is the number that I would predict to be double mutants and confirms that mutation of *igu* rescues expression of *ptc1* in the absence of Shh activity.
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Figure 36: ptcl expression in syu;igu double mutant embryos

Transcripts were revealed by in situ hybridisation with antisense RNA probes to ptcl on 22 hpf progeny of syu^q252/+; igu/+ parents (A - C) and syu^4/+; igu/+ parents (B - F). In both cases three phenotypic classes of progeny could be identified on the basis of ptcl expression: wild-type (A & B); igu-like i.e. expanded ptcl expression (C & D); and syu-like i.e. substantially reduced ptcl expression (E & F).

Figure 37: PCR identification of syu^4 homozygotes amongst the igu phenotypic class

Individual embryos derived from syu^4/+; igu/+ parents were selected on the basis of their ptcl expression patterns and subjected to PCR amplification: the short fragment amplified from the 3' region of the shh locus is indicated by the long white arrow. The control fragment amplified from the flk3 is indicted by the short white arrow.

Lanes 1 and 21 show a 1 KB DNA ladder; lanes 2, 22 and 28 show the absence of the shh specific band in three syu^4 homozygotes (S) identified by their loss of ptcl expression; lanes 3 - 20 and 23 - 27 show the products obtained from each of the embryos that had expanded ptcl expression (and therefore resembled igu homozygotes). Six of these embryos, indicated with *s, are missing the shh band and therefore correspond to embryos that are homozygous for syu^4.
Figure 37
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A comparative analysis of syu⁴²⁵² and syu⁴

syu⁴²⁵² was the first allele of syu isolated: all the other alleles were identified in a separate allele screen, or in the case of syu⁴ occurred spontaneously (van Eeden, F. et al. 1996; Schauerte, H. E. et al. 1998). When I started to investigate ptc expression in the mutants Heike Schauerte had already shown that syu⁴²⁵² was genetically linked to shh but no mutations had been found in the coding sequence of shh, and Freek van Eeden had shown that shh was still expressed in syu⁴²⁵² homozygotes. However, syu⁴ had also just been shown to be a deletion of the shh locus that did not complement syu²⁵². While Heike Schauerte tried to characterise the molecular basis of the different syu mutations, I examined ptc expression in these two alleles to try to establish the degree to which they affected Hh signalling and whether this differed between the two different mutations. Ideally I would have liked to have compared all of the alleles of syu but lack of time and problems with establishing stocks meant that it was only possible to compare these two alleles.

ptc expression is not reduced in either of these syu alleles at bud stage (fig. 38 B and 31 A) but as somitogenesis progresses ptc expression becomes down-regulated in both alleles, especially in the trunk (fig. 38 D & E). By 9 - 10 somites the expression of ptc is strongly reduced in syu⁴ and syu⁴²⁵² homozygous embryos, but more so in the former. With both alleles the reduction in expression is much more severe in the trunk than in the head or the tail bud, but in particular the expression of ptc in the tail buds of embryos homozygous for syu²⁵² is much more substantial than in embryos homozygous for syu⁴ (fig. 38 D & E).

By 18-20 somites the expression of ptc in syu⁴ embryos is still more substantially reduced than in syu²⁵² embryos, especially in certain regions of the embryo (fig. 38 cf. I to K). For example in embryos homozygous for syu²⁵² there is still reasonably high expression of ptc in the tailbud whereas in embryos homozygous for syu⁴ there is no expression of ptc in the tailbud. The trunk and head expression in embryos homozygous for syu²⁵², while substantially reduced compared to wild type embryos, is still stronger than in embryos homozygous for syu⁴. The ventral neural tube expression is also stronger and there is more evidence of weak expression in the somites (fig. 38 I & K).

The differences in ptc expression between these two alleles can be explained if there is residual activity of shh in the syu²⁵² allele and therefore these results suggest that syu²⁵² is a regulatory allele that makes functional protein. Consistent with this, and

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Unlike in syu^14 homozygotes where shh is never expressed, shh is initially expressed in syu^tq252 mutant embryos at what appears to be normal levels. Embryos at bud stage can not be identified as mutant or wild type on the basis of their expression of shh (fig. 38 A). This continues throughout early somitogenesis and it is only by about 18 somites that a down-regulation of shh expression can be seen in syu^tq252. (However, as ptc1 expression is reduced earlier than this the level of Shh activity probably decreases before this stage). Convincingly, the remaining expression of shh at the 18 - 20 somite stage corresponds to the regions of the embryo where there is more substantial expression of ptc1 in syu^tq252 homozygotes than in syu^14 homozygotes, most notably the tailbud (fig. 38 cf. F to G). This spatial correlation between the reduced expression of ptc1 and the reduction of shh RNA in syu^tq252 suggests that syu^tq252 forms functional Shh protein, but that the levels of the protein are reduced at certain stages, in a similar manner to the RNA. This is consistent with the DNA sequence of the mutation which suggests that it is a point mutation in the untranslated sequence, 226 bp. upstream of the start codon (Schauerte, H. E. et al. 1998).
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Figure 38: Comparing Different Alleles of syu

Transcripts were revealed by in situ hybridisation with antisense RNA probes to ptcI (B-E & H-K) or shh (A, F & G) and in addition immunolocalisation of Engrailed proteins using the 4D9 antibody was undertaken after the in situ hybridisation procedure in (F-I) to confirm the genotype of embryos. (A & B) show embryos at the end of gastrulation; (C-E) show embryos at 10-12 somites and (F-K) show embryos at 18-20 somites.

syu\textsuperscript{t4} is a deletion of the shh locus so shh is never expressed in syu\textsuperscript{t4} homozygous embryos. In contrast shh is expressed normally in syu\textsuperscript{q252} embryos at the end of gastrulation (A) and the levels of expression are only visibly reduced by the 18-20 somite stage (cf. (G) which is a homozygous mutant embryo, confirmed by its lack of 4D9 labelled MPs, to (F) which is a wild type sibling, confirmed by the presence of MPs. Both were processed in the same tube). There is still considerable expression of shh in the heads and tailbud of syu\textsuperscript{q252} homoyzygous embryos at 18-20 somites but the expression in the trunk is substantially reduced (G).

ptcI is expressed normally in syu\textsuperscript{q252} homozygotes at the end of gastrulation (A), but it is also expressed normally at this stage in syu\textsuperscript{t4} homozygotes (fig. 31A). By 10-12 somites ptcI expression is reduced in both syu\textsuperscript{t4} and syu\textsuperscript{q252} homozygotes, most notably in the trunk (E and D respectively and cf. C). This is also the case at 18-20 somites (K & I and cf. J & H respectively). However at both of these stages there is considerably more expression of ptcI around the tailbud of syu\textsuperscript{q252} homozygote embryos than in syu\textsuperscript{t4} embryos. At the 18-20 somite stage there is considerably more expression of ptcI in syu\textsuperscript{q252} homozygotes than syu\textsuperscript{t4} homozygotes, and this remaining expression of ptcI in syu\textsuperscript{q252} homozygotes corresponds to the areas where shh is still being expressed in this mutant (cf. I to G).
Figure 38

18-20 somites *ptc1*

18-20s *shh*

10-12s *ptc1*

*bud suy*^{lq252}
Analysis of zebrafish mutations

ptc2 expression

In contrast to ptc1, ptc2 expression is hardly affected in any of the mutants discussed above, including embryos homozygous for syu

For example, at late somitogenesis stages ptc2 is still expressed throughout its normal expression domain, but its expression is slightly weaker all over in syu

This is also the case for embryos homozygous for con (fig. 40 Q). In embryos homozygous for yot expression of ptc2 is also very slightly weaker, but less so than in embryos homozygous for con or syu

The reduction in ptc2 expression at 24 hpf is subtle, especially in the heads. However, there is no expression of ptc2 in the rostral somites of these embryos (fig. 40 S), which is a more severe somite effect than in embryos homozygous for con or syu

where weak somite expression of ptc2 persists. There are no obvious differences in ptc2 expression in embryos homozygous for you, ubo or detour. In igu homozygotes the only obvious difference in ptc2 expression by 24 hpf is a very slight difference in the pattern of expression in the heads [29/96] (fig. 40 T).

Fin bud expression of ptc1 and ptc2

To investigate whether the remaining expression of ptc2 in these mutants could be due to redundancy between shh and the other hh genes, I examined ptc expression in the pectoral fin buds of embryos homozygous for syu

as shh is normally expressed in these fin buds (which are homologous to the limbs in other vertebrates) whereas ehh and twhh are not. I found that neither ptc gene is expressed in the fin buds of syu

homzygotes (in situ were done at 32 hpf, (4 separate in situ); 33 hpf; 31 hpf; 30 hpf; and 37 hpf. In total 0/57 mutant embryos had fin bud expression of ptc1 and 0/24 mutant embryos had ptc2 fin bud expression (fig. 40 B & G). To establish that the fin buds do form in syu

homzygotes I also did an in situ on embryos at 33 hpf with a nucleolin probe that is expressed in the fin buds (Qiao, T. 1997). I observed fin bud staining in 4/4 mutant embryos, though it was weaker and / or slightly smaller, and closer to the flank than in wild type embryos (fig. 40 E & J). This agrees with previous observations that the fin buds are established in this mutant but fail to grow out (Schäuerle, H. E. et al. 1998).

I also investigated the expression of both ptc genes in the pectoral fin buds of other mutations to see if there was a correlation between Hh signalling and phenotype in the fin buds. Both yot and you homozygotes have no fin bud phenotype and both ptc1 and ptc2
are expressed normally in the fin buds of these embryos. *ubo* homozygotes have irregularly indented fin buds but again *ptc1* and *ptc2* are expressed normally in the pectoral fins of these embryos. However, the fin bud expression of both *ptc* genes is weaker in *igu* homozygotes, though *ptc1* expression is reduced more than *ptc2*, and unlike in other embryonic regions the expression of *ptc1* is not obviously expanded in the fin buds (fig. 40 D & I). The expression of both *ptc* genes is also reduced in the fin buds of *con* homozygotes: *ptc1* expression is weaker and smaller and the expression of *ptc2* is weaker but to less of an extent (fig. 40 C & H). This is consistent with the variably reduced phenotype of the pectoral fins in *con* homozygotes, which is very similar to that in weaker alleles of *syu*.

**Figure 39: ptc expression in syu homozygotes**

Transcripts were revealed by *in situ* hybridisation with antisense RNA probes to *ptc1* (A & C) or *ptc2* (B & D) on 18-20 somite embryos. Mutations homozygous for *syu* have a dramatic reduction in *ptc1* expression (C and cf. to (A) which shows wild type *ptc1* expression); but hardly any reduction in *ptc2* expression (D and cf. B which shows wild type *ptc2* expression).
Figure 39
Analysis of zebrafish mutations

Figure 40: ptc expression in the fin buds and ptc2 expression in the u mutants

(A-J) show fin bud expression of different genes in wild type and mutant embryos at 32 hpf and (K - Q ) show ptc2 expression in wild type and mutant embryos at 24 hpf. Transcripts were revealed by in situ hybridisation with antisense RNA probes to ptc1 (A - D) or ptc2 ( F - I & K - T) or nucleolin C (E & J). Both ptc1 and ptc2 are expressed in wild type fin buds but ptc2 is expressed more broadly and weakly than ptc1 (A & F). In contrast neither gene is expressed in the fin buds of syu homozygotes (B & G) even though the finbuds do still form and express nucleolin C (E & J). Both ptc1 and ptc2 expression is reduced in the fin buds of con homozygotes (C & H) and ign homozygotes (D & I) though in both cases ptc1 expression is reduced more than ptc2 expression.

At 24 hpf ptc2 expression is only slightly affected by mutations in the u mutants. Wild type sibling embryos processed in parallel are shown for each example in this section of the figure (K- O) as staining levels vary between different ptc2 in situ ptc2 expression is slightly reduced throughout its normal expression domain in syu and con homozygous embryos (P & Q) and has a slightly different expression pattern in the brains of ign homozygotes (T). In yot homozygotes ptc2 expression is very slightly weaker than in wild type embryos (R) but less so than in con or syu homozygotes, with the exception of the anterior somites which have no expression of ptc1 in yot homozygotes (S), even though they still weakly express ptc1 in syu and con homozygotes.
Figure 40
Analysis of zebrafish mutations

B. Preliminary analysis of ptc expression in other mutants from the Tübingen screen

Background and general results

I also examined ptc expression in a number of other mutations discovered in the Tübingen screen. These included the other mutations that had abnormal floor plate development like con, yot, dtr, igu and syu: schmalhans (smhh); schmalspur (sur); and monorail (mol) (Brand, M. et al. 1996). I also investigated a few mutations which had some of the other phenotypic characteristics that I might predict for a Hh pathway mutation: J02A, a curly tail mutant that was thought for a while to be allelic to iguana (but more extensive analysis showed that it complements igu); fused somites which forms irregular somite boundaries; white tail (wit) which also forms irregular somite boundaries caudally and has an increased number of primary motoneurons; and knollnase (kas) which affects the development of the roof plate in the anterior brain (telencephalon) (van Eeden, F. et al. 1996; Jiang, Y. J. et al. 1996; Heisenberg, C. P. et al. 1996). With all of these mutations I collected progeny from identified heterozygous fish during my visits to Pascal Haffter's laboratory in Tübingen. I saw no obvious changes in ptc expression in embryos from mol at 22 hpf [0/54]; J02A at 24 hpf [0/37]; and no obvious changes in either ptc1 expression or ptc2 expression in smhh embryos at 24 hpf; white tail (wit) embryos at bud stage or 24 hpf or kas embryos at 1-2 somites or 24 hpf. However I did see altered patterns of ptc expression in schmalspur (sur) and fused somites (fss) and I will describe these below. I did not have time to pursue any of this analysis in any more depth.

ptc expression in sur homozygotes

In sur homozygotes both of the ptc genes are expressed normally in the trunk and the anterior of the head, but expression in the mid and hind brain is strongly reduced (fig. 41) [ptc1 12/56; ptc2 12/48]. Initially I was interested in examining ptc expression in this mutant because its floor plate and ventral brain phenotype made it a possible candidate for a mutation in twhh. Embryos homozygous for sur have a curly tail; do not develop normal floor plates; have deletions of ventral CNS tissue in the mid and hind brain, but not in the forebrain; and have eyes that are turned inwards posteriorly like the eyes in embryos homozygous for dtr, yot, con, and igu (Brand, M. et al. 1996). Further analysis of this mutant has shown that it is not a mutation in a hh gene, but seems to belong to the same class of mutations as oep and cyc. oep, cyc and sur are all required for the
Analysis of zebrafish mutations

development of the prechordal plate, but sur is required specifically for the posterior prechordal plate whereas the other two mutants are required for the development of the whole prechordal plate. This is consistent with the expression of ptc1 and ptc2 in this mutant, which resembles the loss of expression seen in the brain of cyclops embryos (described in chapter 7), with the difference that ptc1 and ptc2 are expressed normally in the anterior brain of sur homozygotes presumably because of the normal expression of shh in this region (fig. 41C, D, G & H and cf. fig. 26; Brand, M. et al. 1996).

*ptc1* expression in *fss* homozygotes

At 22 somites fss homozygotes have disturbed somite expression of ptc1 [16/71]. Lateral views show that the somite staining is irregular and missing in patches along the rostro-caudal axis of the embryos. In many of the embryos there are stretches, especially towards the tail, where the normal anterior-posterior restriction of ptc1 expression within the somites is lost and instead there is continuous expression of ptc1 in the somitic mesoderm along the rostral caudal axis (fig. 42C & D). This is similar to the expression of myoD and snail1 in fss homozygotes and correlates with later anterior-posterior patterning defects in the vertebrae (van Eeden, F. et al. 1996).

*ptc1* expression in *syu/ubo* double mutant embryos

Heike Schauerte generated fish heterozygous for both syu\*\* and ubo and sent me embryos from a cross between these fish fixed at 24 hpf. I observed three different classes of ptc1 expression in these embryos. One class had strong wild type like expression of ptc1 [27/45] (fig. 43A); one class had a curled body shape reminiscent of syu homozygotes and had weak expression of ptc1 in the ventral brain and ventral neural tube [10/45] (fig. 43C); and the third class had straight bodies but almost no expression of ptc1, with just a very little bit of expression near the tail and at the very anterior of the head [8/45] (fig. 43B. This was a surprising result as ubo homozygotes usually express ptc1 normally, so I had expected to see the same result as with a syu single mutant cross: 3/4 of the embryos with normal expression of ptc1, and 1/4 with curled body shapes and reduced expression of ptc1. The numbers of embryos are too small to come to any firm conclusions but the results suggest that there might be an interaction or some redundancy between syu and ubo. It would be interesting to investigate this double mutant combination further. Unfortunately I was unable to obtain any more embryos to repeat or extend this analysis.
C. Brief Summary and Conclusions.

The analysis presented in this chapter suggests a wide range of conclusions, which I will discuss in depth in the following chapter. However, in brief, one of the main conclusions that my analysis suggests is that con, you, yot, igu and dtr are all required for at least some aspects of Hh signalling, thought the precise spatial and temporal requirements for these different genes seem to differ. In addition my results confirm that Hh signals are required for correct muscle cell type specification in the Zebrafish; and suggest that the syu<sup>q<sub>252</sub></sup> allele of syu makes functional Shh protein, but that the levels of the protein are reduced compared to wild type embryos, at certain stages of development.
Analysis of zebrafish mutations

Figure 41: ptc1 and ptc2 expression in sur homozygotes

Transcripts were revealed by in situ hybridisation using antisense probes to ptc1 (A - D) or ptc2 (E - H) on embryos at 24 hpf. Both ptc genes are expressed normally in the trunks of sur homozygotes but there is a dramatic loss of expression in the mid brain and hindbrain. (C, D, G & H show sur homozygotes, cf. to A, B, E & F which show wt sibling embryos).

Figure 42: Expression of ptc1 in fss homozygotes

Transcripts were revealed by in situ hybridisation using antisense probes to ptc1 on embryos at 20 hpf. The somite expression of ptc1 in fss homozygotes is disorganised: somitic cells either express ptc1 throughout somitic regions without any anterior -posterior restriction within what would normally be a somitic unit, and / or have regions of the trunk where there is very little somitic expression of ptc1 (see C and D and cf. A and B)

Figure 43: ptc1 expression in syu/ubo double mutant embryos

in situ hybridisation of embryos from ubo^{+/}; syu^{+/} parents at 24 hpf, with an antisense probe to ptc1, revealed three classes of expression pattern: wild type like (A); almost complete loss of expression (B) and reduced expression, with the somitic expression being more obviously reduced than the ventral neural tube expression (C). The embryos with hardly any ptc1 expression (B) had a similar straight body shape to the wild type embryos (A) whereas the embryos with a reduction in expression had a curled body shape (C).
A. Conclusions about ptcl and ptc2.

Sequence and structural conclusions

One of the main conclusions of the research described in chapter three is that there are at least two ptc genes in zebrafish, ptcl and ptc2. These do not derive from a simple tandem duplication of the locus since linkage analysis reveals that the two genes map to different chromosomes. Moreover, while ptcl is closely linked to twhh, ptc2 is on a different linkage group to shh: this suggests that the duplication event giving rise to ptcl and ptc2 occurred significantly earlier than the relatively recent partial duplication of the zebrafish genome thought to have generated the closely related shh and twhh genes (Postlethwaite, J. H. et al. 1998; Zardoya, R. et al. 1996). Consistent with this, reports of a second ptc gene in both newt and mouse have subsequently been published, indicating that duplication of the ptc genes most likely occurred prior to the vertebrate radiation (Takabatake, T. et al. 1997; Motoyama, J. et al. 1998).

My analysis of the protein sequence of different ptc genes in chapter three suggests that they all have a very similar structure with twelve putative transmembrane domains, and that they fall into either two or three classes on the basis of sequence conservation, with zebrafish ptc2 belonging in the same class as the ptc genes initially described in mouse, chick and human (mptcl, cptcl, HPTCl). The shorter carboxyl terminal domains of both of the zebrafish genes aswell as mptc2, and the sequence diversity in the intracellular domain of ptc between the 6th and 7th transmembrane regions suggests that at least parts of both of these domains are either dispensable for Ptc function (or in the case of the central domain only have structural significance) or have species specific functions. All of the Ptc proteins are roughly equally similar to mouse NPC suggesting that the similarity of the two domains of homology is likely to be functionally significant.

Functional comparisons between different ptc genes

It is hard to determine at the moment whether the different ptc genes fall into different classes in terms of functional homology, in the same way as they do with respect to
sequence homology, as there is hardly any data that compares different \textit{ptc} genes in the same organism. For example loss of function data in mouse has only been described for \textit{mptc1}, and in zebrafish I have only been able to investigate the results of over-expressing \textit{ZF ptc1}. Also, since no comprehensive descriptions of the expression patterns of the \textit{ptc2} genes in other species have yet been published it is difficult to make comparisons between the spatial deployment of the different \textit{ptc} genes in various species (Motoyama, J. et al. 1998; Yang, Y. et al. 1997). Both \textit{ZF-ptc1} and \textit{ptc2} are expressed in similar embryonic regions to all of the other vertebrate \textit{ptc} genes, though in most cases \textit{mptc1} and \textit{cptc1} seem to resemble \textit{ZF-ptc1} more than \textit{ZF-ptc2}, in that they are not as broadly expressed as \textit{ZF-ptc2} (for example in the developing limbs). One exception is the neural tube where later expression of \textit{mptc1} and \textit{cptc1} extends quite far dorsally (Marigo, V. and Tabin, C. J. 1996; Goodrich, L. V. et al. 1996). The description of \textit{mptc2} expression is so far restricted to its expression around developing teeth buds, hair and whisker cells, in the lung epithelium, and some preliminary reports of whole embryo expression in wild type and \textit{Doublefoot} embryos (Motoyama, J. et al. 1998; Yang, Y. et al. 1998; St-Jacques, B. et al. 1998; Pepicelli, C. V. et al. 1998). However, in comparison to their expression in early development where \textit{mptc1} and \textit{mptc2} expression patterns overlap, in epidermal development the two genes are differentially expressed: \textit{mptc2} is co-expressed with \textit{shh} in the invaginating epithelial cells whereas \textit{mptc1} is expressed in mesenchymal condensations surrounding these invaginating cells (Motoyama, J. et al. 1998). Recent analysis of hair development in mice lacking Shh function, suggests that this \textit{mptc1} expression is regulated by Shh signals, but that the \textit{mptc2} expression is not (St-Jacques, B. et al. 1998). In addition \textit{mptc-2} expression in the developing lung epithelium is also not altered by loss of Shh function in the epithelium, whereas \textit{mptc-1} expression in the mesenchyme is reduced (Pepicelli, C. V. et al. 1998). \textit{ZF-ptc2} has a different expression pattern to \textit{ZF-ptc1}, especially at early stages but generally the expression of the two genes overlaps with \textit{ptc2} being expressed more broadly than \textit{ptc1}. I have seen no evidence of one of the zebrafish \textit{ptc} genes being co- expressed with \textit{shh} while also being complementary to the expression of the other \textit{ptc} gene, though in rostral somites at 24 hpf, \textit{ptc1} and \textit{ptc2} are expressed in complementary medial - lateral domains of the somites. Further comparisons between the different \textit{ptc} genes in different organisms will have to await more detailed characterisation of \textit{ptc2} in other species.
Regulation of \textit{ptc1} and \textit{ptc2} expression by Hh signals

My analysis of \textit{ptc} expression in wild type and mutant embryos, and in embryos exposed to ectopic Hh signalling, demonstrates that both \textit{ptc1} and \textit{ptc2} are transcriptionally regulated by Hh signals. Several aspects of my results suggest that there is no specificity between particular Hh proteins and the transcriptional regulation of particular \textit{ptc} genes, but that the expression of both \textit{ptc} genes responds to overall levels of Hh signal; with \textit{ptc1} only being expressed at high levels close to the sources of Hh signals, in cells which presumably receive high levels of Hh activity and \textit{ptc2} being expressed more widely in response to lower levels of Hh signals. Firstly Hh activity is required for expression of both \textit{ptc} genes, as in cases where all \textit{hh} expression is missing (most of the head in \textit{cyc} homozygotes, the midbrain and hindbrain in \textit{sur} homozygotes, the trunk mesoderm in late somitogenesis stage \textit{flh} homozygotes, the pectoral fin buds of \textit{syu} homozygotes) there is no expression of either \textit{ptc} gene. Secondly Shh is sufficient at some times and in some places (and hence Ehh and Twhh are not required) for the expression of both \textit{ptc} genes, but, at least at some stages of development, Shh is not required for the expression of either gene. That Shh activity is sufficient is shown by the wild type expression of both \textit{ptc} genes in the pectoral fin buds where \textit{shh} is the only \textit{hh} gene expressed, and by the expression of both genes around the perpendicular stripe of \textit{shh} expression in the heads of \textit{cyc} homozygotes at 24 hpf. However, the expression of \textit{ptc2} in \textit{syu} homozygotes shows that Shh is not required for most of the expression of \textit{ptc2}. In contrast, the expression of \textit{ptc1} in \textit{syu} homozygotes suggests that Shh is required for most, but not all, of the expression of \textit{ptc1} at late somitogenesis stages, and in particular that Twhh can compensate, but only slightly, for the loss of Shh in the neural tube and head (the expression of \textit{ptc1} in these regions is still a lot weaker than in wild type embryos), but that Ehh is not sufficient for \textit{ptc1} expression at these stages (there is no \textit{ptc1} expression in the somites around the notochord). However, at the end of gastrulation there is no noticeable effect on either \textit{ptc1} or \textit{ptc2} expression in embryos homozygous for \textit{syu}, showing that at earlier stages Shh is not required for expression of either of the \textit{ptc} genes.

My results in chapters 4 and 7 show that Ehh is not required for either \textit{ptc} gene to be expressed at high levels: both \textit{ptc} genes are expressed in the heads of wild type embryos and the trunks of \textit{flh} homozygotes, where there is no expression of \textit{ehh}. However, the loss of expression of both \textit{ptc} genes in the anterior somites of \textit{flh} homozygotes suggests that \textit{ehh} and/or sustained \textit{shh} expression is required for this expression. It also shows
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that floor plate expression of \textit{twhh} and \textit{shh} is not sufficient to maintain somite expression of the two \textit{ptc} genes.

I also show in chapter 5 that overexpression of any of the \textit{hh} genes or of a dominant negative subunit of PKA has the same effect on the expression patterns of \textit{ptcl} and \textit{ptc2}. This again argues against any specificity between the activity of particular \textit{hh} genes and the transcription of particular \textit{ptc} genes. However, in addition, lower concentrations of \textit{hh} RNA affect \textit{ptc2} expression more than \textit{ptcl} expression. This suggests that \textit{ptc2} expression responds to lower levels of Hh activity than \textit{ptcl} expression. This is consistent with the expression of the two genes, both in wild type and in mutant embryos, where \textit{ptc2} is expressed farther away from cells expressing \textit{hh} than \textit{ptcl} (for example the neural tube, somite and pectoral fin bud expression in wild type embryos and the neural tube expression around the small groups of floor plate cells in \textit{flh} homozygotes), and hence can presumably be transcriptionally up-regulated by lower concentrations of Hh signal.

This lack of specificity between particular \textit{hh} genes and transcription of particular \textit{ptc} genes is consistent with the available data on \textit{ptc} genes in other vertebrates: \textit{cptcl} and \textit{mptcl} are expressed around sites of expression of all three \textit{hh} genes, \textit{ihh}, \textit{dhh} and \textit{shh}; both \textit{mptcl} and \textit{mptc2} seem to be up-regulated by ectopic \textit{Ihh} activity (Yang, Y. et al. 1998); newt (Cynops) \textit{ptcl} and \textit{ptc2} are both up-regulated by Shh (Takabatake, T. et al. 1997); and both mouse \textit{ptc} genes are down-regulated in \textit{gli2} homozygotes (Motoyama, J. et al. 1998). However, as mentioned above, there is recent evidence that \textit{mptc2} expression in the epithelium of the lung and the skin is not regulated by Shh (Pepicelli, C. V. et al. 1998; St-Jacques, B. et al. 1998). In addition, my results do not address the question of whether there is any specificity between individual Hh ligands and Ptc receptors: it is still possible that all Hh proteins can act similarly to induce high level expression of both \textit{ptc} genes but that individual Hh proteins interact more specifically with particular Ptc proteins.

My overexpression experiments also raise a couple of other interesting points. First of all they show that the transcription of \textit{ptcl} and \textit{ptc2} responds differently to high concentrations of ectopic Hh signals: high levels of Hh signal induce ectopic expression of \textit{ptcl} in the head and trunk, but while they induce high levels of \textit{ptc2} in the head they suppress \textit{ptc2} expression in the ventral trunk. In the trunks of embryos that have been exposed to very high ectopic \textit{hh} expression the only \textit{ptc2} expression that remains is in the dorsal neural tube, which is the region of the embryo farthest away from the endogenous Hh signals. In contrast to this I saw no evidence that \textit{ptcl} expression is down-regulated
by high levels of ectopic Hh. Secondly, the expression of *ptc1* in *hh* injected embryos is very similar to normal *ptc2* expression but stronger. It is also very similar to the expression of *ptc1* in *iguana* homozygotes at 24 hpf. This suggests that this expanded *ptc1* expression pattern and the normal *ptc2* expression pattern may demarcate the area of competence for *ptc* expression. Consistent with this the expression pattern of *ptc2* correlates well with areas that are known to be capable of responding to Hh signals (for example the whole dorso-ventral extent of the somites and neural tube).

My analysis of the expression of *ptc1* and *ptc2* also raises some interesting points about somite development. I show in chapter 4 that *MyoD, ptc1* and *ptc2* are expressed in complementary regions of the somites when they first form and this continues to be true for caudal somites at later stages. Previous analysis has suggested that these lateral stripes of *MyoD* in the somites are independent of Hh activity (Weinberg, E. S. et al. 1996; van Eeden, F. et al. 1996; Schauerte, H. E. et al. 1998), which in combination with my results, suggests that newly forming somites are pre-patterned along their anteroposterior axis in terms of their competence to respond to Hh signals. Even though the expression of both *ptc* genes moves from anterior to posterior within the somites as they develop, both *ptc* genes continue to be co-expressed, and even in cases where *ptc1* expression is expanded in the trunk (*iguana* homozygotes and *hh* overexpression studies) its expression is still restricted along the anterior posterior axis of the somites. This suggests that the somite continues to be divided into cells that can respond to Hh signals and cells that can not, although the anterior-posterior localisation of this competence varies along the rostro-caudal axis of the embryo. There is some evidence that the lateral stripes of *MyoD* expression do require Hh signals to maintain their normal level of expression at later stages of somitogenesis (chapter 8; van Eeden, F. et al. 1996) which is also consistent with the overlapping expression of *ptc* genes and *myoD* in rostral somites at these stages (*ptc1* in now posteriorly localised and *MyoD* is expressed throughout most of the anterior-posterior extent of the somite medially and is posteriorly restricted in more lateral regions (Weinberg, E. S. et al. 1996)).

Another interesting observation is that *ptc2* somite expression at 24 hpf closely resembles the expression of slow muscle fibre markers. Intriguingly the expression of *ptc2* in *yot* homozygotes, that lack all slow muscle fibre types, is down regulated in the rostral somites, whereas it is still expressed in the somites of *con* homozygotes, that have
normal slow muscle. However, \textit{ptc2} is also still expressed in the somites of \textit{syu} homozygotes, and dorsal views at 24 hpf show the same pattern of expression as in wild type embryos, even though \textit{syu} homozygotes have a dramatic (but not complete) reduction in slow muscle fibre types. It is therefore difficult to deduce the significance of this \textit{ptc2} expression. It would be interesting to detect \textit{ptc2} transcript and slow muscle markers simultaneously at different stages of somitogenesis to determine if the same cells are expressing both, and if they are, at what stages this occurs. Ultimately, when the technology is available, it would be interesting to create \textit{ptc2} GFP transgenics and see if the dynamic expression of \textit{ptc2} in the somites is due to cell movement or to cells changing their levels of \textit{ptc2} transcript. This could also be addressed by cell labelling experiments.

\textit{ptc1} overexpression

My \textit{ptc1} overexpression experiments, described in chapter 6, suggest that Ptc function has been conserved between \textit{Drosophila} and vertebrates. In \textit{Drosophila}, Ptc activity suppresses the intracellular pathway that transduces the Hh signal. Activation of Hh targets is achieved by the inhibition of Ptc activity, either by Hh binding to Ptc or by mutation of the \textit{ptc} gene (Ingham, P. W. et al. 1991). Conversely, over-expression of \textit{ptc} can block the effects of Hh activity, resulting in a Hh loss of function phenotype (Schuske, K. et al. 1994; Johnson, R. L. et al. 1995). Similar effects of \textit{ptc} inactivation on Hh signalling have been observed in mice and humans (Goodrich, L. et al. 1997; Gailani, M. R. et al. 1996), and there are preliminary reports that overexpression of \textit{mptc1} can dorsalise the CNS (L. Goodrich, L. Milenkovic, K. Higgins and M. Scott, personal communication) which also suggests that the function of the Ptc proteins has been conserved between invertebrates and vertebrates. In chapter 6 I show that ectopic expression of synthetic \textit{ptc1} mRNA can produce embryos that have morphologically abnormal somites: specifically they lack muscle pioneer cells and have reduced \textit{myoD} expression in their adaxial cells even at the onset of somitogenesis. In addition Pete Currie has injected \textit{ptc1} RNA that I synthesised and he finds that slow muscle is reduced in injected embryos at 24 hpf. The effects vary between individual embryos but slow muscle is often severely reduced and one embryo had an almost complete absence of slow muscle on one side of the midline and normal slow muscle on the other side (Lewis, K. E. et al. 1998). I also found that overexpression of \textit{ptc1} causes varying degrees of cyclopia; reduced expression of a ventral neural tube marker; and less distinct floor plate morphology in some regions.
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All of these phenotypes are consistent with reductions in Hh signalling and the severity of some of the phenotypes (complete cyclopia, complete loss of myoD adaxial expression and of slow muscle cells) are consistent with Ptc1 inhibiting all Hh signals, as they are more severe than the phenotype of syu14 homozygotes. The phenotypes obtained by over-expressing ptc1 are also interesting because ptc1 is reasonably divergent from the ptc genes that have been shown by mutational analysis to repress Hh signalling in other vertebrates (mptcl and HPTC1) (Goodrich, L. et al. 1997; Gailani, M. R. et al. 1996): zebrafish ptc2 is more closely related to these other ptc genes. Therefore it was interesting to see that Zf-Ptc1 is also capable of repressing Hh activity. It is also interesting, given the multiple hh and ptc genes in zebrafish, to see that Ptc1 is capable of inhibiting more than just Shh activity. This is the first example where a particular Ptc protein has been shown to inhibit the activity of more than one Hh: inactivation of Ptc function in the mouse produces a phenotype so severe that it is hard to analyse the effects on Dhh and Ihh signalling in affected embryos (as both of these proteins are required later in development than Shh).

Significance / Function of the two ptc genes in zebrafish

My analysis has not produced any clear indication as to whether Ptc1 and Ptc2 have different functions in zebrafish, nor what their distinct functions might be. The differing expression patterns suggest that there may be differences in the spatial requirements for the two proteins but there is no evidence yet that they provide substantially different functions. To answer this question further experimental analysis is needed, and in particular we need to isolate mutations in both of these genes. However it would also be informative to investigate the consequences of over-expressing ptc2 and to determine the binding coefficients for each of the possible combinations of Hh and Ptc proteins. It will also be interesting to see if the analysis of ptc2 in other vertebrates provides any clues as to possible differences in function between different ptc genes.

B. Candidate mutations for components of the Hh pathway.

Our strategy of using ptc1 expression to screen existing mutations for possible involvement in the Hh pathway was successful, most notably because it identified iguana and detour as candidate mediators of Hh signalling, but also because it distinguished
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between different u mutations. I was, however, unable to analyse as many different mutations as I would have liked because of lack of time. For example I would have liked to investigate more mutations that had aspects of the phenotype that we might expect for a ptc mutation (such as small eyes, defects in neural tube patterning, or overgrown pectoral fins). However, as the mapping of existing mutations proceeds this becomes a more reliable way of identifying possible candidate mutations in ptc, as both of the ptc genes are now mapped.

dtr, con, you, yot and igu are possible components of a Hh pathway.

The original characterisation of the u-type mutants revealed a common defect in all members of this class, namely the absence of muscle pioneer cells; a finding that led to the suggestion that these mutations disrupt the generation or transduction of the Hh signals implicated in muscle pioneer induction (van Eeden, F. et al. 1996). To explore this possibility further, I analysed the expression of the Hh target gene ptcl in these and other midline mutant embryos.

My analysis of ptcl expression, discussed in chapter 8, suggests that dtr, con, you, yot and igu are components of a Hh pathway but that ubo is a component of a parallel or downstream pathway also involved in somite patterning. ptcl expression is altered in you, yot, con, igu and dtr homozygotes, consistent with these embryos receiving altered Hh signals, but it is expressed normally in ubo homozygotes. None of these mutations are likely to be in hh genes themselves, or in upstream regulators of hh gene expression, as hh genes are expressed normally in homozygote embryos and only igu is genetically linked to a hh gene, a linkage that is unlikely to be significant (discussed below). Therefore dtr, con, you, yot and igu are likely to be genetically downstream of hh genes, somewhere between hh expression and hh induced transcription of ptcl. In addition, my epistasis analysis confirms that igu is genetically downstream of shh as igu/syu double mutant embryos have the same ptcl expression phenotype as igu homozygotes. con, you, yot and dtr have reduced levels of ptcl transcription suggesting that these gene products are required for Hh signalling activity (as opposed to components of the Hh pathway like Ptc and Costal-2 that normally act to repress the Hh signalling pathway). igu however, is more complicated as some aspects of its phenotype are consistent with a reduction in Hh signalling while other aspects are consistent with a de-repression of the Hh pathway. I will discuss this in greater detail below.
Consistent with all of these conclusions there are preliminary reports that \textit{yot} is a mutation in \textit{gli2} and that \textit{dtr} is a mutation in \textit{gli1} (Karlstrom and Schier, personal information). The other mutations may identify novel components of the Hh pathway or they may be mutations in zebrafish homologues of components already discovered in \textit{Drosophila}. To verify this, the mutations will need to be cloned, or zebrafish homologues of Hh pathway genes will need to be cloned and mapped relative to these mutations. However, there are probably many other possible Hh pathway mutations that have not been identified yet in zebrafish, as there are now eleven proteins that have been implicated in the pathway in \textit{Drosophila} (see chapter one).

\textbf{The uboot mutation}

Unlike all the other mutations originally assigned to the u class, \textit{ptcl} expression is unaffected by the \textit{ubo} mutation. \textit{ubo} was originally assigned to the u mutant class on the basis of its morphological somite phenotype, and was shown to be required in the somites like \textit{yot}, but there are several phenotypic differences between \textit{ubo} and the other u mutants. For example \textit{ubo} does not affect the formation of the dorsal aorta, and all its fins are irregularly indented, whereas the other u mutants form a defective dorsal aorta and either have no fin phenotype or have a specific reduction of the pectoral fins. The expression of Engrailed (Eng.) proteins in the somites also differs between \textit{ubo} and the other u mutants. Whereas \textit{yot}, \textit{con}, \textit{syu} and \textit{you} have a reduction in the number of Eng. expressing cells in the trunk, \textit{ubo} has the same number of Eng. positive cells as wild type fish, but in \textit{ubo} the cells all express a level of Eng. proteins intermediate between normal muscle pioneers and the cloud of weaker expressing cells that is normally seen around muscle pioneer cells. This all suggests that \textit{uboot} is in a different signalling pathway to the other genes in the u mutant class. However, \textit{ubo} does have a slow muscle phenotype which resembles the phenotype seen in \textit{igu} homozygotes (discussed below): there is an expansion of slow muscle markers in anterior somites, but these markers are co-expressed with fast muscle markers (P. Currie's data in Lewis, K. E. et al. 1998). This suggests that \textit{uboot} could be a mutation in a downstream repressor of responses to Hh signals. In this scenario \textit{ubo} would be downstream of the Hh pathway, because unlike in \textit{igu}, \textit{ptcl} and \textit{myoD} expression are not affected in \textit{ubo} homozygotes. Du and colleagues have shown that a BMP like molecule can act downstream of Shh signals to repress Shh induction of muscle pioneers, and they propose that inhibitory signals may oppose the action of Shh signals on myotome cells, resembling what has been suggested for the neural tube (Du, S. J. et al. 1997; Placzek, M. and Furley, A. 1996 and references
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therein). This suggests the intriguing possibility that *ubo* might be a mutation in such an inhibitory action. Alternatively, or in addition, the expression of Eng. proteins suggests that *ubo* might be required for selecting individual adaxial cells to form muscle pioneers by a process akin to lateral inhibition. The results of my analysis of embryos from fish heterozygous for both *syu* and *ubo* are also intriguing, even though they are preliminary, as they suggest that *ubo* and *syu* might functionally interact or that *ubo* may have a subtle role in the Hh pathway that is only revealed when levels of Hh activity are lowered. It will therefore be interesting to determine the molecular nature of the *ubo* mutation.

*igu* is unlikely to be a mutation in *ehh*

Heike Schauerte and Pascal Haffter established that *igu* is very closely linked genetically to *ehh*, and this was also independently established by Alex Schier's laboratory, but none of these researchers were able to find any mutations in the *ehh* coding sequence from *igu* homozygotes (personal information). There are also several reasons that make it unlikely that *igu* is a mutation in *ehh*. For example *igu* has defects in the head but *ehh* is not expressed in the head in wild type embryos; and I have found no evidence for ectopic expression of *ehh* in *igu* homozygotes. Also the *ptc1* phenotype of *igu* homozygotes suggests that these embryos have ectopic Hh signalling and *igu* is unlikely to be a gain of function mutation as there is no evidence of a heterozygote phenotype, and two separate alleles of *igu* have been identified that have very similar phenotypes, which would be unusual for a gain of function allele. I have also shown that *igu* is epistatic to *syu* suggesting that *igu* is genetically downstream of *shh*. Subsequently, radiation hybrid mapping has in any event now separated the *igu* locus from *ehh* (Heike Schauerte & Pascal Haffter, unpublished data).

Some Hh activity remains in all the candidate Hh pathway mutations

*con, you, yot* and *dtr* are all strong candidates for Hh pathway components, but their phenotypes differ in major and subtle ways. Also, even though mutations in these genes strongly reduce the levels of *ptc1* transcript at late somitogenesis stages, none of these mutations have as dramatic an effect on all *ptc* expression (and in particular on *ptc2* expression) as the regions of *cyc, sur* and *flh* embryos that have no expression of *hh* genes. This suggests that there is still some Hh activity in all of these mutations. This is not surprising for *syu* because the other *hh* genes are still expressed in embryos homozygous for this mutation, but it is more surprising for the other mutations as we
might have expected that these would be downstream of all hh genes and hence would remove all Hh activity. There are several reasons that could explain this observation: there could be functional redundancy between different components in a common pathway; the mutations could be hypomorphs rather than a complete loss of function; there could be precise spatial and temporal requirements for particular components; or some of the components could be maternally provided. In addition Peter Currie found significant differences between the slow muscle phenotypes of these different mutations. Slow-muscle-specific, myosin heavy chain expression is completely eliminated in yot homozygotes (n=11) and significantly reduced in you (n=17); however, some expression persists in syu (n=10) while in con homozygotes, expression appears normal apart from the loss of the MP cells themselves (n=12). In all cases, the fast muscle specific myosin heavy chain isoform is expressed at the appropriate time. However, in ignu (n=6) and ubo (n=7) there is an expansion of expression of the slow isoform in the more rostral somites such that the slow and fast isoforms are co-expressed. The differentiation of slow and fast muscle is unaffected in dtr homozygotes (n=12) (P. Currie's data in Lewis, K. E. et al. 1998). I will discuss each of the mutations in turn and deal with some of these issues.

\textit{you}

you homozygotes have more substantial expression of ptcl than either con, yot, or syu homozygotes yet they still have a severe somite phenotype, both morphologically and in terms of the reduction in slow muscle. Interestingly, at 24 hpf the expression of ptcl is most strongly reduced in the somites of these embryos, suggesting that there might be more of a requirement for you activity in the somites than in other embryonic regions. The expression at earlier stages also suggests that you may be a hypomorphic allele and that this might account for the variability in the phenotype between individual embryos, seen in terms of ptcl expression and muscle pioneer cell formation. Muscle pioneers are only ever seen in anterior somites, suggesting that this variability in phenotype is due to slight differences in the time by which Hh signalling reduces to such an extent that muscle pioneers no longer form. This agrees with my analysis of ptcl expression at 5-6 somites where less than a quarter of embryos in the you cross had reduced ptcl expression.

\textit{dtr}

dtr homozygotes also only have a subtle reduction in ptcl expression, but unlike you homozygotes they also have a dorsal expansion of ptcl expression in the neural tube and we have been unable to identify any defects in the myotome. This suggests that dtr is a
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component of the Hh pathway but that its activity is either not required, or is redundant, in the myotome.

**yot**

*yot* shows the strongest reduction in *ptcl* expression (and also in *myoD* expression) of any of these mutations, including *syu*, consistent with it being required to transduce signals from more than one *hh* gene. However, even in *yot* homozygotes, there is initially very weak *ptcl* expression in the adaxial cells, suggesting either that there is still some Hh activity or that a factor other than Hh initiates the adaxial expression of *ptcl*. An interesting observation at this stage (end of gastrulation) is the difference between *myoD* and *ptcl* expression: *ptcl* is very weakly expressed throughout its normal domain, whereas *myoD* is still expressed quite strongly in the most caudal adaxial cells and in the tail bud, but is completely eliminated from more rostral adaxial cells. One possible explanation of these observations would be if *yot* was not a complete null, and enough Hh activity persisted in *yot* homozygotes at this stage to induce and maintain weak *ptcl* expression, but not to maintain *myoD* expression. If *ptcl* and *myoD* expression requires different levels of Hh signalling, this would explain the difference between *ptcl* and *myoD* expression in *yot* homozygotes, regardless of whether Hh signals are needed to induce or maintain the expression of these two genes. It would also be consistent with *ptcl* but not *myoD* being expanded in *igu*, assuming that *igu* homozygotes have low levels of ectopic Hh activity. However this hypothesis is not consistent with *myoD* and *ptcl* expression in *iguana* at tail-bud stage where there is initially a reduction of *ptcl* expression but not of *myoD* expression.

As the initial (80% epiboly) expression of *myoD* in *yot* homozygotes is indistinguishable from wild type this suggests that the initiation of *myoD* expression in the mesoderm may occur independently of Hh signals. In addition, in *you*, *yot*, *con*, *igu* and *syu* homozygotes there is more substantial expression of *myoD* around the tailbud than in the more anterior adaxial cells at about 12 somites of development which would be consistent with *myoD* expression continuing to be induced independently of Hh signals in this region of the embryo. The expression of *myoD* at these later somitogenesis stages in all of the potential Hh pathway mutants, also suggests that Hh signals may have a later maintenance role in the expression of *myoD* in the medial-lateral stripes in the somites, though it is much less dramatic than the role in maintaining adaxial expression.

At later stages the reduction of *ptcl* expression in *yot* homozygotes is much more dramatic in the trunk than in the head, and there is more *ptcl* expression in the heads of
yot homozygotes than in the heads of syu homozygotes. This suggests that either yot is only required for Hh signalling in the trunk, or that there is more redundancy between different components of the pathway in the head than the trunk. In addition to this the expression of both of the ptc genes suggests that yot is required more in the somites than in the neural tube as in contrast to the somites, there is still occasionally weak ptc1 expression in the ventral neural tube of yot homozygotes, and ptc2 expression is specifically lost from the rostral somites of yot homozygotes, whereas it is only slightly reduced in the neural tube.

con

The effects of con mutations on ptc1 expression at late somitogenesis stages are similar to those of either yot or syu, expression being almost completely eliminated from the CNS and somites. Yet at earlier stages of development con mutant embryos, like syu, exhibit a much weaker effect on ptc1 and myoD expression than is seen in yot homozygotes. This apparent difference in the temporal requirement for con function is more difficult to rationalise than that for syu or you: unlike syu, con does not map to any known hh gene nor does it affect the expression of the three hh genes expressed in the midline structures, and unlike you, con has a dramatic reduction of ptc1 expression at later stages. Thus, like yot, con most likely acts downstream of Hh signals, yet the requirement for its activity seems greater at later stages of embryogenesis. Given the similarities between the effects of syu and con on ptc1 and myoD expression, a simple explanation could be that con is specifically required for the transduction of Shh activity. Against this, however, the effects of con mutations on muscle differentiation are strikingly different from those of syu. Like the other u-type mutants, con homozygotes lack most MPs, but in contrast to the other mutants the differentiation and migratory behaviour of all other slow muscle cells appears completely normal. This phenotype is intriguing since it separates the specification of slow muscle cells in general from the induction of MPs in particular, and implies that con may be specifically required only for the latter process. Previous studies have shown that in ntl and flh mutants, muscle pioneers fail to differentiate despite the specification of slow muscle cells (Halpern, M. E. et al. 1993; Halpern, M. et al. 1995; Currie, P. D. and Ingham, P. W. 1996; Odenthal, J. et al. 1996; Stemple, D. et al. 1996; Blagden, C. S. et al. 1997); and since axial midline cells in ntl and flh embryos express shh but not ehh, it was proposed that ehh is specifically required for MP induction (Currie, P. D. and Ingham, P. W. 1996). Thus according to this model, con might be specifically required for ehh signalling. However, this is unlikely to account for the observed loss of ptc1 expression in the CNS and in the pectoral fin buds where shh but
not ehh is expressed. An alternative explanation may be that con is maternally expressed - in this case, the effects of zygotic loss would only be manifest relatively late in development, for instance, after slow muscle specification but prior to MP induction.

At late somitogenesis stages, ptcl is expressed at higher levels in the heads than in the trunks of homozygous con embryos, in a similar way to yot. However, at 32 hpf ptcl expression is reduced in the brains of con homozygotes - unlike in yot which at this stage has normal expression of ptcl in the brain. The reduction of ptcl expression in con brains is most noticeable in the hindbrain, whereas the reduction in syu homozygotes is more severe in the forebrain. This difference is presumably due to the expression of twhh, which does not completely mirror shh expression in the heads at this stage, and it also suggests that con is not a complete loss of function, or that there is functional redundancy between con and other components of the pathway in the brain. This is also the case in the pectoral fin buds, where unlike in syu homozygotes, the expression of ptcl and ptc2 is reduced but not eliminated.

igu

The effects of mutations in igu on both Hh target gene expression and muscle development are strikingly different from those of the u-type mutants. As in you, syu and con homozygous embryos, expression of myoD is initially normal in igu homozygotes but by early somitogenesis the levels in the adaxial cells are reduced compared to wild type, an effect consistent with a partial loss of Hh signalling. By contrast, expression of ptcl is already aberrant at the bud stage, being at once reduced in level but expanded in its spatial domain, the latter effect being suggestive of a partial de-repression of Hh signalling. This ectopic expression of ptcl persists and increases in intensity as development proceeds, being particularly striking towards the completion of somitogenesis.

There are at least two plausible explanations of the igu phenotype: one possibility is that igu identifies a gene encoding a downstream negative regulator of the pathway such as Ptc, Protein Kinase A (PKA) or the Costal-2 (Cos-2) protein. In Drosophila, mutation of the genes encoding each of these proteins causes the de-repression of the Hh pathway, (reviewed in Ingham, P. W. 1998a; Johnson, R. L. and Scott, M. P. 1998). However, both ptcl and a second zebrafish ptc homologue, ptc2, map to different linkage groups from igu, so both are ruled out as candidate genes for the igu locus. However, it remains possible that igu could correspond to either a zebrafish homologue of cos-2 or indeed to a PKA catalytic subunit. Alternatively, igu may identify a novel component without any
known counterpart in Drosophila. Either way, if this hypothesis is correct, then the reduced expression of myoD is puzzling, as we would predict it to also be expanded, rather than reduced.

An alternative, more complex scenario would be that the igu mutants are hypomorphemic alleles of a positive regulator of the Hh pathway. In this event, the ectopic expression of ptcl could ensue as a consequence of the initially low levels of Ptc1 protein failing to sequester Shh and/or Ehh protein efficiently, thus allowing it to diffuse further and activate ptcl transcription inappropriately, a phenomenon comparable to that observed in Drosophila when the Hh receptor component Smo is inactivated (Chen, Y. and Struhl, G. 1996). In addition, other morphological aspects of the igu phenotype suggest a reduction in Hh signalling. For example igu homozygotes sometimes form an ectopic lens in the position of the mouth and they have an ipsilateral rectinotectal phenotype like yot; their eyes are closer together as are those in yot and con; and they have impaired floor plate development (specifically they lack the lateral floor plate cells (Odenthal, J. et al. 1998)) and a curly tail body shape which resembles the phenotypes of yot, syu, con and you (van Eeden, F. et al. 1996). However, the epistasis of igu over syu mutations indicates that the phenotype cannot be due to the inappropriate diffusion of Shh protein. It might be possible to test whether the ptcl expression in igu homozygotes requires Hh protein by examining ptcl expression in bozozok/igu double mutants or flh/igu double mutants, as both flh and bozozok lack ehh and have reduced shh expression.

Spatial differences in the requirements for particular genes.

There is no evidence from any of these mutations that separate pathways exist for different hh genes, just as there is no evidence for separate pathways from my investigations of the regulation of ptc gene transcription by different Hh proteins. However there is evidence that there are spatial differences in the requirements for particular components within the same pathway. For example con, igu and yot can all be implicated in shh signalling as con and igu both have reduced ptcl expression in the pectoral fin buds; iguana is epistatic to syu; and the severity of the yot phenotype suggests that it is required for shh signalling (loss of ehh in ntl homozygotes does not produce such a dramatic reduction of slow muscle) as well as for additional Hh signalling (its phenotype is more severe than syu). However yot is not required in the pectoral fins, and may not be required in the brain, even though con is required in both of these places.
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One possible explanation of why so many of these mutations affect the somites more than the neural tube, and the trunks more than the heads could be that the activity of ehh is lower than that of twhh or shh at least at late somitogenesis stages. In this scenario there would be more Hh activity in the heads and the ventral neural tube of embryos and hence in mutations that are not complete nulls, there would be more expression of ptc1 in these areas. However the early expression of ptc1 in syu homozygotes suggests that ehh activity is high enough at tail bud and very early somitogenesis stages to maintain adaxial ptc1 expression even in the absence of shh expression. Also, as con homozygotes have reduced ptc1 expression in the brain at 32 hpf but yot homozygotes do not, yot is probably not required in the brain.

C. The role of Hh signals in patterning the myotome

Shh is required for most, but not all slow muscle formation

Pete Currie analysed the muscle fibre phenotype of syu homozygotes and showed that Shh is required for the formation of most, but not all, of the slow muscle fibres in zebrafish somites and is not required for the development of fast muscle fibres. In addition my own analysis, and that of others, confirms a role for Shh in the induction of muscle pioneers (chapter 8; van Eeden, F. J. M. 1997; Schauerte, H. E. et al. 1998; Lewis, K. E. et al. 1998). This is all consistent with conclusions from earlier studies that examined mutants that affect the development of the notochord, and hence reduce the amount and quality of Hh signal present in embryos, as well as examining the effects of over-expressing hh genes or PKA (Blagden, C. S. et al. 1997; Du, S. J. et al. 1997).

It is still possible that the induction of slow muscle precursors depends on a high concentration of general Hh activity rather than on Shh per se, but this would imply that shh normally provides much higher levels of Hh activity than ehh, as in contrast to syu homozygotes, mutations that affect the development of the notochord and hence have no ehh expression, but retain shh expression, still form substantial slow muscle (Blagden, C. S. et al. 1997). However, ptc1 expression in the different Hh pathway mutants, also (as discussed above) suggests that ehh may have less activity, at least by mid to late somitogenesis stages, as assayed by ptc1 expression, than twhh or shh. This hypothesis, that the overall level of Hh signal is crucial, rather than qualitatively distinct signals from
particular hh genes, is also consistent with the residual slow muscle that still forms in syu homozygotes, which we argue below is due to signalling by other Hh proteins.

Surprisingly, my analysis of myoD and ptc1 expression shows that, although Shh is required for the formation of most of the slow muscle (P. Currie's data in Lewis, K. E. et al. 1998) it is not required for the initial expression of ptc1 or myoD, though it rapidly becomes required for the maintenance of the expression of these two genes (both are beginning to be reduced by 5 somites, which is less than two hours after tail-bud stage). This also suggests that initial expression of myoD in the adaxial cells is not sufficient for correct formation of slow muscle, but that continued high level expression is needed, probably for at least 2 hours after gastrulation.

Conclusions from igu

The opposing effects of igu mutations on slow muscle markers and myoD expression also pose an interesting paradox. Since overexpression of Hh activity can induce both ectopic ptc1 expression and supernumerary muscle pioneer differentiation throughout the somites, the finding that igu homozygotes are characterised by both ectopic ptc1 expression and supernumerary muscle pioneers is not entirely surprising. Previous analyses have, however, suggested that a pre-requisite for such an expansion of muscle pioneer cell fate throughout the myotome is the precocious expression of myoD throughout the myogenic mesoderm (Currie, P. D. and Ingham, P. W. 1996). igu mutant embryos, on the contrary exhibit no such expansion of myoD expression and indeed show levels of expression within the adaxial cells comparable to those seen in con or you homozygotes. Yet despite this, Pete Currie found that the slow variant myosin heavy chain isoform is in fact ectopically expressed throughout the myotome of the somites in igu embryos - intriguingly, cells simultaneously express both the slow and fast isoforms, as if the cells now have a mixed character, a situation never observed in wild type embryos or indeed following mis-expression of Hh proteins by mRNA injection. This may indicate that a partial de-repression of the Hh pathway is sufficient to activate expression of the slow myosin heavy chain gene but not sufficient to repress transcription of the fast isoform, and may imply that myosin heavy chain gene expression is regulated by Hh signalling independently of the activity of MyoD, or that MyoD activity promotes the development of slow muscle fates by suppressing fast muscle development rather than by directly inducing slow muscle fates.
Another interesting aspect of the *igu* phenotype is that the expansion of slow muscle and of muscle pioneers is limited to the anterior somites. This is reminiscent of the effect of *shh* injection, which can affect muscle pioneers more substantially in anterior somites, maybe because less *shh* RNA is still present when the more posterior somites are forming muscle pioneers (Du, S. J. et al. 1997). If ectopic Hh signalling in *iguana* decays with time this would produce a similar effect. An intriguing possibility is that the increasing expression of *ptc* throughout development in *igu* homozygotes could produce this reduction in the ectopic Hh activity. Ptc normally acts to repress the Hh pathway in the absence of Hh signals and it is possible that there is a balance between Ptc repression of the pathway and Hh activation. Maybe by later stages there is enough Ptc in *iguana* homozygotes to repress the weak ectopic Hh activation and prevent ectopic slow muscle or muscle pioneers from forming in the tail. Alternatively, if these aspects of the phenotype are caused by an abnormal distribution of Hh proteins in the somites, by these later stages the expression of *ptc* may be substantial enough to correctly localise Hh protein to the medial region of the somites.

Another intriguing aspect of the *iguana* phenotype is that the ectopic muscle pioneers in *iguana* also form mainly in the ventral somite. This could suggest that there is a dorsal-ventral difference in the requirement for *igu* in the somites, but in this case I would also expect *ptc1* expression to show a dorsal-ventral difference, which it does not. Alternatively it could suggest that there is a dorsal repressor of Hh activity, downstream of *ptc1* expression, that is capable of suppressing weak ectopic Hh activity. Consistent with this, ectopic Hh activity can also preferentially induce ectopic MPs in the ventral somite (Currie, P. D. and Ingham, P. W. 1996); and Devoto and Du suggest that BMPs act as a dorsal repressor of muscle pioneer formation and they show that a bmp like molecule can repress muscle pioneer cell formation and that it appears to act downstream of *shh* (Du, S. J. et al. 1997).

**Small amounts of slow muscle can be induced by other *hh* genes**

Several lines of evidence suggest that the slow muscle that still forms in *syu* homozygotes is due to the activity of other *hh* genes. Firstly I show in chapter 6 that over expression of *ptc1* can completely eliminate slow muscle from some segments and can produce a concomitant loss of *myoD* expression in the adaxial cells even at the onset of somitogenesis, in contrast to the reduced but continuous expression of *myoD* observed in *syu* mutants at the equivalent stages. This is consistent with *ptc1* inhibiting all Hh
signals, and with the residual slow muscle and \textit{myoD} expression in \textit{syu} homozygotes being due to other \textit{hh} genes, most probably to \textit{ehh} as this is also expressed in the notochord, and hence in close proximity to the adaxial cells. This also agrees with earlier experiments that produced a similar severe loss of slow muscle fibre types by overexpressing constitute active PKA (Du, S. J. et al. 1997) and with the phenotype of \textit{yot}, which has a much more severe reduction of slow muscle and of early \textit{myoD} expression than \textit{syu} homozygotes, presumably because mutations in \textit{yot} reduce signalling from both \textit{shh} and other \textit{hh} genes. These conclusions are also consistent with the previous finding that overexpression of Ehh can induce ectopic \textit{myoD} expression albeit much less efficiently than Shh (Currie, P. D. and Ingham, P. W. 1996).
BACKGROUND

In this chapter I will introduce the $talpid^3 (ta^3)$ chicken mutation and describe the analysis of this mutation that I undertook in collaboration with Cheryll Tickle and Litsa (G) Drossopoulou and Katie Robertson in her laboratory. I became interested in the $ta^3$ mutation after reading (Francis-West, P. H. et al. 1995) as the results described in this paper suggested to me that the $ta^3$ phenotype might be due to a mutation in $ptc$. Cheryll Tickle was my University of London supervisor, and our laboratory was already collaborating with Cliff Tabin's laboratory on vertebrate $ptc$ so I proposed looking at $ptc$ expression in $ta^3$. Just as I obtained my first results problems developed with the flock in Edinburgh and 18 months elapsed before we could obtain any more embryos. Luckily, the flock survived and the supply of embryos resumed in October 1997. However, even when the flock is healthy, many of the eggs laid are infertile, so the number of $ta^3$ embryos that we have been able to work with is very small: often a batch of about 50 eggs contains only 3-5 fertile $ta^3$ embryos.

Unless otherwise noted, I did the in situ hybridisations and the primary analysis of the results described in this chapter, but the grafts, and insertion of Shh beads, were all done by Litsa (G) Drossopoulou. Both Litsa and myself fixed and processed embryos. When I initially started this analysis Katie Robertson fixed wild type and $ta^3$ embryos for me.

INTRODUCTION

$Talpid^3 (ta^3)$ is a pleiotropic, recessive, autosomal, embryonic-lethal chicken mutation in an unknown gene. It was discovered by Hunton in about 1960 in the Light Sussex Flock at Wye College and named for its similarity to the $talpid$ mutation discovered in 1936 and described by Cole in 1942 (Ede, D. A. and Kelly, W. A. 1964; Hunton, P. 1960; Cole, R. K. 1942). $ta^3$ is now maintained in Dave Burt's laboratory at the Roslin Institute in Edinburgh.

The first $talpid$ mutation was named after the short spade like / mushroom shaped wings that were thought to resemble the forelimbs of the mole ($talpa$) but this mutation was already lost by 1959. A second $talpid$ mutation $talpid^2$ was discovered in 1953 in America (Abbott, U. K. et al. 1959). All the $talpid$ mutations have similar phenotypes and are
"talpid\(^3\)"

autosomal, recessive mutations in single genes, but the complementation has never been tested so its not known whether they correspond to the same gene or not. A comparison of the descriptions of the different phenotypes suggests that talpid\(^3\)>talpid\(^1\)>talpid\(^2\) in severity. For example ta\(^3\) usually dies after about 5 - 6 days; ta\(^1\) after about 8-10 days and ta\(^2\) after about 10-14 days; and ta\(^2\) is reported to have a normal brain though it has an abnormal beak and face, whereas ta\(^3\) and ta\(^1\) have more severe head phenotypes with abnormal faces and abnormalities in the brain such as multiple epiphysis and lenses (Cole, R. K. 1942; Abbott, U. W. et al. 1960; Ede, D. A. and Kelly, W. A. 1964; Ede, D. A. and Kelly, W. A. 1964).

As mentioned above talpid\(^3\) is pleiotropic. For example its eyes are closer together than normal and sometimes fuse, the prechordal mesoderm is continuous across the midline in the head, there is no upper beak, ectopic lenses and multiple epiphyses sometimes form, there is abnormal feather development if embryos survive that long, the vertebral column is shortened and adjacent vertebrae sometimes fuse, the neural tube is misshapen / ovoid, the myotome seems to be disorientated and to mingle with the sclerotome and the dermis is considerably reduced, and no cartilage replacement bone forms. The morphological descriptions describe the neural tube as looking like "it has been forced up dorsally": the spinal ganglia are high up dorsally; the central canal does not extend as far ventrally and the floor plate is misshapen and looks rounded up. I have also seen double neural tubes in the posterior of mutant embryos. The embryos also have extreme ectopia and subcutaneous oedema (Ede, D. A. and Kelly, W. A. 1964; Ede, D. A. and Kelly, W. A. 1964).

The limbs of talpid embryos have been more extensively analysed than the rest of the embryo and are the only regions of the embryo that have been characterised molecularly. Talpid limbs are polydactylous. Initially the limb buds are short and broad and eventually (if the embryos survive long enough) in ta\(^3\) there are about 7 digits in both the fore limb and hind limb which are all fused (compared to 3 digits in the wild type wing and 4 digits in the wild type hind limb) and look morphologically similar. The forelimb is recognisable as ta\(^3\) before the hind limb - the forelimb can be reliably identified by stage 22 but the hind limb can not be reliably identified till stage 25. There is no obvious morphological anterior-posterior polarity in either limb (Hinchliffe, J. R. and Ede, D. A. 1967; Ede, D. A. and Kelly, W. A. 1964).

However some anterior-posterior pattern is still identifiable in talpid\(^2\) limbs. In the legs there are 7-10 digits that all look like digit 3, but in the wing it is often possible to
talpid³

distinguish the most posterior digit from the rest (though none of the digits look like a normal digit), and the radius is always distinguishable form the ulna. Also, if a wild type ZPA is grafted into the anterior of a talpid² limb bud the radius can be respecified to form a second ulna. The number of digits varies in talpid² wings, even between different sides of the same embryo, but ranges from 5-10 (Dvorak, L. and Fallon, J. F. 1991; Dvorak, L. and Fallon, J. F. 1992).

Another striking aspect of the talpid limb phenotype, in both ta² and ta³, is the absence of the normal areas of anterior and posterior cell death (anterior and posterior necrotic zones). This may add to the morphologically broader limb phenotype but it does not completely explain this phenotype, as the talpid limb buds are already broader than in wild type embryos before the necrotic zones normally form (Dvorak, L. and Fallon, J. F. 1991; Hinchliffe, J. R. and Ede, D. A. 1967).

Recombination experiments - recombining wild type mesoderm and talpid ectoderm and vice versa showed that the defect in ta² and ta³ is in the mesoderm: recombinant limbs develop with the phenotype determined by the mesoderm and not the ectoderm (Goetinck, P. F. and Abbott, U. K. 1964; Ede, D. A. 1980; Ede, D. A. and Shamslahidjani, M. 1983).

Previous Molecular Analysis of talpid Limbs

Before I started analysing the ta³ mutation, the expression of several genes had already been investigated in ta³ limbs and it was primarily the results of this work that inspired me to work with this mutation. Several genes that are normally posteriorly restricted in the chicken limb bud, and that have been postulated to be downstream targets for Shh signalling, are expressed throughout the anterior posterior axis of ta³ limb buds. These include bmp2 and bmp7; FGF4; Hox 4.6, 4.8 and d-13; and cek-8 (which is a gene that is thought to be downstream of Bmp2). However shh expression is still posteriorly restricted (Izpisúa-Belmonte, J.-C. et al 1992; Francis-West, P. H. et al. 1995; Patel, K. et al. 1996). This suggests that the ta³ gene acts somewhere between shh transcription and bmp and HoxD gene expression. This is consistent with the phenotype of ta³, which as well as affecting the development of the limbs also, as described above, includes abnormalities in many other patterning processes where Shh signalling has been implicated, such as dorsal/ventral patterning of the neural tube and somites, correct spacing of the eyes and development of the face and feathers (Ede, D. A. and Kelly, W. 180
A. 1964; Ede, D. A. and Kelly, W. A. 1964). However, as discussed in chapter one, it is unclear whether *bmp* and 5' *HoxD* genes are direct targets of Shh signalling or are further downstream, and therefore it was unclear at which point in these developmental signalling cascades *ta* normally acts. I was therefore interested in investigating *ptc* expression in *ta*. Only one homologue of *ptc* has been cloned so far in chicken, so all the analysis of *ptc* described in this and the following chapter is with this single *ptc* gene. *ptc* is a direct target of Hh signalling in Drosophila (Ingham, P. W. 1993; Forbes, A. J. et al. 1993; Alexandre C. et al. 1996; Strigini, M. and Cohen, S. M. 1997; Struhl, G. et al. 1997b) and all the evidence so far suggests that this is also the case in vertebrates (Goodrich, L. V. et al. 1996; Marigo, V. et al. 1996; Concordet, J.-P. et al. 1996; Goodrich, L. et al. 1997; chapters 4, 5, 7, 8, 11). Thus the pattern of *ptc* expression in *ta* should show whether *ta* acts in the Shh pathway, or downstream of Shh signalling. *ptc* was also a possible candidate for *ta*, because as Ptc represses Hh pathways in the absence of Hh signals, mutations in *ptc* usually result in ectopic activation of Hh pathways throughout the embryonic fields that are competent to respond to Hh signals (Ingham, P. W. et al. 1991; Goodrich, L. et al. 1997). Therefore I would expect a *ptc* mutation in chicks to have a similar phenotype to *ta*: normal expression of *shh* but expanded expression of Shh target genes, such as *bmps* and 5' *HoxD* genes.

Molecular analysis of *talpid* has produced similar findings to analysis of *ta*. In *ta* embryos *Hox d-11, Hox d-12* and *Hox d-13* are no longer posteriorly restricted; *bmp2* is uniformly distributed around the rim of the limb bud; and *FGF-4* is expressed throughout the AER but *shh* expression is still normal (Rodriguez, C. et al. 1996). Similarly *GHox-7* which is normally expressed in a graded manner across the anterior posterior axis with higher levels anterior, is expressed uniformly across the anterior-posterior axis of *talpid* limbs, at a low level that resembles the expression normally found in the posterior of the limb bud; *GHox-8* which is expressed in the anterior mesoderm and the AER of wild type limb buds is not expressed in the mesoderm of *talpid* limb buds, though it is still expressed in the AER; and *GHox-4.6* which normally has a posteriorly restricted expression pattern in limb buds is also expressed in anterior mesoderm in *talpid* (Krabbenhoft, K. M. and Fallon, J. F. 1992; Coelho, C. N. D. et al. 1992).

Similar expression patterns of *Hox d-11, Hox d-12, Hox d-13, bmp2, FGF-4* and *shh* are also seen in embryos homozygous for two other chicken polydactylous mutations - *diplopodia* and *diplopodia*, which both have ectopic digits pre-axially in addition to...
talpid<sup>3</sup>

normal digits, and therefore are more reminiscent of limbs that have been exposed to ectopic polarising activity in the anterior of the limb than ta<sup>3</sup> embryos (Rodriguez, C. et al. 1996). talpid<sup>2</sup> and diplopodia are unlinked and the double mutants look most like ta<sup>2</sup> (Abbott, U. W. et al. 1960).

Despite <i>shh</i> being expressed normally the distribution of polarising activity in ta<sup>3</sup> limbs is abnormal. Previous experiments have established that there is a gradient of polarising activity in ta<sup>3</sup> limbs: the posterior has polarising activity like wild type limbs, though it is weaker and/or more variable in ta<sup>3</sup> limbs (an ectopic digit 4 was only seen in 55% of ZPA grafts to the anterior of wild type limbs); but the apical and anterior regions of the limb bud also have weak polarising activity. However, this analysis also suggested that polarising activity decreases with age in ta<sup>3</sup> (Francis-West, P. H. et al. 1995).

Despite the gradient of polarising activity across the ta<sup>3</sup> limb, grafting a wild type ZPA to the anterior of a ta<sup>3</sup> limb bud does not induce any more digits to form, however the digits become more separated, though they are still unidentifiable. In contrast if a low dose of Retinoic Acid, which would cause digit duplications in wild type limbs, is applied to the anterior of a ta<sup>3</sup> limb bud it reduces the number of digits (Francis-West, P. H. et al. 1995).

My analysis of <i>ptc</i> expression in ta<sup>3</sup> suggested that ta<sup>3</sup> acts between Shh and the transcription of <i>ptc</i>, but linkage analysis showed that the ta<sup>3</sup> phenotype is not due to a mutation in <i>ptc</i> itself. Therefore I examined the expression of other components of the Shh pathway in ta<sup>3</sup> embryos. We also investigated whether the defect in ta<sup>3</sup> was in the signalling cells or the responding cells by determining whether ta<sup>3</sup> limbs could respond to purified recombinant Shh protein and whether ta<sup>3</sup> mesoderm was capable of signalling to wild type mesoderm. All of this analysis is described in this chapter. Where numbers are given these usually refer to the number of ta<sup>3</sup> limbs analysed, or the number of experimental manipulations performed.
**Results**

*ptc expression in talpid*<sup>3</sup> *limbs*

As discussed above, *shh* is normally expressed in *ta*<sup>3</sup> limbs (confirmed in these experiments for 7 legs and 3 wings, fig. 44B; first described in Francis-West, P. H. et al. 1995). However, in contrast *ptc* expression is clearly very different in normal and *ta*<sup>3</sup> limb buds. In wild type chick limbs, *ptc* is expressed at high levels in the posterior mesenchyme, its expression decreasing in a graded manner towards the anterior of the limb (fig. 44C shows a strongly stained wing), but high levels of *ptc* expression are never detected in *ta*<sup>3</sup> limbs (0/26, 15 wings and 11 legs - these numbers include limbs that had a Shh bead inserted into the anterior - see below). At stages 19 - 21 *ptc* is expressed at very low, uniform, levels throughout *ta*<sup>3</sup> limbs except right under the AER (fig. 44D). This resembles the level of expression in the very anterior of wild type limbs that have been stained for a similar length of time to the *ta*<sup>3</sup> limbs (cf. fig. 44C to D). In *ta*<sup>3</sup> limbs at stage 27 *ptc* expression is still very weak but has resolved into a wide distal band and a more medial proximal band that resemble the fused mesenchymal condensations that form in *ta*<sup>3</sup> limbs (Hinchliffe, J. R. and Ede, D. A. 1967) (2 wings and 2 legs - included in the total of 26 above; fig. 44F). In contrast wild type limbs still have strong posterior expression of *ptc* at this stage, but they are also beginning to express *ptc* anteriorly, also around developing skeletal elements (Marigo, V. et al. 1996; fig. 44E).

*ptc expression in the branchial arches and the mouths of *ta*<sup>3</sup> embryos*

In normal chick embryos, high levels of *ptc* expression are also clearly visible in the branchial arches and the mouth. The branchial arches are misshapen in *ta*<sup>3</sup> embryos, and consistent with our observations in the limbs, there is no high level expression of *ptc* in either the branchial arches or the mouth in *ta*<sup>3</sup> embryos (0/5) (cf. fig. 44 G to H). This shows that in a number of different embryonic regions, normal high level expression of *ptc* is missing in *ta*<sup>3</sup> embryos.
Figure 44: shh and ptc expression in wild type and talpid^3 embryos

Transcripts were revealed by in situ hybridisation with antisense RNA probes to shh (A & B) or ptc (C - J). (A, C, E, G & I) show expression in wild type embryos and (B, D, F, H & J) show expression in ta^3 embryos.

(A & B) ventral views of stage 20 wings: shh expression is still posteriorly restricted in ta^3 limbs. (C & D) dorsal views of stage 20 wings, both stained for a similar length of time (several hours). ptc is only expressed at very low levels in ta^3 limbs (D), resembling the low basal levels of ptc expression in the anterior of wild type limbs (C). (E & F) dorsal views of stage 27 legs, the ta^3 leg was stained for much longer than the wild type leg. ptc is just beginning to be expressed in the anterior of the proximal wild type leg (E) where mesenchymal condensations are occurring and Ihh is beginning to be expressed (very weak ptc expression is just about visible underneath the *), and in the ta^3 limb (F) low level expression of ptc seems to be pre-figuring the fused condensations that form in ta^3 limbs. (G & H) branchial arches at stage 20/21. The branchial arches are misshapen in ta^3 and do not have the normal high level expression of ptc that occurs in the wild type arches. (I & J) wings that had a Shh bead implanted into the anterior, were then grafted to a wild type host, and fixed 18 - 23 hours later. Recombinant Shh induces high level expression of ptc in wild type (I) but not in ta^3 limbs (J).
talpid  

wild type

shh

ptc
Does the \( ta^3 \) mutation result in an altered Shh signal or an altered response to the Shh signal?

This lack of high level \( ptc \) expression in \( ta^3 \) embryos suggested that there was either a defect in the ability of \( ta^3 \) tissue to respond to Shh signals, or a difference in the Shh signal itself (\( shh \) RNA is expressed normally in \( ta^3 \) but it was possible that there was a defect in the processing of the Shh protein). We therefore investigated both of these possibilities.

a.) Recombinant Shh protein does not induce high level expression of \( ptc \) in \( ta^3 \) limbs.

To determine whether the ability of mesodermal cells to respond to Shh was affected in \( ta^3 \) embryos we investigated whether it was possible to induce high level expression of \( ptc \) in \( ta^3 \) limbs with purified Shh protein. Litsa Drossopoulou inserted beads soaked in Shh protein into the anterior of \( ta^3 \) limbs and I analysed \( ptc \) expression 18-24 hours later. In one case a bead was implanted into the wing of a \( ta^3 \) embryo and the embryo was fixed 24 hours later. The contra-lateral wing and the wing with the implanted Shh bead had identical low level expression of \( ptc \). In a number of other cases the manipulated \( ta^3 \) limb was grafted to a wild type chicken embryo incase the \( ta^3 \) embryo died before I could assay for \( ptc \) induction. \( ptc \) expression was also never induced in these \( ta^3 \) limbs (0/7 - 3 legs and 4 wings). However, in these cases, there was clear induction of \( ptc \) in the flank of the wild type host, suggesting that the Shh protein from the bead was active and had diffused across the \( ta^3 \) limb (fig. 44J).

Beads were also inserted into wild type wings and these wings were grafted to different wild type hosts to check that the grafting procedure did not interfere with the ability of limb tissue to respond to ectopic Shh protein. In these cases \( ptc \) was strongly induced in the wild type limbs (2/2). There was also weak expression of \( ptc \) induced in the flank of the host embryo (fig. 44I).

These experiments show that wild type and \( ta^3 \) limbs respond differently to Shh beads: unlike in wild type limbs, purified Shh protein does not induce high level expression of \( ptc \) in \( ta^3 \) limbs. This suggests that there is a defect in the response to Shh signals in \( ta^3 \) mesoderm. However, it was still possible that the endogenous Shh signal was also altered in \( ta^3 \), so we investigated the ability of \( ta^3 \) posterior mesoderm to induce \( ptc \) expression in wild type tissue.
**Posterar ta³ limb tissue can induce ptc in wild type tissue**

Litsa Drossopoulou grafted mesodermal tissue from the posterior of ta³ limbs to the anterior of wild type limbs and I assayed ptc expression after 18 hours (fig. 46). I found that posterior ta³ mesenchyme can induce high level expression of ptc in wild type limb tissue [7/8: 4 grafts showed strong induction and 3 showed weaker induction; see fig. 45A for an example of strong induction]. However, the grafted ta³ tissue still did not express high levels of ptc. Litsa Drossopoulou also showed that grafts of wild type posterior tissue induced high level ptc expression in the wild type host tissue [4/4: 3 grafts showed strong induction and 1 showed weaker induction; see fig. 45E].

Un-manipulated ta³ limbs grafted to wild type chicken embryos also induced ptc expression in the host flank near the posterior of the grafted ta³ limb, despite there still being no high level expression of ptc in the grafted ta³ limbs themselves (2/2, assayed 25-26 hours after grafting; fig. 45I). However ptc was never induced in the host flank when we grafted wild type limbs to wild type hosts (0/4; assayed 19-24 hours after grafting; fig 45H). This suggests that posterior ta³ tissue can induce ptc expression in wild type tissue, and that it can do so over a longer distance than posterior wild type tissue.

**Anterior and apical ta³ tissue can sometimes induce ptc in wild type tissue**

Polarising activity in ta³ limbs is not as posteriorly restricted as in wild type limbs at stages 20-24 (Francis-West, P. H. et al. 1995), so I was interested in investigating whether apical or anterior ta³ tissue could also induce high level ptc expression in wild type tissue. Therefore Litsa Drossopoulou also grafted anterior and apical ta³ mesodermal tissue into the anterior of wild type wings and I assayed for ptc expression (fig. 46). I found that some, but not all, of these grafts induced ptc expression in the wild type host tissue around the graft (2/4 anterior grafts and 1/1 apical graft; fig. 45B-D). In contrast Litsa Drossopoulou showed that grafts of anterior and apical wild type tissue never induced ptc expression (0/3 apical and 0/1 anterior; fig. 45F & G).
talpid^3

Figure 45: ta^3 mesoderm can induce ptc expression in wt tissue

Transcripts were revealed by in situ hybridisation with antisense RNA probes to ptc. (A, B & C) show grafts of ta^3 posterior, apical and anterior mesoderm respectively that induced ptc expression in the anterior of wild type wings. In all cases the induced ptc expression is in wild type mesoderm overlying the graft (confirmed with sections - data not shown). (D) shows an example of a graft of anterior ta^3 mesoderm that did not induce ptc expression. Grafts of wild type posterior mesoderm (E) induced high level ptc expression and grafts of apical (F) and anterior (G) wild type mesoderm did not induce any expression of ptc. In (F & G) the contra lateral wing is also shown for comparison. In all cases the grafts of mesodermal tissue are indicated with the yellow arrows.

(H & I) show wings that were grafted to a wild type host and fixed 24 - 26 hours later. ta^3 wings induced high level ptc expression in the wild type flank ((I); indicated with yellow arrow) unlike wild type wings grafted in the same way (H).
Figure 46: A Schematic of the Tissue Graft Experiments

Posterior, apical and anterior mesenchyme was removed from talpid or wild type limb buds as shown and grafted to the anterior of a host wing, under the anterior apical ridge. In all of these cases the ectoderm was removed from the donor limb bud after subjecting the mesenchymal tissue to trypsinisation for 1 hour.
Expression of gli genes in ta\(^3\) limbs

ci, the Drosophila homologue of the vertebrate gli genes, has been implicated as a transcription factor in the Drosophila Hh pathway and in particular as a transcription factor for the induction of high level ptc expression by Hh. In vertebrates Gli can also induce high level ptc expression but in addition the expression of the gli genes is regulated by Shh signals. For example an activated gli construct can induce ptc expression in the AER when retrovirally infected into the anterior of wild type chicken limbs, and shh mis-expressed in the anterior of wild type chicken limbs can induce high levels of gli expression and down-regulate gli3 expression (Marigo, V. et al. 1996). I therefore examined the expression of the two gli genes characterised so far in chicken - gli and gli3 (Marigo, V. et al. 1996) in ta\(^3\) limbs.

In normal limbs gli is expressed in the posterior in a domain similar to ptc (Marigo, V. et al. 1996; fig. 47A). However, in ta\(^3\) limbs there is no high level expression of gli: just very weak expression throughout the limb except at the distal edge underneath the AER (14/14 - 10 legs and 4 wings, stages 20-24; fig 47B). Again, as for ptc, the level of expression of gli in ta\(^3\) limbs resembles the most anterior expression in wild type limbs (cf. fig. 47B to 3A).

In contrast to ptc and gli, gli3 expression is expanded in ta\(^3\) limbs (7/7, 3 wings 4 legs, stages 20-23; see fig 47D & F). In wild type limbs, there is a clear area in the posterior of the limb where gli3 is not expressed (Marigo, V. et al. 1996; fig. 47C & E). This is not present in ta\(^3\) limbs: expression of gli3 extends to the very posterior of the limb and is strongest just under the AER, which is similar to what is seen in wild type limbs. In some limbs (4/7) there was a small posterior, proximal region in which gli3 expression was weaker ventrally, but the area of weaker expression was always very small and much less distinct than the clear region in wild type limbs. An area of weaker gli3 expression was only visible dorsally, in one of the limbs (a stage 22 leg; fig 47F).

These changes in the expression of gli and gli3 in ta\(^3\) limbs are consistent with my observations of ptc expression: the expression of all three of these genes suggests that ta\(^3\) limbs are not able to respond to Shh signalling.
talpid

The expression of \textit{bmp-2} is expanded in \textit{ta\textsuperscript{3}} limbs

It had been reported previously that \textit{bmp-2} is expanded in \textit{ta\textsuperscript{3}} limbs and expressed around the rim of the bud (Francis-West, P. H. et al. 1995). My results agreed with this with respect to the AER in \textit{ta\textsuperscript{3}} limbs, which have a very strong anterior expansion of \textit{bmp-2} expression, resulting in symmetrical expression of \textit{bmp-2} right around the limb bud (8/8, 4 legs, 4 wings, stages 21-24; fig. 47 H - K. Numbers do not include limbs that had a Shh bead inserted into them, see below). However, my analysis revealed further subtleties in the \textit{bmp-2} expression in the mesoderm of \textit{ta\textsuperscript{3}} limbs. \textit{bmp-2} expression is expanded in the mesoderm of \textit{ta\textsuperscript{3}} limbs, but it is weaker than the AER expression and there is always a thin band where \textit{bmp-2} is not expressed, just under the AER. Despite its expression domain being expanded, \textit{bmp-2} is sometimes still expressed at slightly higher levels in the posterior of the limb (6/8 limbs, fig. 47H & J): in one leg (stage 21/22) \textit{bmp-2} was expressed in a very clear, wide, anterior-posterior stripe of expression in the proximal limb bud that was stronger posteriorly and decreased anteriorly (fig. 47H), and in the other 5 limbs this pattern could also be distinguished as a slightly higher level of expression underlying expression throughout almost all the limb.

As \textit{bmp-2} is expressed in \textit{ta\textsuperscript{3}} limbs, unlike \textit{ptc} and \textit{gli}, this suggests that even though transcription of all these genes can be increased by Shh signals in normal limbs, these genes are regulated independently in \textit{ta\textsuperscript{3}} limbs. We therefore tested whether \textit{bmp-2} expression in \textit{ta\textsuperscript{3}} limbs is changed by inserting a bead soaked in Shh protein into the anterior of the limb. \textit{bmp-2} was expressed throughout these limbs in a similar manner to un-manipulated \textit{ta\textsuperscript{3}} limbs (5/5; assayed 22-23 hours after inserting the Shh bead). 4 of these limbs were grafted onto wild type hosts after the bead was inserted, but one was left to grow \textit{in situ} and survived, and in this case there were no obvious differences in expression levels between the wing with the bead and the contra lateral wing (fig. 47I).
**Figure 47: Expression of gli, gli3 and bmp2 in talpid\textsuperscript{3} limbs**

Transcripts were revealed by *in situ* hybridisation with antisense RNA probes to *gli* (A & B); *gli3* (C - F); or *bmp2* (G - K). (A, C, E & G) show wild type limbs and (B, D, F & H - K) show limbs from *ta\textsuperscript{3}* embryos.

(A & B) ventral views of stage 21+ wings from normal and *ta\textsuperscript{3}* embryos respectively, stained for the same length of time (several hours) in the same tube: *gli* is only expressed very weakly in *ta\textsuperscript{3}* limbs, at a similar level to the weak basal expression in the anterior of wild type limbs. (C & D) ventral views of stage 20 wings, note the posterior expansion of *gli3* expression in *ta\textsuperscript{3}* limbs. (E & F) dorsal views of a stage 25 leg (E) and a stage 22 leg (F). The *ta\textsuperscript{3}* leg (F) does have a very small posterior area without *gli3* expression, but this clear area is smaller and less distinct that in younger or older wild type limbs (cf. F to C & to E); (G & H) ventral views of stage 21 / 22 wing (G) and leg (H). Note anterior expansion of the normal mesodermal *bmp-2* expression domain in the *ta\textsuperscript{3}* leg (H). (I) shows a wing which had a Shh bead inserted into the anterior, and the contra lateral wing, fixed 23 hrs after the bead was inserted (so now about stage 24). The arrow shows the position of the bead, but both wings have identical expression of *bmp-2* (differences between the wings in the photo are due to the angle of the views being slightly different as the wing with the Shh has a more planar morphology). (J & K) ventral views of a stage 20 wing (J) and a stage 24 leg (K), both with *bmp-2* expression throughout the mesoderm, though in (J) slightly higher levels of expression can be distinguished in the posterior of the wing.
Apical and anterior \( ta^3 \) tissue can induce \( bmp-2 \) in wild type tissue

We also tested whether the ectopic expression of \( bmp-2 \) in \( ta^3 \) tissue was maintained in a wild type environment. Litsa Drossopoulou grafted tissue from the apical and the anterior of \( ta^3 \) limbs into the anterior of wild type wings (fig. 46) and I examined the expression of \( bmp-2 \) 18-20 hours later. In all cases \( bmp-2 \) was ectopically expressed in the wild type, host AER near the graft of \( ta^3 \) tissue and there was some remaining mesodermal expression of \( bmp-2 \) in the edges of the graft itself, but the central regions of all of the grafts were clear, and the remaining expression in the edges of the grafts was weaker than the ectopic AER expression or the wild type expression of \( bmp-2 \) in the posterior mesoderm of the limbs (3 apical grafts and 1 anterior graft; fig. 48). In contrast Litsa Drossopoulou showed that grafts of posterior wild type tissue induced \( bmp-2 \) expression in the neighbouring AER and in the host mesoderm around the graft, but grafts of apical wild type tissue did not (2/2 posterior grafts and 0/1 apical graft; data not shown).

Figure 48: \( talpid^3 \) mesoderm can induce \( bmp2 \) expression in wt tissue

Transcripts were revealed by \textit{in situ} hybridisation with antisense RNA probes to \textit{bmp2}. (A & C) show a wild type wing where apical \( ta^3 \) mesodermal tissue was grafted into the anterior of the wing and (B) shows a wild type wing where anterior \( ta^3 \) mesodermal tissue was grafted into the anterior of the wing. All three limbs were fixed 18-20 hours after the grafting procedure. In all three cases strong ectopic \( bmp-2 \) expression can be seen in the anterior AER and weaker \( bmp-2 \) expression can be seen in the mesoderm at the edges of the graft.
grafts of *talpid* tissue

Figure 48
Expression of coupTFII in wild type and ta^3 limbs

coupTFII is a direct target gene of Shh signalling in vitro but unlike ptc its transcription is probably not mediated by Gli (Krishnan, V. et al. 1997). In ta^3 limbs the expression of some target genes of Shh signalling is expanded (Francis-West, P. H. et al. 1995 and this chapter), but transcription of gli, gli3 and ptc no longer seems to respond to Shh signalling. I was therefore interested in determining whether the expression of coupTFII in ta^3 limbs followed either of these two patterns. It has been reported that coupTFII is expressed in the limbs of chickens but no details were given (Lutz B et al. 1994). I found that at stages 21 - 24 coupTFII is expressed in the centre of the limbs in the wild type chicken, though the wing expression is broader proximally and extends slightly more distal than the leg expression (fig. 49A & B).

In ta^3 limbs coupTFII expression is expanded. coupTFII is expressed throughout ta^3 wings, except for a thin band of no expression proximal to the AER (3/3, stage 20-26; fig. 49C & E). In younger ta^3 legs the expression of coupTFII is similar to ta^3 wings (2/2, stage 20; fig 49D) but in contrast, in older ta^3 legs, there is no expression of coupTFII in the posterior mesoderm (3/3 stage 25-26; fig. 49F) and there is a wider band of distal cells that does not express coupTFII than at younger stages, or in ta^3 wings of the same stage (cf. fig. 49E to 49F).

The expansion of coupTFII expression in ta^3 limbs suggests that this gene is regulated in a similar way to the normally posteriorly expressed bmp and Hox-D genes. However, the expression of coupTFII in wild type limbs is more anterior than these other Shh target genes and the experiments that suggest that coupTFII expression is induced by Shh signalling have been conducted either in vitro or in the neural tube (Lutz B et al. 1994; Krishnan, V. et al. 1997). Therefore, to establish if, and how, coupTFII can be regulated by Shh in the limbs we inserted Shh beads into the anterior of wild type wings, and looked at the expression of coupTFII after 18-19.5 hours. In most cases I observed an increase in coupTFII expression in the wing (4/5; fig 49G), though in a couple of cases (2/5) there was less high level expression around the bead than elsewhere in the limb (fig. 49H). One wing (fig. 49G) had an expression pattern that was very reminiscent of coupTFII expression in ta^3 wings. Therefore coupTFII expression can respond to Shh signalling in the limb and the expression of coupTFII in ta^3 limbs is consistent with an expansion in Shh signalling.
Figure 49: *coup TF II* expression in wild type and *talpid*\(^3\) embryos

Transcripts were revealed by *in situ* hybridisation with antisense RNA probes to *coup TFII*. (A, C, E) are all dorsal views of wings and (B, D, F) are all dorsal views of legs. (A & B) show limbs from wild type embryos and (C - F) show limbs from *ta^3* embryos. (A - D) show limbs at stage 20 - 21 and (E & F) show limbs from the same stage 26 embryo. Expression of *coupTFII* is expanded throughout almost all the limb bud mesoderm in *ta^3* embryos, except for the distal and posterior mesoderm in older legs.

(G & H) show wild type wing buds that had a Shh bead inserted into the anterior together with the contra lateral wings, fixed 18 - 19.5 hrs after the bead was inserted. In both cases, the arrow points to the position of the bead. In (G) the expression of *coup TFII* in the wing exposed to Shh resembles the expression of *coup TFII* in a *ta^3* wing (cf. C). In (H) the wing exposed to Shh has ectopic expression of *coupTFII* but the expression to the right of the bead is not as strong as elsewhere in the limb.
Figure 49
talpid³

The $ta^3$ gene is not $ptc$, $gli$, $shh$, or smoothened

Our analysis of gene expression in the $ta^3$ limb bud suggested that $ta^3$ is a component of the Shh pathway so Dave Burt's laboratory investigated several genes in the Shh pathway to see if they co-segregated with the $ta^3$ phenotype. They mapped the genetic location of these genes and then used SSCP polymorphisms that they had detected in the $ptc$, $shh$, $gli$ and smoothened ($smo$) genes for co-segregation analysis. $ta^3$ heterozygote parents ($n = 13$) and $ta^3$ progeny ($n = 19$) were genotyped for the polymorphisms in the candidate genes. The $ta^3$ phenotype is inherited as an autosomal, recessive lethal gene. $ptc$ was mapped to the Z sex chromosome and so clearly excluded as a potential candidate for the $ta^3$ defective gene. The mutant allele in all the $ta^3$ carriers is derived from a single grand sire (D. W. Burt, personal information). Therefore if the $ta^3$ gene defect were in one of these genes, then all $ta^3$ homozygous progeny should be homozygous for one of the candidate gene polymorphisms. Conversely, if there is no association then all genotypes for the candidate gene will be found within $ta^3$ homozygotes. Two SSCP alleles were detected for the $smo$, $shh$ and $gli$ gene markers. All classes of genotypes were found in the $ta^3$ mutants, thus excluding $smo$, $shh$ and $gli$ as the defective gene in the $ta^3$ phenotype. Unfortunately Dave Burt's laboratory were unable to extend this analysis to $gli3$ as they were unable to detect any polymorphisms in this gene.

Investigating gene expression in the trunk of $ta^3$ embryos

coupTFII is normally strongly expressed in two domains in the ventral neural tube (Lutz B et al. 1994; fig. 50A), and it has been implicated in the induction of motoneurons by Shh. Therefore I investigated whether coupTFII expression was altered in $ta^3$ neural tubes. I found that, unlike in $ta^3$ limbs, where coupTFII expression is expanded, coupTFII is not expressed in the neural tubes of $ta^3$ embryos (stages 21-24, different anterior-posterior levels), though it is still expressed at high levels in the lateral somites (fig. 50B and cf. 50A). However, the effect of the $ta^3$ mutation on shh expression also differs between the limb and the neural tube. In $ta^3$ limbs shh is expressed normally, but shh expression in the ventral neural tube is discontinuous and reduced in both the trunk and in the head, suggesting that the floor plate in $ta^3$ embryos is patchy and discontinuous (cf. fig. 50C-D & 50E-G). However, shh is still expressed normally in the notochords of $ta^3$ embryos (fig. 50G).

I also examined the expression of $ptc$, $gli$ and $gli3$ in the trunks of $ta^3$ embryos. $ptc$ is still expressed in the medial neural tube, though at weaker levels than in wild type embryos.
However, compared to wild type embryos, there is hardly any expression of ptc around the notochord in the ventral somites of ta<sup>3</sup> embryos (fig. 50H & I). gli is also still expressed in the neural tubes of ta<sup>3</sup> embryos but like ptc there is less expression of gli in the somites of ta<sup>3</sup> embryos (fig. 50J & K). In contrast gli<sub>3</sub> is strongly expressed in the neural tube and the somites in ta<sup>3</sup> embryos. Compared to wild type embryos the neural tube expression of gli<sub>3</sub> is expanded ventrally and appears stronger than in wild type embryos (fig. 50 cf. M to L).

I also observed characteristic morphological abnormalities in the ta<sup>3</sup> trunks. In agreement with earlier reports, the neural tubes were usually ovoid in appearance and had larger lumens than wild type neural tubes (Ede, D. A. and Kelly, W. A. 1964). In addition I observed a splitting of the main neural tube into two adjacent neural tubes in the posterior of the axis in two separate embryos.

Attempts to assay the distribution of Shh protein

I also tried to assay the distribution of Shh protein in ta<sup>3</sup> limbs directly using a Shh antibody (Marti, E. et al. 1995). Unfortunately I could not get the antibody to work reliably on wild type embryos and hence did not try it on ta<sup>3</sup> embryos. I either got no DAB staining, or staining only in the notochord and floor plate, or occasionally staining in the legs but not the wings. I tried a variety of different fixation times and tried both dehydrating embryos into EtOH before the antibody procedure and not dehydrating the embryos, but I still did not get reliable results. I also tried antibody staining on sections a couple of times, but again this did not work. If our predictions are correct (see discussion) and Shh protein is more widely distributed in ta<sup>3</sup> limbs then we might not be able to see this using the antibody, even if it is working reliably. However, in this case we hoped to be able to use the antibody on a wild type and ta<sup>3</sup> limb processed together, and see high level posterior staining in the wild type limb but not in the ta<sup>3</sup> limb. This experiment would still be interesting to do if we can get the Shh antibody to reliably work on wild type embryos.

Brief Summary and Conclusions

In summary, in this chapter I demonstrate that the ta<sup>3</sup> defect is in responding cells and not in the production of the Shh signal, and that genes that are regulated by Shh in the chicken limb behave in one of two abnormal ways in ta<sup>3</sup>: the transcription of ptc, gli and
**talpid^3**

gli3 no longer responds to Shh signalling, suggesting that ta^3 acts in the Shh pathway; but in contrast the expression of bmp, Hox-D and coupTFII genes is expanded. However, in the trunk, floor plate development and associated shh expression in the ventral neural tube is impaired, and coupTFII expression is lost. In addition, I present genetic evidence from Dave Burt's laboratory, that demonstrates that the ta^3 phenotype is not caused by mutations in ptc, gli, smo or shh. I will discuss these results, and the different conclusions that they suggest, in more detail in chapter twelve.

*Figure 50: Gene Expression in wild type and talpid^3 trunks*

Transcripts were revealed by whole mount in situ hybridisation with antisense RNA probes to coup TFII (A & B); shh (C - G); ptc (H & I); gli (J & K); and gli3 (L & M) and transverse sections were then cut by hand. (A, C, E, H, J & L) show expression in wild type embryos and (B, D, F, G, I, K & M) show expression in ta^3 embryos. Unlike in wild type embryos coupTFII is not expressed in the ventral neural tubes of ta^3 embryos (cf. B to A), shh is expressed normally in the notochord of younger ta^3 embryos (G and cf. E) but, in contrast to wild type embryos, it is already reduced and discontinuous in the ventral neural tube (compare F & G to E). (C, D, & F) are all taken from stages where Shh is no longer expressed in the notochord in either ta^3 or wild type embryos but it is still expressed in the floor plate. ptc and gli are no longer expressed at high levels in the ventral somites around the notochord in ta^3 embryos (cf. I & K to H & J) and gli3 expression is expanded ventrally in the neural tube (cf. M to L).
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Background

We were interested in establishing when \( ptc \) is first induced by ectopic Shh protein in the anterior of chicken limbs. Initially we were interested in this question because we were trying to establish if we could induce \( ptc \) with ectopic Shh in \( ta^3 \) limbs, and we wanted to know if \( ptc \) is normally induced quickly enough to allow us to insert beads into \( ta^3 \) embryos and then test for \( ptc \) induction before the embryos died. However, this is an interesting question in itself, partly because if \( ptc \) is a direct target of Shh signalling in vertebrates we would predict that its expression would be quickly increased in response to ectopic Shh signalling, and also in the context of the response of anterior limb tissue to Shh, Retinoic Acid (RA) and ZPA grafts (discussed below). Also, once we started looking at early responses of \( ptc \) to ectopic Shh (2-8 hrs) we became interested in the spatial aspects of the induction of \( ptc \) expression by Shh, as the pattern of induction was very different from what we had expected. We were also interested in establishing whether early \( ptc \) induction required the continued presence of Shh to be maintained and if so, when, if ever, \( ptc \) expression became independent of Shh. Again this is an interesting question in its own right, and also because of the results of previous experiments that I will describe below.

Introduction

Beads soaked in RA or Shh protein, cells expressing various forms of \( shh \) (full length, \( N-Shh \), and a membrane tethered form of \( shh \)), and ZPA grafts can all induce mirror image duplications of the digits when grafted into the anterior of chicken limbs (Yang, Y. et al. 1997; Tickle, C. et al. 1975; Tickle, C. et al. 1985; Eichele, G. et al. 1985; Riddle, R. et al. 1993; Lopez-Martinez, A. et al. 1995). The character of the ectopic digits depends upon the dose or number of cells used and the length of time of exposure to the ectopic polarising activity. Very low doses of Shh or RA or small numbers of polarising region or \( shh \) expressing cells have no effect on the digit pattern, and then as the dose or number of cells is increased an ectopic digit 2 (the most anterior digit in a normal wing) is produced; then an ectopic digit 3; and finally, at high concentrations of Shh or RA or with
Time Course Experiments

large numbers of polarising region or \textit{shh} expressing cells, an ectopic digit 4 is induced. If the source of ectopic polarising activity is removed again after different lengths of time, then a similar graded response can be seen with respect to length of exposure, with longer exposure producing digits that are more posterior in character (Smith, J. C. 1980; Tickle, C. 1981; Tickle, C. et al. 1985; Eichele, G. et al. 1985; Wanek, N. et al. 1991; Yang, Y. et al. 1997; fig. 51). The dose and time interact: both a low concentration of Shh left for a long time and a higher concentration of Shh left for shorter time can induce a digit 2 (Yang, Y. et al. 1997). However, with all of these manipulations there is an initial period of about 12-15 hours that is necessary for duplications to occur later, but which itself has no irreversible effect on wing digit patterns (fig. 51). If the source of polarising activity is removed after 12-15 hours a normal digit pattern is produced, but if a control bead is used for 12 hours and then replaced with a Retinoic Acid bead then digit duplications do not occur (Eichele, G. et al. 1985). This raises the question of what happens during this time period. Interestingly, this requirement for about 12 hours of exposure to polarising activity before any irreversible changes are produced is very similar to what has been reported in the trunk. Both notochord and floor plate can induce neural explants to express floor plate markers in vitro, but only when contact is maintained between the notochord/floor plate and the explants for more than 12 hours. (12 hours of contact did not produce induction but 18 hours of contact did - all the explants were cultured for a total period of 100 hours of which either 12, 18 or 24 hours were in contact with the inducing tissue) (Placzek, M. et al. 1993).

The role of Retinoic Acid in this digit duplication process is also unclear. At least some of the polarising activity of Retinoic Acid is probably mediated by Shh, as RA can induce expression of \textit{shh} in the anterior of the limb bud and may be required for normal \textit{shh} expression in the ZPA (Helms, J. et al. 1996; Stratford, T. et al. 1996; Riddle, R. et al. 1993), but there are a number of observations that suggest that this may not account for all of its activity. For example, beads soaked in low concentrations of RA can induce digit duplications and expression of \textit{shh}, but the mesoderm next to the bead does not acquire polarising activity. However, beads soaked in a high concentration of RA do result in the adjacent mesoderm acquiring polarising activity, but only after 12 hours (after 12 hours there is no polarising activity but by 18 hours there is) (Helms, J. et al. 1994). Similarly cell lines expressing \textit{shh} that do not induce full duplications on their own, can induce full duplications when they have been cultured with RA or when they are transiently transfected with a constitutive active RA receptor (Ogura, T. et al. 1996). In addition the timing of these re-patterning events suggest that the story may not be as
**Time Course Experiments**

simple as RA induces *shh* and then Shh re-patterns the limb. For example, beads soaked in RA or N-Shh need similar lengths of time to produce digit duplications (20 - 24 hrs for a full duplication - see fig. 51; Yang, Y. et al. 1997; Eichele, G. et al. 1985). This all suggests that RA and *shh* may act synergistically to establish ZPA like activity.

One problem with comparing the time required for different sources of polarising activity to produce irreversible cell fate changes is that we do not know the comparative concentrations of polarising activity produced by different types of source. Another complication is that endogenous Shh may be modified in different ways, such as being tethered to cholesterol and possibly also palmitic acid, which acts to increase the potency of the Shh signal presumably by tightly localising Shh to the membrane of expressing cells (Porter, J. et al. 1996; Pepinsky, R. B. et al. 1998). Therefore Retinoic Acid beads may produce changes faster than would be expected for a linear progression of RA - induces *shh* - induces ectopic digits, if RA has a very high comparative concentration (and induces more *shh* than Shh sources provide) or because the *shh* that it induces in neighbouring cells is correctly modified and hence more potent. However even low concentrations of RA can induce full digit duplications as fast as Shh beads (fig. 51).

All of this suggests that it would be interesting to compare the effects of RA and of Shh during this period of about 24 hours during which irreversible fate changes occur. A careful comparison of gene expression at different time intervals could help to establish if all the effects of RA are mediated by Shh, or if these two sources of polarising activity also act in parallel. Our investigations do not address this question, but by starting to analyse early responses of genes to Shh we have started to identify things that could be compared between RA and Shh.
Figure 51 A comparison of different sources of polarising activity.

A comparison of the different published results on the dosage and temporal response of anterior limb mesoderm to different sources of polarising activity. These are all averaged figures, taken, or calculated from the sources cited. The data refers to the time or dose at which most wings produce an ectopic digit of that identity or at which a curve of percentage respecification = 25%, 50% or 100% (Tickle, C. et al. 1985). For more detail about individual results see the papers.

**Dosage Response to Polarising activity**

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<td>10 - 100 µg/ml</td>
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**Temporal Response to Polarising activity**

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<tr>
<th>DIGIT 2</th>
<th>15 hrs</th>
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<th>15-16 hrs</th>
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<td>17 hrs</td>
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<td>DIGIT 4</td>
<td>(didn't see any - but probably removing this mesoderm)</td>
<td>24 hrs</td>
<td>20-24 hrs</td>
<td>19 hrs</td>
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* but cells then transplanted after differing lengths of time to test polarising activity.
Time Course Experiments

Results

Induction of ptc expression by ectopic Shh

To address the question of when and where ptc expression is first induced in response to ectopic Shh protein, we grafted beads soaked in N-Shh protein into the anterior of chicken wings at about stage 20, and then fixed these embryos at different time points. We then examined the expression of ptc using in situ hybridisation. In all of these cases the grafting was performed by Litsa Drossopoulou and apart from where noted to the contrary I performed the in situ hybridisations and subsequent analysis. I examined ptc expression in embryos fixed 2 hours, 4 hours, 6 hours and 8 hours after the Shh bead had been implanted. In all these cases, most of the embryos had higher levels of ptc in the operated wing than in the contra lateral wing, though the degree of ectopic ptc varied [3/4 after 2 hours; 5/6 after 4 hours; 3/3 after 6 hours; and 3/3 after 8 hours] (fig. 52 A-D). However, the pattern of ptc induction was different to what we had expected. Instead of high level ptc expression around the bead, low level ptc expression was seen to expand anteriorly from the normal wild type domain of ptc. In two cases fixed after 6 hours and one case fixed after 8 hours there was also a very small domain of ptc induced just over the bead. Litsa Drossopoulou also inserted Shh beads and fixed embryos 16 hours later and in all cases (3/3) there was strong ectopic expression around the bead, and weaker ectopic expression in the rest of the limb.

To investigate whether this increase in ptc expression in these limbs was simply a consequence of the operation, we repeated the experiments with beads soaked in buffer. These control manipulations and the subsequent in situ analyses were performed by Litsa Drossopoulou; she found no increase in ptc expression [0/3 at 4 hours; 0/3 at 16 hrs and 0/3 at 24 hours] (fig. 52 E - I).

The changes in the expression of ptc, in wings that have been exposed to ectopic N-Shh, could reflect the way in which N-Shh is released from Shh beads. To test this possibility Litsa Drossopoulou examined the pattern of ptc transcripts 4, 8, 16 and 24 hours after polarising region grafts. She saw ptc expression in the graft and ectopic expression in the anterior of the limb around the graft after 4 hours [5/5], ptc expression around the graft but no longer in the graft after 8 hrs [4/4]; around the graft [2/2] and in the graft [1/2] after 16 hours; and in the graft and strongly above the graft after 24 hours [4/4: 3 with very strong ectopic expression and 1 with weaker expression] (fig. 53). However, unlike
**Time Course Experiments**

the experiments with the Shh beads, the ectopic expression was always localised around the graft and did not increase first in the posterior of the limb bud.

**The initial expression of ptc needs Shh for its maintenance**

As described above Shh is needed continuously for about 15 - 18 hours for digit duplications to occur, so it is likely that any early response to Shh needs the continued presence of Shh to be maintained. Therefore we tested the reversibility of this early induction of ptc, by removing Shh beads 4-24 hours after they had been implanted into the anterior of the limb, and fixing the embryos several hours later. In one experiment we inserted beads, removed them after 4 hours and fixed the embryos 12 hours later (16 hours after bead insertion). The manipulated wings were flatter and more symmetrical in shape than the control wings, but any effects on ptc expression were subtle. 4/6 looked like they had slightly more expression of ptc anteriorly than the control wings (fig. 54C).

In a second experiment we inserted Shh beads, removed them after 16 hours and fixed the embryos 8 hours later (24 hours after bead insertion). Again the manipulated wings were rounder in shape than the control wings, resembling a ta^3 limb. However I saw no ectopic expression of ptc in these limbs. More surprisingly, in all of these limbs I saw a reduction in the normal ptc expression domain - so the manipulated wings had less ptc expression at this stage than the control wings [4/4]. However Litsa Drossopoulou repeated these experiments with three more embryos and she found that 2/3 had normal expression of ptc in the manipulated wing and the 3rd wing had a normal posterior domain of ptc expression but in addition it had a small amount of ectopic ptc expression just above where the bead had been. Therefore in total 1/7 of these wings maintained ectopic expression of ptc (fig. 54 D - F). Litsa Drossopoulou also inserted Shh beads, removed them after 24 hours and fixed after 36 hours. One embryo maintained high level ectopic expression of ptc (fig 54G) and the other embryo did not, but this second embryo was also not very healthy.

Litsa Drossopoulou also did some control experiments where she inserted beads soaked in buffer and then removed them and fixed at a later stage. Embryos where beads were removed after 16 hours and the embryos were fixed after 24 hours showed no ectopic expression of ptc and normal posterior expression of ptc [0/3]. One embryo where the beads was removed after 4 hours and the embryo fixed after 16 hours may have had very slightly more staining in the posterior, but if so it was very subtle [1/3] (fig. 54A, B).
Time Course Experiments

Brief Summary and Conclusions

In summary, the analysis that I present in this chapter demonstrates that recombinant N-Shh and polarising region grafts can both rapidly increase ptc expression in the chick limb bud, but continued exposure to Shh signals is required, at least initially, for this ptc expression to be maintained. In addition, we identify a difference in the spatial distribution of this increased ptc transcription, depending on whether we inserted a Shh bead, or grafted polarising region cells, into the anterior of the limb bud. This analysis and the conclusions that it suggests will be discussed in more detail in the following chapter.

Figure 52: Induction of ptc expression by ectopic Shh

Transcripts were revealed by in situ hybridisation with antisense probes to ptc. A bead soaked in N-terminal Shh protein was inserted into the anterior of wild type chicken wings and the embryo was then fixed 2-8 hours later. ptc expression was increased in the posterior of the limb bud after 2 hours (A) and after 4 hours (B). After 6 hours (C) ectopic expression of ptc extended right up to the Shh bead and by 8 hours (D) ptc was expressed throughout the manipulated limb. By 16 hours (G) or 18 hours (H & I) the ectopic expression of ptc was beginning to resolve to the region around the bead. Control experiments were done with beads soaked in buffer and these embryos were fixed after 4 hours (E) and 6 hours (F), but there was no ectopic expression of ptc. The arrows in (B, D, & G-I) point to the position of the bead.
Figure 52

<table>
<thead>
<tr>
<th>shh bead</th>
<th>ptc expression</th>
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<tr>
<td>A 2hrs</td>
<td>E 4hrs</td>
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<tr>
<td>B 4hrs</td>
<td>F 16hrs</td>
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<tr>
<td>C 6hrs</td>
<td>G 16hrs</td>
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<tr>
<td>D 8hrs</td>
<td>H 18hrs</td>
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control bead

Image links to shh bead and control bead at different time intervals.
**Time Course Experiments**

**Figure 53: Induction of ptc expression by polarising region grafts**

Transcripts were revealed by *in situ* hybridisation with antisense probes to *ptc*. Polarising regions (ZPA) from wild type embryos were grafted into the anterior of wild type chicken wings and the expression of *ptc* was then assayed 4 - 24 hours later. *ptc* was expressed in, and around the graft after 4 hours (A & B), around the graft but no longer in the graft after 8 hrs (C & D); around the graft (E & F) and sometimes in the graft (F) after 16 hours; and in the graft and strongly above the graft after 24 hours (G).

**Figure 54: Investigating if ptc expression becomes independent of Shh**

Transcripts were revealed by *in situ* hybridisation with antisense probes to *ptc*. A bead soaked in N-terminal Shh protein was inserted into the anterior of wild type chicken wings, then it was removed again after 4, 16 or 24 hours and the embryo was then fixed 8-12 hours later (C-G). Control experiments were done with beads soaked in buffer (A & B). One set of experiments removed the bead after 4 hours and fixed the embryos after a further 12 hours (A, C); another set removed the bead after 16 hours and fixed the embryos after a further 8 hours (B & D-F) and a final set of experiments removed the bead after 24 hours and fixed the embryos after a further 12 hours (G). In all cases an arrow indicates where the bead was inserted. The expression of *ptc* was still very slightly expanded in wings where the bead had been present for 4 hours and the wings were fixed 12 hours later (C). In embryos where the bead was removed after 16 hours and the embryos fixed 8 hours later some embryos seemed to have lower levels of endogenous *ptc* expression in the manipulated limb (D); some had normal *ptc* expression in the manipulated limb (E) and one example had a very small patch of ectopic *ptc* expression where the bead had been (F - indicated with the arrow). In one case where the bead was removed after 24 hours and the embryos fixed 12 hours later very strong ectopic expression of *ptc* persisted where the bead had been (G, indicated by the arrow).
24 hours
fixed after 16 hours
8 hours
fixed after 4 hours

In 16 hr; Fix 24 hr
In 4 hr; Fix 16 hr
In 16; Fix 24
In 4 hr; Fix 16 hr

Polarizing region grafts

Figures 53 and 54
A. Ectopic Induction of ptc Expression

The experiments described in chapter eleven demonstrate that ptc expression is rapidly up-regulated in response to ectopic Shh signalling in the chicken limb, suggesting that, like in Drosophila, ptc is a direct target of Shh signalling in vertebrates. This ectopic ptc expression initially requires continued Shh signalling but the results described in chapter eleven suggests that it may become independent of Shh signal after 16 - 24 hours (1/7 wings that had been exposed to ectopic Shh for 16 hours still had ectopic ptc expression 8 hours later and 1/2 wings that had been exposed to ectopic Shh for 24 hours still had expression 12 hours later). This initial requirement for continuous exposure to Shh activity for ptc expression is not surprising given that cells need to be exposed to polarising activity for at least 12 hours for irreversible fate changes to occur. However, we also found that 4/6 limbs exposed to ectopic Shh for only four hours still had slightly higher levels of ptc expression 12 hours later. Therefore, there may be a time lag between the removal of Shh activity and the loss of ectopic ptc expression. It would be interesting to investigate this in more depth as it bears on the interpretation of normal ptc expression patterns. If cells continue to express ptc for a number of hours after they were last exposed to Shh signals, then as a tissue such as the limb grows, cells that move away from sources of Shh will still express ptc for a number of hours. In this case the domain of high level ptc expression will demarcate cells that have seen Shh signals as well as cells currently responding to Shh signals, and a graded expression of ptc could at least partly reflect the differences in time since cells last responded to Shh signals.

We observed spatial differences in the ectopic ptc expression, depending on whether we grafted polarising region cells, or inserted a bead soaked in N-Shh, to the anterior of the limb; suggesting that Shh quickly diffuses a long way away from the bead, and maybe in addition, that the posterior of the chicken limb can be induced to express ptc ectopically more rapidly than the anterior of the limb. This would be consistent with an anterior repressor of Shh signals having to be down-regulated / inactivated before cells can respond to Shh signals, and Gli3 would be a good candidate for such a repressor. This could also explain why the anterior limb needs to be exposed to ectopic polarising activity for a minimum of 12-16 hours for any irreversible fate changes to occur, if this is the time required to overcome an anterior repression of Shh signalling. However, this would
Discussion and Conclusions of Chicken Experiments

also imply that the induction of ptc expression in the anterior limb is not an immediate early response to ectopic Shh. In addition, ectopic ptc is induced in the anterior of the limb in response to N-Shh or polarising region grafts more rapidly than this hypothesis would suggest: ectopic expression of ptc can be detected after only 4 - 6 hours of exposure to Shh, suggesting that if 12 + hours are needed to inactivate a repressor, this repressor must act downstream of ptc expression, and can't be responsible for the spatial distribution of ectopic ptc transcription caused by Shh-beads.

After about 16 - 24 hours the ectopic expression of ptc in wings exposed to ectopic Shh, becomes much more tightly localised around the bead (in a similar way to the ectopic expression induced by polarising grafts). This is probably the result of the ectopic Ptc induced around the bead sequestering the N-Shh and limiting its diffusion away from the bead, which suggests that N-Shh can be effectively localised by Ptc protein, even when it is not been post transcriptionally modified with cholesterol or palmitic acid.

B. Conclusions from the analysis of talpid^3

talpid^3 is probably a component of the Shh pathway, required in cells responding to Shh signalling

shh is expressed normally in ta^3 limbs but, as I show in chapter ten, high level expression of ptc and gli is not induced in ta^3 limbs, and the normal posterior repression of gli3 expression does not occur. This suggests that the product of the ta^3 gene acts between shh expression and Shh regulation of ptc, gli and gli3 transcription. It is unlikely that ta^3 is required for the correct processing of Shh protein, as, in contrast to wild type limbs, purified recombinant Shh protein cannot induce ectopic ptc expression in ta^3 limbs; ta^3 tissue grafted into wild type limbs continues to express low levels of ptc, even when surrounding wild type tissue is expressing a higher level of ptc; and posterior ta^3 tissue can induce ptc expression in wild type tissue. This all suggests that the ta^3 gene product is required for the response to Shh signals. The pleiotropic morphological phenotype of ta^3 embryos is also consistent with ta^3 being required for correct Shh signalling as abnormalities have been observed in most of the embryonic regions in which Shh signalling has been implicated. In two of these regions (the branchial arches and the mouth) there is also an absence of high level ptc expression. However, Dave
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Burt's laboratory have ruled out several of the known components of the Shh pathway, (gli, shh, smo, ptc) as candidate genes for ta^3.

Comparing the talpid^3 phenotype to other mutations

ta^3 has a pleiotropic phenotype that affects many different regions of the embryo that are thought to be patterned at least partly by Shh signals. Some of the aspects of the ta^3 phenotype resemble other vertebrate mutations that have a reduction in Shh signalling, for example in ta^3 embryos the eyes are variably closer together and in extreme cases fuse, which is reminiscent of the holoprosencephaly and cyclopia that is seen in humans and mice with shh mutations (Chiang, C. et al. 1996; Roessler, E. et al. 1996; Belloni, E. et al. 1996). My analysis of gene expression in the neural tube of ta^3 embryos suggests that the neural tube phenotype also resembles what we would expect from a loss of Shh signalling, as expression of shh is reduced in the ventral neural tube; the motoneuron domain of coupTFII expression is lost; and there is lower expression of ptc and gli and expanded expression of gli3. This is reminiscent of mice lacking Shh function in which no morphologically distinct floor plate can be identified, and it contrasts with mice lacking Ptc function in which floor plate characteristics, including expression of shh, are expanded dorsally (Chiang, C. et al. 1996; Goodrich, L. et al. 1997).

However, the morphological limb phenotype of ta^3, and especially the polydactyly of the digits, more closely resembles mutant phenotypes that arise because of increased Shh signalling (or a reduction in Ptc activity). Unfortunately neither the ptc nor the shh targeted loss of function mice are very informative for considering anterior posterior pattern in the limb as the embryos either die very early in development in the case of ptc^-/- embryos (between embryonic day 9 and 10.5)(Goodrich, L. et al. 1997) or lack distal limb structures in the case of shh^-/- embryos, (these also die at or just before birth)(Chiang, C. et al. 1996). However, loss of Shh activity in zebrafish affects the pectoral fins, which are the equivalent of the limbs in the tetrapod. These are either variably reduced or fail to grow out, depending on the severity of the allele (Schauerte, H. E. et al. 1998). Humans and mice that are heterozygous for loss of function alleles of shh show no limb phenotype (Roessler, E. et al. 1996; Chiang, C. et al. 1996) but, in contrast, both mice and humans heterozygous for a mutation in ptc have phenotypes that occasionally include polydactyly, presumably due to a partial increase in Shh signalling: a very small number of ptc^-/+ mouse embryos have extra digits in the hindlimb and in humans, Gorlin's syndrome (also called nevoid basal cell carcinoma syndrome
(NBCCS)), a dominant phenotype caused by heterozygous germ-line mutations in ptc, is
sometimes associated with extra digits (roughly 4% of cases - examples of pre-axial and
post axial polydactyly, and syndactyly have all been seen) (Gorlin, R. J. 1995; Hahn, H.
preliminary evidence that ptc¹⁻ mice that are rescued from early lethality by over­
expression of a ptc transgene also show polydactyly (L. Goodrich, L. Milenkovic, K.
Higgins and M. Scott, personal communication). Even more convincingly, transgenic
mice that ectopically express shh specifically in their skin show polydactyly of both their
fore and hind limbs: each limb now forms about 8 morphologically similar digits, and the
authors suggest that these limbs resemble ta³ limbs (Oro, A. et al. 1997).

Most of the polydactylous mutations in the mouse investigated so far (Strong's luxoid,
hemimelic extra toes, extra toes, recombinant induced mutant 4, luxate, X-linked
polydactyly, Alx-4) are also associated with ectopic expression of shh in the anterior of
the limb bud (Buscher, D. and Ruther, U. 1998; Buscher, D. et al. 1997; Chan, S. C. et
review see Niswander, L. 1997). One exception is the doublefoot mutation, which has
normal expression of shh, and unlike the other mouse mutants does not have mirror
image duplication of its digits: most of its digits appear identical like ta³. However, unlike
ta³, doublefoot has a dominant phenotype and it has ectopic expression of all the target
genes of Shh signalling that have been examined so far (ptc, ptc2, gli, bmp2, FGF4 and
HoxD-13)(Hayes, C. et al. 1998; Yang, Y. et al. 1998). This suggests that the
Doublefoot phenotype is also due to ectopic activation of the Shh pathway, either because
of a mutation in an, as yet unknown component of the pathway (Hayes, C. et al. 1998)
or more likely because of premature mis-expression of another hh gene, ihh (Yang, Y. et
al. 1998). I examined the expression of ihh in ta³ limbs and found no evidence of
premature expression (data not shown), but as we have shown that ta³ is required for the
response to Shh signals, it is very unlikely that the phenotype is due to ectopic or
premature expression of a hh gene.

Despite the morphological similarities between the mouse polydactyly mutants and ta³, in
all the situations where Shh signalling is known to be ectopically activated, ptc and gli are
ectopically expressed at high levels, which is not the case in ta³ limbs. In addition, unlike
mouse and human mutations in ptc, and several of the mouse mutations with polydactyly
including doublefoot, we don't see a heterozygous phenotype in ta³ embryos. This,
along with the paradox that some aspects of the ta³ resemble a loss of Shh signalling and
some resemble a gain of Shh signalling suggests that something fundamentally different
is happening in ta³.
The talpid\textsuperscript{3} phenotype suggests a bifurcation in the Shh signalling pathway.

The ta\textsuperscript{3} gene product is required for high level expression of ptc and gli and for the posterior repression of gli\textsuperscript{3} expression in the limb, but it is not required for the expression of the normally posteriorly expressed bmp, 5\textsuperscript{'} HoxD, or FGF4 genes. Also, as discussed above, the phenotype of ta\textsuperscript{3} limbs most resembles what I would expect from a gain of Shh signalling, but the expression of ptc and gli genes suggests a loss of Shh signalling. Recent experiments in cell culture and Drosophila have suggested that the Hh pathway is not necessarily linear and that gli genes are not the only transcription factors for Shh signalling (Ohlmeyer, J. T. and Kalderon, D. 1997; Lessing, D. and Nusse, R. 1998; Krishnan, V. et al. 1997; for a recent review also see Ingham, P. W. 1998a); and this is also suggested by recent analysis of mice lacking Gli1 and Gli2 function (Matise, M. P. et al. 1998). There is considerable evidence that Gli proteins are the transcription factors that mediate the Shh up-regulation of ptc transcription (Alexandre, C. et al. 1996; Marigo, V. et al. 1996; Hynes, M. et al. 1997) but expression of coupTFII \textit{in vitro} is not mediated by Gli, even though it is directly induced by Shh (Krishnan, V. et al. 1997). The ta\textsuperscript{3} phenotype could thus be explained if Gli proteins regulate the expression of gli, gli\textsuperscript{3} and ptc; but another transcription factor regulates the expression of bmp2, bmp7, 5\textsuperscript{'} HoxD genes, FGF4 and coupTFII, either directly or indirectly; and ta\textsuperscript{3} is only required for the Gli branch of the pathway (fig. 55). A bifurcation in the Shh pathway could also explain why some aspects of the ta\textsuperscript{3} phenotype most resemble what we would expect from a loss of Shh signalling and others more resemble a gain of Shh signalling. This is what I would predict if different branches of the pathway are required to different extents in different tissues. This could also explain the lack of coupTFII expression in the neural tube of ta\textsuperscript{3} embryos. Based on my observations in the limb, I would have expected coupTFII expression to be expanded in ta\textsuperscript{3} trunks, but instead it is lost. However, this could be a secondary effect of the reduced floor plate in ta\textsuperscript{3} embryos. The vertebrate trunk is an unusual site of Shh activity in that very high levels of Shh activity are required to induce the floor plate, and hence an additional source of Shh expression (Roelink, H. et al. 1995; Marti, E. et al. 1995). Floor plate development and associated expression of shh is disturbed in ta\textsuperscript{3} embryos, probably because the Gli branch of the pathway is explicitly needed for the development of the floor plate (Sasaki, H. et al. 1997; Ruiz i Altaba, A. 1998; Ding, Q. et al. 1998; Matise, M. P. et al. 1998; Lee, J. et al. 1997) and
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this could result in an overall level of Shh signal too low for coupTFII expression to be induced in the ventral neural tube.

A bifurcation in the Shh pathway is also consistent with other established differences between the induction of these different Shh target genes in the limb. Shh can induce all of these genes, but either the AER or FGF protein is also required for the induction of bmp2 or 5' Hox D genes, whereas Shh can induce gli and ptc expression in the absence of the AER and FGF proteins. Also Shh can induce FGF4 in the AER but if this is a direct response it can not be mediated through either of the gli genes as these are not expressed in the AER. However, my analysis does not address whether bmp, FGF4 or HoxD genes are direct or indirect targets of Shh.

It is also possible that ta^3 is a simple loss of Shh signalling mutation and that something other than the Shh pathway is responsible for the expression of bmp and Hox D genes in ta^3, but this doesn't explain why these genes are not still expressed normally, but are instead ectopically expressed in ta^3 limbs. In support of the idea that something other than Shh can induce the expression of these genes, the limbless chicken mutation has limb buds that do not express detectable levels of shh, but still express HoxD genes and bmp4, and bmp2 can be induced in response to an FGF4 bead, even in cases where no shh induction can be detected (Ros, M. A. et al. 1996). (It is however possible that these limbs are expressing a very low level of shh that is not detected - but in this case bmp2 must be able to respond to very low concentrations of Shh. Alternatively FGF4 and some other factor in posterior mesoderm must be able to act in concert to induce bmp2 in the absence of shh (we know that an FGF bead on its own in the anterior of a limb can not do this (Laufer, E. et al. 1994)). However, in limbless limb buds, unlike in talpid limb buds, this expression of bmp2 and 5' Hox-D genes is still posteriorly restricted. In contrast, re-aggregates of dissociated mesodermal cells from the anterior of chicken legs don't express shh but have a later expression of bmp2 and Hox D genes across their anterior posterior axis (though these re-aggregates, unlike ta^3 limbs, do not express FGF4 in the AER) (Hardy, A. et al. 1995).
Figure 55: A model of the possible bifurcation in the Shh pathway

A possible model of Shh regulation of different target genes, based on the talpid phenotype. The Shh pathway bifurcates, with Gli proteins controlling the transcription of gli genes, ptc and HNF3B; and unknown transcription factor(s) controlling the expression of bmp, Hox-D and coup TFII genes. In this model talpid would only be required for the Gli branch of the pathway. The transcriptional regulation of the second group of genes need not be a direct result of Shh signalling.
The regulation of Ci in *Drosophila* suggests another possibility, which still implies a bifurcation in the Shh pathway, but at the level of the transcription factor, Gli3. The *ci* gene produces at least two protein forms, one that predominates in the absence of Hh signals and acts as a transcriptional repressor, repressing some Hh target genes in the absence of Hh signals, such as *dpp*, the *Drosophila* *bmp* 2/4 homologue; and one that increases when cells have received Hh signals and is presumed to act as a transcriptional activator for some Hh targets such as *ptc* and *wg* (Aza-Blanc, P. et al. 1997). Consistent with this, loss of Ci function can result in cells that express ectopic *dpp* but do not up-regulate *ptc*, which is similar to what we see in *ta*^3^ limbs (Dominguez, M. et al. 1996; for a review see Kalderon, D. 1997; also discussed in Ingham, P. W. 1998a). If Gli3 is post transcriptionally regulated like Ci and has similar functions to Ci, then it could act as a transcriptional activator for *gli* and *ptc* in the presence of Shh signals, but as a transcriptional repressor of all the targets in the other branch (directly or indirectly) in the absence of Shh signals. In this case a mutation in *gU3* would mean that *gli* and *ptc* could not be up-regulated in response to Hh activity and that the other genes would be activated everywhere, which is what we see in *ta*^3^. However, at the moment there is no evidence to suggest that *gli3*, or any of the other *gli* genes are post transcriptionally regulated. Also, in *Drosophila*, the repressor form of Ci also represses Hh mis-expression, and in the mouse *gli3* represses mis-expression of *shh* in the anterior of the limb bud (Dominguez, M. et al. 1996; Masuya, H. et al. 1995; Buscher, D. et al. 1997); but we have seen no evidence of any ectopic *shh* expression in *ta*^3^ limbs. This suggests that either the chick is different to the mouse, or it argues against *ta*^3^ being a mutation in *gli3*. In addition *gli3* is not expressed in the AER, so it can not be responsible for normally repressing Shh targets in the anterior AER. Therefore if *ta*^3^ is a mutation in *gli3*, the ectopic expression of genes in the AER of *ta*^3^ limbs must be via a mesodermal factor such as *bmp2*.

The expanded expression of some Shh target genes could be due to Shh protein diffusing farther

The absence of high level expression of *ptc* in *ta*^3^ may produce secondary phenotypic effects. High level *ptc* expression, at least in *Drosophila*, normally sequesters Hh protein and prevents its diffusion (Chen, Y. and Struhl, G. 1996). Therefore in the absence of high level expression of *ptc* in the *ta*^3^ limb it is possible that Shh protein is diffusing farther, and can act over a longer distance, than normal. This possibility is suggested by the induction of *ptc* expression in the wild type flanks of host embryos when we grafted *ta*^3^ limbs to them but not when we grafted wild type limbs, and the induction of *ptc* in
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wild type tissue by grafts of anterior and apical ta^3 tissue. A more widespread distribution of Shh protein would also explain the anterior expansion of normally posterior gene expression and polarising activity in ta^3 limbs (Francis-West, P. H. et al. 1995). It could even account for the subtleties of the expression of bmp2 in ta^3 limbs - where in the mesoderm I see an anterior expansion of expression but expression is still occasionally stronger in the posterior of the limb. Consistent with the idea that aspects of the ta^3 phenotype are caused by a wider distribution of Shh protein, re-aggregates of dis-aggregated leg mesoderm from a whole leg or just the posterior third of a leg, which have ZPA cells (and hence shh expression and protein) distributed randomly throughout the limb, also show many similarities to ta^3 limbs: the digits that form are often identical to each other and Hox-D genes, bmp2 and FGF-4 are expressed across the whole anterior posterior axis of the re-aggregated limb bud (Hardy, A. et al. 1995). Also the number of digits that form in ta^3 limbs (7) is equivalent to the maximum number of digits that is ever seen after a ZPA graft, and the widened limb bud in ta^3 embryos is reminiscent of limb buds that have been exposed to a ZPA graft or ectopic Shh protein in the anterior of the limb (Tickle, C. et al. 1975).

As mentioned above, a greater diffusion of Shh protein could account for the ectopic polarising activity that is observed in ta^3 limbs and for the induction of ptc in wild type tissue by anterior and apical ta^3 tissue grafts. However this does not explain why the polarising activity in ta^3 appears to decrease with time. The expression of gli3 in ta^3 limbs suggests a possible explanation for this aspect of the phenotype. The mouse gli3 mutant extra toes has ectopic expression of shh, suggesting that gli3 normally acts to repress shh expression (Masuya, H. et al. 1995; Buscher, D. et al. 1997). Therefore it is possible that the expanded expression of gli3 in ta^3 limbs eventually reduces the expression of shh. We have seen no evidence for a lower expression of shh in ta^3 limbs, but it is possible that there are subtle changes that we have not identified by in situ hybridisation.

An alternative explanation for the expansion in polarising activity in ta^3 limbs is that it is due to the expansion in bmp2 expression as ectopic bmp2 has weak polarising activity: when mis-expressed in the anterior of a chick limb bud it can induce an extra digit 2 or a bifurcation of digit 3 aswell as ectopic expression of Hox D-11, HoxD-13, FGF-4 and additional expression of bmp2 (Duprez, D. M. et al. 1996; Francis, P. H. et al. 1994). ta^3 ectopically expresses a whole cacophony of normally posteriorly restricted genes, including other bmps so it is possible that this combination of ectopic proteins is responsible for the polarising activity. However several lines of evidence suggest that while these proteins may account for some of the polarising activity they are unlikely to
account for it all. Firstly, as mentioned above, our anterior and apical grafts of ta$^3$ tissue sometimes induced ptc expression, which is something that I would expect Shh but not BMPs to do; secondly re-aggregated anterior leg mesoderm expresses Hod D-13 and bmp2 but has no significant polarising activity (Hardy, A. et al. 1995). The grafts of ta$^3$ tissue where I assayed bmp2 expression also suggest that the ectopic polarising activity in ta$^3$ mesoderm is due to a diffusible factor, and are consistent with this being Shh. bmp2 was often expressed around the edge of the grafted tissue but not in the centre of the graft. This would be consistent with Shh diffusing out of the graft, but being sequestered at the interface between the graft and the host tissue as ptc expression is induced in the host tissue. However, these grafts were also able to act at a larger distance to induce robust expression of bmp2 in the anterior AER, but it is possible that this could be via lower levels of diffusing ectopic Shh, or Shh that has diffused before the wild type tissue expressed ptc, and/or BMP proteins.

My analysis of ta$^3$ does not directly address whether Shh protein normally diffuses from the wild type ZPA and acts directly as a morphogen to specify different fates. However, it suggests that at least a lot of the Shh protein produced in wild type limbs is localised close to its source by ptc, but that in the absence of high level expression of ptc this Shh protein can diffuse farther than it does normally, and that this produces abnormal anterior posterior patterning in the limb, and in particular results in posterior character being at least partially acquired across the limb bud. The posterior characteristics of even anterior mesodermal cells in ta$^3$ limb buds, along with our result that grafts of anterior ta$^3$ mesoderm sometimes induce ptc in wild type host tissue suggests that in ta$^3$ embryos Shh diffuses across the whole limb bud. This implies that none of the normal modifications of Shh protein, such as cholesterol linkage, are sufficient to localise Shh in the absence of ptc. However, the ta$^3$ phenotype could be caused either by higher than normal levels of Shh protein diffusing to the anterior limb bud (if Shh does act directly as a morphogen), or by anterior mesoderm being abnormally exposed to Shh protein (if Shh normally only acts on cells adjacent to its source). However, high level expression of ptc extends quite far anterior in wild type limbs, and definitely extends more than a couple of cells away from cells expressing shh, which suggests that at least some Shh normally diffuses or is transported away from the ZPA. The evidence against this is that a membrane bound form of Shh still induces digit duplications if inserted into the anterior of the limb (Yang, Y. et al. 1997). However, it is possible that something could be releasing this Shh or actively transporting it in vivo. For example Shh itself is proposed to have a proteolytic activity based on its crystal structure (Hall, T. M. T. et al. 1995). There is also
considerable evidence that the correct localisation and control of the diffusion or sequestration of Hh proteins is important for at least some aspects of Hh signalling. Shh is covalently coupled to cholesterol during auto proteolysis of full length Shh to the N-terminal signalling moiety, and N-Shh may also in some instances be palmitoylated (Porter, J. et al. 1996; Pepinsky, R. B. et al. 1998). In addition several predicted membrane bound proteins can influence the diffusion of Hh proteins: Ptc sequesters Hh protein (Chen, Y. and Struhl, G. 1996), Tout velou is necessary for Hh diffusion in Drosophila (Bellaiche, Y. et al. 1998) and Hip is another membrane protein (so far only found in vertebrates) that binds Hh (personal information A. McMahon).

The sensitivity of cells to Hh signals may also increase in the absence of high level expression of ptc. Ptc normally represses Hh signalling pathways in the absence of Hh signals, but it is also possible that the high levels of ptc that are normally induced by Shh dampen the effect of the Shh signal. Evidence that a precise balance between Hh and Ptc is required for correct development, at least in some cases, comes from experiments that show that over-expression of ptc can reduce Hh signalling (Schuske, K. et al. 1994; Johnson, R. L. et al. 1995; chapter six; personal information L. Goodrich, L. Milenkovic, K. Higgins and M. Scott) as well as analysis of Gorlin's syndrome and human holoprosencephaly, which are caused by heterozygosity for loss of function PTCH or SHH mutations respectively (Johnson, R. L. et al. 1996; Hahn, H. et al. 1996; Belloni, E. et al. 1996; Roessler, E. et al. 1996). The lack of this "dampening" effect could explain how ta^3 limbs acquire posterior characteristics across the limb bud as assayed by gene expression and the similar morphology of the digits, despite there still being a gradient of polarising activity and presumably also a gradient of Shh protein in ta^3 limbs. In the absence of ptc up-regulation, less Shh signal may be required to specify posterior fates, and therefore enough Shh may be diffusing even to anterior regions of the limb for the whole limb bud to be converted to a posterior fate.

A combination of Ptc sequestering Shh protein and/or dampening Shh signals could also explain the difference between the ta^3 phenotype and the phenotype that is obtained from placing beads soaked in N-Shh in the anterior of the limb. Previous experiments have suggested that N-Shh diffuses a long way from the bead in these experiments, yet this results in symmetrical digit duplications rather than posterior identity across the limb bud (Yang, Y. et al. 1997). However, these results can be reconciled, because even though high levels of Shh protein diffuse away from the bead initially and up-regulate ptc expression throughout a large area (probably partly because there is no ptc expression around the bead, and partly because the N-Shh is not correctly lipid modified) (Yang, Y.
et al. 1997; Porter, J. et al. 1996); I would predict that as \( ptc \) expression is induced, \( Ptc \) protein in cells around the bead begins to sequester the N-Shh. Therefore cells far away from the bead initially receive high levels of Shh activity but then see lower levels of Shh signal but still, at least initially, have high levels of \( ptc \) expression. In contrast Shh activity in \( ta^3 \) limbs is not localised or dampened by \( ptc \) expression.

This sort of interaction between Hh and Ptc may also explain why, in contrast to the \( ta^3 \) polydactylous phenotype where all the digits are morphologically the same, mouse polydactylous mutations (as discussed earlier) and also the chicken \emph{diplopodia} mutations, all specify a sequence of different ectopic digits (Buscher, D. and Ruther, U. 1998; Buscher, D. et al. 1997; Chan, S. C. et al. 1995; Masuya, H. et al. 1995; Masuya, H. et al. 1997; Qu, S. et al. 1997; Niswander, L. 1997; Taylor, L. W. and Gunns, C. A. 1947; MacCape, J. A. et al. 1975). I predict that these mutants all ectopically express \( ptc \), and that this spatially restricts and hence polarises any ectopic Shh pathway activity. Consistent with this, ectopic \( ptc \) expression has already been shown in some of these mutations: for example \emph{extra-toes}, and \emph{Strong's luxoid} (Goodrich, L. V. et al. 1996; Platt, K. A. et al. 1997; Buscher, D. et al. 1997). In contrast to these mutations \emph{Doublefoot} and mice with ectopic \emph{shh} expression in their skin have a series of identical digits even though they ectopically express \( ptc \). However this can be explained by the more widespread, relatively uniform, expression of either \emph{shh} or \emph{ihh} in these mice (Yang, Y. et al. 1998). In contrast to these mutations I would predict that the other \emph{talpid} mutation \emph{talpid}^2 (which has never been tested for complementation to \( ta^3 \) but shares many aspects of its phenotype, including digits that are very similar to each other)(Dvorak, L. and Fallon, J. F. 1991; Dvorak, L. and Fallon, J. F. 1992; Rodriguez, C. et al. 1996; Abbott, U. K. et al. 1959; Krabbenhoft, K. M. and Fallon, J. F. 1992) does not have ectopic expression of \( ptc \).

\emph{coupTFII} expression.

I also describe the expression of \emph{coupTFII} (chicken ovalbumin upstream promoter transcription factor II) in the limbs of the chicken in chapter ten. \emph{coupTFII} is an orphan nuclear receptor transcription factor which is also known as ARPI. Homologues have been cloned from a variety of other vertebrates including mouse, human, zebrafish and frog, but while its expression has been described in detail in the central nervous system of the brain and the trunk, its expression pattern in the limb has been reported in the mouse and the chick but not described (Lutz B et al. 1994; Jonk, L. J. C. et al. 1994). In
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Chapter ten I show that *coupTFII* is expressed in the centre of both limbs and that its expression differs slightly between the wing and the leg. This expression pattern of *coupTFII* in the limbs suggests that it might not be a direct target of Shh in the limb, as it is expressed very centrally and it is not expressed posteriorly. It is possible that it might be responding to low levels of Shh (and high levels of Shh may repress it or induce another gene that represses it) - but this would require Shh to be acting directly over quite a long distance - which as discussed above is still a controversial suggestion. However, I show that Shh can induce ectopic expression of *coupTFII* in the limbs, and that anterior expression of Shh in wild type limbs can produce an expression pattern of *coupTFII* that resembles its expression in *ta*\(^3\) limbs, suggesting that the pattern of *coupTFII* expression in *ta*\(^3\) limbs is due to ectopic Shh pathway activity, and that, if there is a bifurcation of the pathway, *coupTFII* is in the same Gli independent branch, as the *bmp* and *HoxD* genes.

The expression of *coupTFII* in limbs is also intriguing because of the postulated role of Coup TF proteins in potentiating and/or repressing responses to retinoids. CoupTF proteins can repress Retinoic Acid (RA) induced transcription and can sensitise cells to a RA response by repressing the basal activity of promoters that have a Retinoic Acid Response Element (RARE); either by binding the same DNA recognition sequences (RAREs) as retinoid X receptors (RXR's) and/or by forming dimers with RXR's (Cooney, A. J. et al. 1993; Tran, P. et al. 1992; Widom, R. L. et al. 1992; Wu, Q. et al. 1997). In addition the expression of *coupTF* genes is altered by RA in several systems (Qiu, Y. et al. 1996; and references therein). As I discussed in the introduction to chapter eleven, RA has also been postulated to have a role in anterior posterior patterning of the chicken limb, and while at least some of its activity is probably mediated by Shh, I proposed in chapter eleven that RA and Shh may also act synergistically to establish ZPA like activity. It will therefore be interesting to analyse the role of *coupTFII* in the limb and investigate if it interacts with retinoid pathways, and if its normal expression is due to Shh.

A final historical note.

When I was reading about earlier research into the *talpid* mutations I was interested to see how similar our present model for how the *talpid* phenotype arises is to earlier ideas. As early as 1964, Goetinck and Abbott suggested that the *talpid*\(^2\) phenotype might be due to an altered distribution of a mesodermal "apical ectoderm maintenance factor" (Goetinck, 227
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P. F. and Abbott, U. K. 1964). This mesodermal AER maintenance factor was postulated to exist by Zwilling and Hansborough in 1956, who also, based on experiments with two different polydactyloous chicken mutations, hypothesised that polydactyly was caused by an expanded distribution of this factor (Zwilling, E. and Hansborough, L. A. 1956). Goetinck and Abbott suggested that the \( ta^2 \) phenotype might be due to the AEMF being altered due to a larger area of production of AEMF, production for longer, or enhanced transmission of the AEMF, so that the entire limb mesoderm could maintain the AER. Hinchliffe and Ede also suggested this as an explanation for the \( talpid^3 \) phenotype in 1967 (Hinchliffe, J. R. and Ede, D. A. 1967). Then, in 1977, Cairns postulated that the "\( talpid \) wing bud mesoderm permits a more rapid anterior movement of the polarising factor and that it is this factor that is involved in both the conversion of distal to proximal\(^1\) and in the survival in \( talpid \) buds of the cells of the anterior necrotic zone" (Cairns, J. M. 1977).

\(^1\)In \( talpid^3 \), but not \( talpid^2 \) limb buds, proximal cells resemble distal cells in that they are larger and more adhesive.
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Spatial regulation of a zebrafish *patched* homologue reflects the roles of *sonic hedgehog* and protein kinase A in neural tube and somite patterning

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**SUMMARY**

Signalling by members of the Hedgehog family of secreted proteins plays a central role in the development of vertebrate and invertebrate embryos. In *Drosophila*, transduction of the Hedgehog signal is intimately associated with the activity of protein kinase A and the product of the segment polarity gene *patched*. We have cloned a homologue of *patched* from the zebrafish *Danio rerio* and analysed the spatiotemporal regulation of its transcription during embryonic development in both wild-type and mutant animals. We find a striking correlation between the accumulation of *patched* transcripts and cells responding to *sonic hedgehog* activity both in the neurectoderm and mesoderm, suggesting that like its *Drosophila* counterpart, *patched1* is regulated by *sonic hedgehog* activity. Consistent with this interpretation, mis-expression of *sonic hedgehog* results in ectopic activation of *patched1* transcription. Using dominant negative and constitutively active forms of the protein kinase A subunits, we also show that expression of *patched1* as well as of other *sonic hedgehog* targets, is regulated by protein kinase A activity. Taken together, our findings suggest that the mechanism of signalling by Hedgehog family proteins has been highly conserved during evolution.

Key words: induction, midline signalling, *patched*, *sonic hedgehog*, protein kinase A, zebrafish

**INTRODUCTION**

In *Drosophila*, cell patterning is controlled by the segment polarity genes, a molecularly heterogeneous group that includes the components of two signal transduction pathways (Klingensmith and Nusse, 1994; Ingham, 1995). One of these pathways is responsible for transducing the activity of the secreted protein Hedgehog, itself encoded by a member of the segment polarity class (Lee et al., 1992; Mohler and Van, 1992; Tabata et al., 1992; Tabata and Kornberg, 1994). Several genes closely related to *hedgehog* (*hh*) have now been described in various vertebrate species, the best characterised of these being *sonic hedgehog* (*shh*) (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994). The spatiotemporal deployment of *shh* is highly conserved from fish to mouse: expression is initiated during gastrulation in the embryonic shield or node and persists in the midline mesoderm, the notochord precursor, as the main body axis extends. Subsequently, expression of *shh* is activated in the floorplate cells of the ventral neural tube that overlie the notochord, where it continues to be expressed throughout somitogenesis (reviewed by Fietz et al., 1994).

Experimental manipulations have identified both the notochord and floorplate as sources of inducing activities that control the patterning of the neural tube and somites (van Straaten et al., 1989; Placzek et al., 1990, 1991; Yamada et al., 1991; Pourquié et al., 1993; Goulding et al., 1994) and several lines of evidence suggest that *shh* is a major component of such activities. First, in the zebrafish, loss of midline signalling in various mutants is closely correlated with loss of *shh* expression (Krauss et al., 1993; Ekker et al., 1995; Macdonald et al., 1995). Second, ectopic expression of *shh* in zebrafish (Krauss et al., 1993; Barth and Wilson, 1995; Macdonald et al., 1995), as well as in mouse and frog (Echelard et al., 1993; Ruiz i Altaba et al., 1995), leads to the inappropriate expression of floorplate and/or ventral brain markers. Such ectopic expression has also been shown to result in the inappropriate activation of the sclerotomal and myotomal markers *pax1* and *myoD*, respectively, in the developing somites of the chick (Johnson et al., 1994) as well as of *myoD* in paraxial mesoderm of the fish (Weinberg et al., 1996). Finally, and most definitively, recombinant Shh protein is itself capable of inducing floorplate and motor neuron differentiation in neural plate explants (Marti et al., 1995; Roelink et al., 1995) and sclerotomal or myotomal differentiation in explants of presomitic mesoderm (Fan et al., 1995; Munsterberg et al., 1995).

At later stages of development, transcription of *shh* is activated in the posterior mesenchyme of the developing limb buds,
a region corresponding to the signalling centre known as the zone of polarising activity (ZPA). Mis-expression of shh in the anterior of the limb bud results in digit duplications similar to those induced by heterotopic grafts of ZPA material (Riddle et al., 1993; Chang et al., 1994), suggesting that shh mediates the signalling activity of this region of the vertebrate limb.

Although the importance of shh during vertebrate embryogenesis is thus well established, little is known about the way in which the activity of the Shh protein is transduced. In Drosophila, by contrast, genetic analysis has identified a number of putative components of the hh-signalling pathway, such as the cAMP-dependent protein kinase (PKA) (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995) as well as the products of several segment polarity genes, including patched (ptc) (Hidalgo and Ingham, 1990; Ingham et al., 1991; Ingham and Hidalgo, 1993; Capdevila et al., 1994), a novel multipass membrane spanning protein (Hooper and Scott, 1989; Nakano et al., 1989). Inactivation of either Ptc or PKA in the developing limbs of Drosophila has remarkably similar consequences; in both cases, various hh target genes are inappropriately activated, suggesting that Ptc and PKA normally act to suppress the Hh response pathway. Whilst PKA most likely acts by phosphorylating other components of this pathway, the molecular mechanism of Ptc activity has remained enigmatic. One suggestion, based upon its predicted topology and membrane localisation, is that it functions as a receptor for Hh, binding of the latter antagonising Ptc activity and thus relieving the repression of different target genes (Ingham et al., 1991). Although consistent with the spatial deployment of the two proteins and the interactions between them as deduced by genetic analysis, this model has so far received no direct biochemical support.

Whatever the identity of the Hh receptor, it seems likely to have been highly conserved through evolution since vertebrate hh homologues are capable of activating the response pathway when expressed in transgenic Drosophila (Krauss et al., 1993; Chang et al., 1994; Ingham and Fietz, 1995). Moreover, the discovery that the response of tissue explants to Shh activity can be attenuated by drugs that activate PKA (Fan et al., 1995; Hynes et al., 1995) suggests that at least some of the intracellular components of the pathway may have also been conserved. In this study, we have explored the extent of this conservation further by cloning a zebrafish homologue of ptc and analysing its expression during embryogenesis. In Drosophila, ptc is itself one of the principal targets of hh activity, its transcription being upregulated in hh-responding cells. Using midline mutants and overexpression strategies, we have investigated the relationship between shh activity and transcription of the zebrafish ptc1 gene. Our results support a role for shh and ptc1 in the specification of both neural and mesodermal cell fates in the zebrafish; in addition, we show that both aspects of shh signalling are mediated by PKA activity.

**MATERIALS AND METHODS**

**In situ hybridisation**

In situ hybridisation was performed as described by Oxtoby and Jowett (1993) with the following modifications: hybridisation were performed at 70°C and post-hybridisation washes were as suggested by Henrique et al. (1995). Stained embryos were dehydrated through an ethanol: butanol series, embedded in Fibrowax and sectioned (6-10 μm). Double stainings were performed essentially as described (Jowett and Lettice, 1994).

Probes used for in situ hybridisations were synthesized using the following templates: shh, (Krauss et al., 1993); par-3 (Krauss et al., 1991); mk2,2, (Barth and Wilson, 1995); MyoD (Weinberg et al., 1996). Unincorporated DIG-UTP was removed by centrifugation through a Nuc50 column (Kodak). Specimens were analysed using a Zeiss Axiosplan microscope and photographed with Kodak Ektachrome 64T film. Images were scanned on a Sprintscan 35 slidescanner and processed using Adobe Photoshop software.

**zebrafish stocks**

Wild-type Danio rerio were bred from a founder population obtained from the Goldfish Bowl, Oxford. The fkh1 strain was obtained from T. Jowett (Newcastle University, UK). The cye and ntl strains were obtained from C. Kimmel (University of Oregon, USA). Fish were maintained in a constant recirculating system at 28°C on a 14 hours light/10 hours dark cycle.

**Cloning and sequence analysis**

Two pairs of primers (rev2, rev4 and genie1, genie2, see below) were used separately to perform PCR starting from random-primed cDNA samples synthesised using either bud stage or somitogenesis stage zebrafish RNA.

- **Rev2**: ggaacaattcTATCTCGAGCCTG
- **Rev4**: ggaacaattcTATCTCGAGCCTG
- **Genie1**: ggaacaattcGAYGGNATNATNAAYC
- **Genie2**: ggaacaattcATGAGGTTATGAG

The nucleotide sequence of the zebrafish ptc1 gene described here is deposited in the EMBL database under the accession number: X98883.
Embryo injections

Injections were performed on 2- to 4-cell-stage embryos using backfilled capillaries (Clarks Microelectrical Instruments, Reading) and a pressure-pulsed Narishige microinjector. RNA, synthesized in vitro from linearized plasmids p64Tshh (Krauss et al., 1993), pCS2dn PKA and pCS2ePKA, respectively, was injected at empirically determined concentrations which were of approximately 100 µg/ml. Plasmid DNA, purified using Qiagen columns, was injected at a concentration of approximately 100 µg/ml.

RESULTS

Isolating sequences homologous to Drosophila patched from zebrafish

Vertebrate homologues of ptc were first identified in mouse using a polymerase chain reaction (PCR) strategy based upon sequence comparisons of ptc homologues from distantly related insect species (Goodrich et al., 1996). Two pairs of degenerate oligonucleotide primers based upon sequences conserved between the mouse and insect genes were used to amplify related sequences from zebrafish cDNA (see Materials and Methods). Two fragments of approximately 320 bp and one fragment of around 330 bp, whose sequences proved to be related to both mouse and insect ptc sequences, were amplified. Oligonucleotides based on sequences within these three PCR fragments were then used to amplify

![Fig. 1. Comparison of the amino acid sequences of ptc genes from different organisms. (A) Sequence alignment of the Drosophila and mouse ptc proteins with zebrafish ptc1. Putative transmembrane domains are indicated by black lines above the sequence. Orange shading indicates identities between all three species. Blue shading indicates identities between the two vertebrate proteins. Putative N-glycosylation sites in Drosophila are indicated by pink lines and conserved cysteine residues by asterisks. Zebrafish Ptc1 is significantly shorter at the C terminus than Ptc proteins from other species. We checked the sequence of the cDNA in the region of the stop codon and the position of the stop codon, by amplifying this region from cDNA prepared from zebrafish embryos at somitogenesis stages. (B) Percentage identity and similarity between patched genes from different organisms. Comparisons were done using 'GCG Bestf'. The first number is the percentage identity and the number in brackets is the percentage similarity.]

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<tr>
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<td>Mouse</td>
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two fragments of approximately 1.8 kb and 2.0 kb and each of these fragments was used as a probe to screen a 33-hour-old embryonic zebrafish cDNA library. One full-length cDNA, designated ptcl, was isolated and is described in this paper. Similar clones corresponding to the other ptc homologues, ptc2, have yet to be isolated and this gene will not be considered further here.

The deduced amino acid sequence of ptc1 shows 64% identity to both the mouse and chicken ptc genes and 39% identity to Drosophila ptc. Comparison of the different Ptc sequences reveals that eight cysteine residues are conserved in all four species. The Drosophila protein has seven potential N-glycosylation sites of which three are conserved in mouse and chick but only one is present in zebrafish. Hydropathy analysis predicts that each protein contains 12 potential transmembrane domains (see Fig. 1A). There are two regions in the amino acid sequences that are particularly divergent; in the first of these, there are more conserved segments. (O,P) Show transverse sections through the trunk of two different fish at 48 hours post fertilization (hpf) indicating the position of the floor plate. Arrowheads mark the position of the hindbrain and otic vesicle. The identity of both the mouse and chicken Drosophila ptc genes is considerably shorter than that in Drosophila ptc.

**Fig. 2. Comparison of the ptc1 and shh expression patterns during wild-type embryogenesis.** Transcripts were revealed by in situ hybridization with antisense RNA probes to ptc1 (A,C,E,G,IK,M,O,Q,S,U) or shh (B,D,F,H,J,L,N,P,R,T,V), (A,B) Dorsal views of embryos at the end of gastrulation. The arrowhead indicates a row of hypoblast cells adjacent to the axial mesoderm which express ptc1 at high levels. The asterisk indicates the anterior boundary of the ptc1 low-level expression domain in the presumptive mesoderm. (C,D) Lateral views of 10-somite (C) and 14-somite (D) stage embryos. Arrowheads denote ptc1 expression in somites. Asterisks delimit the expression of ptc1 in the neuroectoderm all along the anteroposterior axis of the embryo. (E,F) 18-somite stage embryos. (G,H) 26-somite stage. Asterisks indicate the dorsal extension of ptc1 and shh expression domains in the diencephalon. Brackets indicate upregulation of ptc1 in rhombomeres 2, 4 and 6, a modulation of expression that is unrelated to shh expression (see also Fig. 5c). (L) Embryos at 48 hours of development. Asterisks indicate the position of the floor plate of the neural tube. Arrowheads indicate expression in the gut. golden mutant embryos, which are defective in pigmentation, were used to facilitate visualisation of ptc1 and shh expression. (K,L) 8-somite stage embryos. Dorsal view of flat preparations showing the complementarity between the paraxial expression of ptc1 and the axial expression of shh in the notochord and the tailbud. (M,N) Transverse sections through the trunk of embryos at 24 hours of development. (N) Sections at two different levels along the axis, the section on the right-hand side being ventral and that on the left-hand side dorsal. (Q,R) Transverse sections through the trunk of 36-hour-old embryos showing the expression of ptc1 and shh in the posterior region of the developing fins. (S,T) Detail of embryos shown in I and J, respectively. Asterisks indicate the position of the floor plate of the neural tube. Arrowheads indicate expression in the foregut. Expression in the hindbrain, when observed dorsally, was found to be restricted to periventricular cells in the midline (data not shown). (U,V) Frontal views of embryos shown in I and J, respectively. Arrowheads indicate expression in the foregut surrounding the developing mouth. Abbreviations: e, eye; fp, floor plate; hb, hindbrain; n, notochord; te, tectum; t, telencephalon; th, tail bud; y, yolk.

The spatial expression pattern of ptc1 during zebrafish embryogenesis

The expression pattern of ptc1 during zebrafish embryogenesis was analysed by in situ hybridisation and compared to that of shh. Transcription of ptc1 is first apparent at around 70% epiboly in the presumptive mesodermal cell layer in two stripes of cells flanking the axial mesoderm, which at this stage already shows robust expression of shh. As the convergence extension movements of gastrulation proceed, low-level ptc1 expression is apparent throughout the presomatic mesoderm while the two stripes of high-level expression, corresponding to the so-called adaxial cells (Thiss et al., 1993), extend along the entire axis; in addition, high levels of ptc1 transcript are detectable in the neuroectoderm overlying the axial mesoderm and in the ventral part of the future brain (Fig. 2A). With the onset of somitogenesis, high-level expression persists in the tissues surrounding the notochord in the trunk as well as in the ventral neuroectoderm of the brain (Fig. 2C). Distinct patches of high-level mesodermal expression become apparent adjacent to the notochord as the somites form (Fig. 2M), a pattern similar to that of α-tropomyosin at the same stage (Thiss et al., 1993). During later stages of somitogenesis when shh expression is lost from the notochord, expression of ptc1 persists throughout the ventral neural tube, except in the floor-plate, which at this stage still expresses shh at high levels. Notably, expression in the mesoderm also persists lateral and ventral to the notochord (Fig. 2G,M-P). In the brain, ptc1 expression extends dorsally in the diencephalon, parallelizing the dorsal extension of shh expression in the same region and shows a modulation in the hindbrain, which becomes more defined at later stages (Fig. 2G; see also Fig. 5C).

The relationship between the ptc1 and shh expression domains in the developing brain was analysed directly using double-labelling techniques to visualise both transcripts simultaneously. At all stages, high-level expression of ptc1 occurs in a domain delimiting the expression of shh. By 24 hours, expression of shh no longer occupies the most ventral part of the rostral forebrain and ptc1 expression can now be detected ventrally to shh-expressing cells (Fig. 3A). High-level expression of ptc1 is also detected around the lumen of the neural tube.

By 36 hours, additional sites of ptc1 transcription appear in the first branchial arch (not shown) and the posterior mesenchyme of the fin buds (Fig. 2Q), in both cases close to domains of shh expression (Fig. 2R; see also Krauss et al., 1993). By 48 hours, both genes exhibit a complex expression pattern in the brain with a persistence of high-level ptc1 expression adjacent to shh-expressing cells. High-level expression of ptc1 is, however, also observed some distance away from cells expressing shh in a number of places, including most notably a domain of intense expression in the hindbrain (Figs 2S,T,3B). Expression of both genes is now obvious in the foregut (Fig. 2S-V) as well as in more posterior domains.
Disruption of midline signalling affects both neural and mesodermal expression of ptc1

The relationship between the patterns of expression of shh and ptc1 described above is highly reminiscent of that between their Drosophila counterparts (Taylor et al., 1993; Tabata and Kornberg, 1994) suggesting that, as in Drosophila, transcription of ptc1 may be induced in response to hh signalling. Since no mutation of shh is currently available in the zebrafish, we took advantage of a number of mutants that eliminate shh expression at different levels along the anteroposterior axis to investigate this possibility.

Mutation of the cyclops (cyc) gene disrupts the specification of the prechordal plate mesoderm (Thisse et al., 1994) and concomitantly the induction of the overlying neuroectoderm, resulting in defective midline signalling in the developing brain which gives rise to the cyclopic phenotype (Hatta et al., 1991; Esker et al., 1995; Macdonald et al., 1995). In addition, cyc mutants lack a floorplate, apparently due to a defect in the response of cells to the inductive signal from the underlying notochord (Hatta et al., 1991). In line with these phenotypic effects, expression of shh is completely absent from both the prechordal plate mesoderm and the overlying neuroectoderm that gives rise to the ventral floor of the brain (Krauss et al., 1993) while, posterior to the midbrain, expression is normal in the axial mesoderm but absent from the ventral neural tube.

Expression of ptc1 in cyc embryos mirrors these changes in shh expression precisely: thus by the time the body axis is fully extended, no ptc1 transcripts are detectable anterior to the domain of expression of pax[b] (Fig. 4A), which marks the future hindbrain (Krauss et al., 1991), and the forebrain and midbrain remain devoid of ptc1 expression throughout the rest of embryogenesis (Fig. 4B). The only exception is a short dorsoventral stripe in the abnormal neural fold of the diencephalon that appears at around 24 hours of development surrounding a stripe of shh-expressing cells that appears at the same stage (Fig. 4B-D). Posterior to the midbrain/hindbrain boundary, ptc1 is expressed normally in the somites and ventral neural tube (Fig. 4B), except that it persists along the ventral midline of the latter, presumably reflecting the failure of the floorplate to differentiate.

In contrast to cyc, mutation of the floating head (flh) gene has no effect on prechordal plate mesoderm but instead disrupts notochord specification; this leads to a premature loss of shh expression from the axial mesoderm posterior to the midbrain/hindbrain boundary and the resultant disruption of floor plate induction is reflected in patchy expression of shh along the ventral midline of the neural tube (Talbot et al., 1995; see Fig. 5D). In addition, muscle pioneer cells that derive from the adaxial cells adjacent to the notochord (Felsenfeld et al., 1991; Thisse et al., 1993) fail to differentiate (Halpern et al., 1995; Talbot et al., 1995). A similar effect on muscle pioneer differentiation is caused by mutation of the notail (ntl) gene, which also lacks a notochord. Unlike flh, however, ntl mutants do not lack the floorplate (Halpern et al., 1993). These differences in phenotype seem to reflect differences in the effects of each mutation on shh expression, which persists longer in the axial midline of ntl embryos (Krauss et al., 1993) than in that of flh embryos (Talbot et al., 1995).

In flh embryos at 24 hours, expression of ptc1, like that of

Fig. 3. Simultaneous localisation of ptc1 and shh transcripts in the head. Whole embryos were hybridized simultaneously with riboprobes to ptc1 and shh revealing ptc1 expression in red staining and that of shh in blue. Lateral views are shown with anterior to the left; yolk and eyes were removed to improve brain visualisation. (A) Embryo at 36 hours of development. (B) Embryo at 48 hours of development. Abbreviations: fp, floor plate; hb, hindbrain; hy, hypothalamus; t, telencephalon.

Fig. 4. Expression of ptc1 and shh in cyclops mutants. ptc1 expression is detected in blue (A,B) or in red (C,D) and shh in blue (C,D). (A) Frontal view of embryos at the 1- to 2-somite stages. Expression of ptc1 is shown in a wild-type sib on the left and in a cyclops mutant on the right. To provide a landmark along the anteroposterior axis, embryos were simultaneously hybridized with a probe for pax-2 highlighting the position of the future posterior midbrain (asterisk). (B) ptc1 expression in a 24-hour-old cyclops mutant. The arrowhead indicates re-expression of ptc1 at high levels in a dorsoventral stripe in the diencephalon. (C) 24-hour-old cyclops embryos. ptc1 expression is revealed in red, shh in dark blue. (D) 24-hour-old wild-type embryo.
shh, is completely normal in the brain and anterior neural tube (Fig. 5C,D). More caudally, however, expression is restricted to small clusters of cells distributed sporadically along the ventral neural tube in a manner reflecting the intermittent expression of shh (Fig. 5; compare C to D and E to F). In addition to the effects on neural expression, the broad stripes of ptc1 expression typical of wild-type somites are absent from flh homozygotes, with only scattered mesodermal cells expressing the gene in close association with expressing cells in the neural tube (Fig. 5E). Thus expression of ptc1 in the mesoderm, as in the neural tube, seems to depend upon proximity to a source of shh activity. To investigate this relationship further, we analysed the expression of ptc1 at earlier stages of development in flh embryos. At the bud stage, shh is still expressed along the axial midline in flh embryos, in cells of uncertain origin (Fig. 5B); at the same stage, ptc1 is expressed in a single stripe of cells along the midline instead of in the two stripes typical of wild type (Fig. 5A). A similar change in the early pattern of ptc1 expression is seen in ntl homozygotes (data not shown). However, at 24 hours, in ntl mutants high-level expression persists in distinct stripes in each somite adjacent to the floorplate that expresses shh (Fig. 5G,H).

**Induction of ptc1 transcription by ectopic shh activity mirrors its effects on neural and adaxial-specific genes**

The analysis of ptc1 expression in midline mutations suggests that expression of shh is necessary for the induction of high-level ptc1 transcription. To test whether shh is sufficient to induce such transcription, we next examined the effects of ectopic shh activity on ptc1 expression. Previous studies have shown that injection of synthetic shh mRNA into the 2- to 4-cell-stage embryo results in the ectopic activation of genes such as axial and nk2.2 whose expression domains are normally restricted to the ventral region of the neural tube and brain (Krauss et al., 1993; Barth and Wilson, 1995). In the brain, the distribution of ptc1 transcripts around the shh expression domain closely mirrors the expression domain of nk2.2 (Fig. 6A,B). To determine whether ptc1 expression can similarly be ectopically induced by shh in the nervous system, embryos from the same cohort were injected with shh mRNA, fixed after 27 hours of development and hybridised with probes for ptc1 and nk2.2. As expected, nk2.2 is ectopically expressed in dorsal and lateral regions of the brain as well as in the eye rudiment of such embryos (Fig. 6D). Similarly, high-level expression of ptc1 is also detected ectopically in the diencephalon and midbrain of shh-injected embryos as well as in the eye rudiments (Fig. 6C). Notably, ectopic expression of ptc1 was never detected in the telencephalon. Similar effects on ptc1 expression were observed in small groups of cells following injection of a shh expression plasmid (data not shown) supporting the interpretation that the induction of transcription is a direct consequence of shh activity.

To examine the effects of ectopic shh activity in the mesoderm, embryos from the same injection cohorts were fixed at the onset of somitogenesis and hybridised with probes for myoD and ptc1. Like ptc1, expression of myoD is restricted to the adaxial cells in the presomitic mesoderm (Fig. 6F) and recent studies have suggested that its expression is regulated by shh (Weinberg et al., 1996). In a significant proportion of injected embryos, we found that the expression domains of both ptc1 (64%; n=112) and myoD (71%; n=90), are expanded so that they occupy most of the lateral mesoderm on one or both sides of the midline (Fig. 6G,H). In addition, ptc1 is ectopically expressed throughout much of the neur ectoderm at this stage following shh injection (Fig. 6G).

**Transcription of ptc1 is regulated by protein kinase A**

In Drosophila, removal of PKA activity from cells mimics their response to hh signalling, activating the transcription of ptc and other effectors of hh activity, suggesting that PKA normally acts to repress expression of hh target genes (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). Since the relationship between

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**Fig. 5. Comparison of ptc1 and shh expression in floating head and no tail mutant embryos.** Expression of ptc1 (A,C,E) and shh (B,D,F) in flh mutant embryos. (A,B) Embryos at the 5-somite stage. (C,D) Embryos at 24 hours of development. Brackets and bars indicate modulation of ptc1 in different rhombomeres which is unrelated to shh expression. (E,F) Lateral view of the trunk of embryos shown in C,D. Expression of ptc1 and shh in ntl mutant embryos. (G) ntl mutant embryos at 24 hours of development. ptc1 expression is revealed in red, shh in blue. (H) Lateral view of the trunk of a 24-hour-old ntl embryo in which ptc1 expression is revealed in blue.
hh signalling and ptc expression appears to be conserved from flies to fish, we wondered whether regulation of ptc1 and other shh targets might also depend upon PKA activity. PKA exists as an inactive heterodimer comprising a catalytic and a cAMP-binding regulatory subunit; binding of cAMP to the latter dissociates the heterodimer allowing the catalytic subunit to phosphorylate its target substrates. To modulate PKA activity, we made use of two previously described dominant mutations of PKA subunits. In the first of these, the cAMP-binding sites in the regulatory subunit are mutated such that it cannot be dissociated from the catalytic subunit (Li et al., 1995). Expression of this mutated subunit, which we refer to as dnPKA, thus results in the inactivation of the catalytic subunit and hence in the reduction or loss of PKA activity. In the second case, the catalytic subunit is mutated such that it cannot bind the regulatory subunit, thus rendering it constitutively active (Orellana and McKnight, 1992); we refer to this mutant form as cPKA.

Amongst embryos injected at the 2- to 4-cell stage with synthetic mRNA encoding the dnPKA mutant and fixed at early somitogenesis stages, more than half (55% n=120) exhibit high-level expression of ptc1 transcription throughout the lateral mesoderm on one or both sides of the midline (Fig. 7C). In addition, a similar proportion of embryos from the same injection cohort exhibit ectopic expression of myoD either unilaterally or bilaterally (Fig. 7D). At later stages (27 hours), injected embryos express both ptc1 (Fig. 7G) and nk2.2 (Fig. 7H) at ectopic locations in the diencephalon and mesencephalon. All of these effects are similar to, though somewhat less robust than, those induced by ectopic shh expression (compare Figs 6 and 7). Similarly, embryos injected with dnPKA mRNA also consistently show ectopic activation of pax6 in the eye, again similar to, though less extensive than, that induced by ectopic shh expression (Fig. 8A-C).

By contrast, injection of embryos with mRNA encoding the cPKA mutant has the opposite effect on the expression of hh target genes. At 27 hours, expression of ptc1 and nk2.2 is almost totally eliminated from the brain and ventral neural tube of injected embryos, with only a stripe of expression of both genes persisting in the diencephalon (Fig. 9D,E). This stripe corresponds to the dorsal extension of the normal diencephalic expression domain, which also persists in cyc mutant embryos (compare with Fig. 4). Like the latter, most cPKA-injected embryos (60%, n=90) also exhibit varying degrees of cyclopia, involving fusion of the retina alone or both the retina and lens (Fig. 9F), a further indication that midline signalling is attenuated or eliminated by the unregulated activity of PKA.

To explore the relationship between shh signalling and PKA
Fig. 8. Expression of pax[b] in wild-type, shh-injected and dnPKA-injected embryos. Figure 8. Expression of pax[b] in uninjected (A), shh-injected (B) and dnPKA-injected (C) 27-hour-old embryos. Note expansion of expression into the eyes in the injected embryos in comparison to the restricted expression in the optic stalk (arrowhead) of the normal embryo.

Further, we co-injected mRNAs encoding shh and cPKA and analysed the eyes of resultant embryos at 27 hours. Injection of shh RNA alone leads to a high proportion of embryos with rudimentary eyes (77% n=183) as previously described (Krauss et al., 1993; Ekker et al., 1995; MacDonald et al., 1995). Amongst embryos co-injected with both shh and cPKA RNAs, only 22% (n=70) exhibited such reduction of the eyes. Moreover, the frequency of cyclopia was much reduced (11%) relative to that induced by injecting cPKA alone (60%), suggesting that the activities of shh and PKA are mutually antagonistic.

DISCUSSION

ptc1 is a target of shh signalling

Signalling by Hh family proteins is used repeatedly and in various contexts during animal development. In Drosophila, signalling by hh in the embryonic segments as well as in the imaginal discs, retina and ovary is intimately associated with the activity of the segment polarity gene ptc (Ingham and Hidalgo, 1993; Ma et al., 1993; Capdevila et al., 1994; Heberlein et al., 1995; Tabata et al., 1995; Forbes et al., 1996a,b). In each instance, the two genes are expressed in complementary domains (Taylor et al., 1993; Tabata and Kornberg, 1994; Capdevila et al., 1994; Forbes et al., 1996b), the activity of ptc suppressing the transcription of hh target genes wherever it is expressed. Thus when ptc activity is eliminated, cells behave as though they have received the hh signal (Ingham and Hidalgo, 1993; Capdevila et al., 1994; Lepage et al., 1995; Tabata et al., 1995), even in the total absence of hh function (Ingham and Hidalgo, 1993). These properties have led to the notion that hh acts by antagonising the activity of ptc (Ingham et al., 1991; Ingham and Hidalgo, 1993; Basler and Struhl, 1994; Tabata and Kornberg, 1994) and, because ptc encodes a transmembrane protein, it has been suggested that it may act as a receptor for the secreted Hh protein (Ingham et al., 1991).

Since the signalling activity of Hh family proteins has been highly conserved during evolution (Krauss et al., 1993; Chang et al., 1994; Ingham and Fietz, 1995), we anticipated that proteins involved in their reception at the cell surface would similarly be well conserved. The isolation of ptc homologues from zebrafish reported here, as well as from mouse (Goodrich et al., 1996) and chicken (Marigo et al., 1996), is in line with this expectation. Whether or not there is a direct interaction between these proteins and the respective Hh family proteins, however, remains a moot point. Although the membrane association of Drosophila Ptc (Taylor et al., 1993) is consistent with its hypothesised role as a receptor, the predicted topology of both the invertebrate and vertebrate Ptc proteins is more typical of that of ion channels or transporter proteins (Hooper and Scott, 1989; Nakano et al., 1989; Goodrich et al., 1996).

A defining feature of Drosophila ptc is its transcriptional up regulation in response to hh signalling. The highest levels of ptc expression are typically found in cells immediately adjacent to those expressing hh (Hooper and Scott, 1989; Nakano et al., 1989; Taylor et al., 1993; Tabata and Kornberg, 1994; Capdevila et al., 1994; Forbes et al., 1996b) and this pattern of expression depends upon hh activity (Hidalgo and Ingham, 1990; Capdevila et al., 1994) whilst ectopic expression of hh induces inappropriate levels of ptc transcrip-
High-level expression of \( ptc \) is thus an indication of a response to \( hh \) signalling that is independent of cell or tissue type. Although the functional significance of this regulatory relationship is currently unclear, one possibility is that \( ptc \) activity acts as a sink for the \( Hh \) protein; in this case, its upregulation in response to \( Hh \) would effectively limit the range of the \( Hh \) signal. Alternatively, the increase in \( ptc \) expression could act as a feedback mechanism that attenuates the response of a cell to the \( Hh \) signal.

Our analysis of \( ptc1 \) expression in the zebrafish embryo does not allow us to distinguish between these or other possibilities; however, it does provide compelling evidence that this regulatory interaction has itself been conserved during evolution and is therefore likely to be fundamental to the mechanism of \( hh \) signalling. Thus as in flies, the highest levels of \( ptc1 \) transcription are invariably associated with \( shh \)-expressing cells; for instance, in the pectoral fin buds and the foregut high-level \( ptc1 \) expression is found immediately adjacent to cells expressing \( shh \), while in the CNS, the levels of \( ptc1 \) transcript are at their highest in the ventral region of the neural tube adjacent to the \( shh \)-expressing cells of the axial mesoderm and floor plate. A similar relationship exists between the expression domains of \( ptc \) homologues and \( shh \) both in the chick (Marigo et al., 1996) and mouse (Goodrich et al., 1996). However, in each of these organisms, additional sites of elevated \( ptc \) expression are observed in tissues associated with the expression of two other \( hh \) family members, \( Indian (Ihh) \) and \( Desert (Dhh) \) hedgehog (Goodrich et al., 1996; Marigo et al., 1996; Bitgood et al., 1996). In zebrafish, the expression of \( tiggy-winkle hedgehog \) (\( twhh \)), the only other \( hh \) family gene characterised to date in this organism, although initiated slightly earlier, is entirely included within the \( shh \) expression domain (Ekker et al., 1995). Thus, in contrast to amniotes, there is no expression of \( ptc1 \) that can be specifically associated with the activity of a \( hh \) family gene other than \( shh \). On the contrary, except in the fin buds, where \( twhh \) is not expressed, it is possible that at least some aspects of the transcriptional regulation of \( ptc1 \) may in part be mediated by \( twhh \).

In midline mutants that lack \( shh \) expression at specific positions along the body axis, we observe changes in the pattern of \( ptc1 \) transcription consistent with a regulatory interaction between the two genes. In \( cye \) mutants, high-level \( ptc1 \) expression is completely absent from the brain, consistent with the lack of \( shh \) expression in the ventral floor of the brain typical of this mutant (Krauss et al., 1993; Ekker et al., 1995; MacDonald et al., 1995). Since \( cye \) embryos also lack \( twhh \) expression along the entire midline (Ekker et al., 1995), we cannot rule out the possibility that this gene may also be necessary (and indeed sufficient) for \( ptc1 \) transcription in the brain. However, the essentially normal expression of \( ptc1 \) along the ventral neural tube of \( cye \) mutants, suggests that in this part of the body at least, \( shh \) activity is sufficient for the regulation of \( ptc1 \) transcription. In \( flh \) mutants by contrast, \( shh \) expression disappears from the axial mesoderm at a relatively early stage, many of the cells along the ventral neural tube failing to differentiate into floorplate presumably as a consequence (Talbot et al., 1995). In line with this widespread loss of \( shh \) expression, transcription of \( ptc1 \) is also severely reduced, being maintained only around the few scattered islands of cells that still express \( shh \). Taken with our finding that transcription of \( ptc1 \) can be induced ectopically in the neural tube by mis-expression of \( shh \), these data strongly suggest that \( shh \) is both sufficient and necessary for transcriptional activation of \( ptc1 \) transcription in the neural tube.

**\( ptc1 \) regulation reflects a role of \( shh \) in somite patterning in the zebrafish**

Whereas low level transcription of \( ptc1 \) is detectable throughout the presomitic mesoderm, its expression is significantly elevated in the adaxial cells that flank the midline mesoderm. Several lines of evidence suggest that this mesodermal expression of \( ptc1 \) depends upon \( shh \) rather than \( twhh \) activity. First, at this stage, expression of \( twhh \) is restricted to the neuroectoderm (Ekker et al., 1995) while \( shh \) is expressed at high levels throughout the axial mesoderm (Krauss et al., 1993). Second, in \( cye \) embryos, which lack \( twhh \) expression along the entire midline (Ekker et al., 1995) but have normal levels of \( shh \) expression in the axial mesoderm, expression of \( ptc1 \) in the somitic mesoderm is normal. Conversely, the absence of \( shh \) expression along the axial midline caused by the \( flh \) mutation, is accompanied by a significant loss of \( ptc1 \) expression in the mesoderm. When considered with our finding that mis-expression of \( shh \) induces high-level transcription of \( ptc1 \) throughout the paraxial mesoderm, these data strongly suggest that the mesodermal expression of \( ptc1 \) is regulated by \( shh \).

Interactions between the notochord and paraxial mesoderm have previously been well documented in higher vertebrates (Dietrich et al., 1993; Koseki et al., 1993; Pourquie et al., 1993; Goulding et al., 1994) and indeed the activity of Shh has been directly implicated in these interactions (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Fan et al., 1995). However, whereas in zebrafish, the induction of \( ptc1 \) expression in the adaxial cells appears to involve a very short-range effect of \( shh \), in the chick, Shh protein acts over relatively long distances (Fan and Tessier-Lavigne, 1994; Fan et al., 1995). Moreover, adaxial cells give rise to a myotomal lineage (Felsenfeld et al., 1991; Thise et al., 1993) and indeed express the muscle-specific transcription factor \( myoD \), which can itself be induced by \( shh \) activity (this report; Weinberg et al., 1996). By contrast, in the mouse and chick, Shh induces expression of the sclerotomal marker \( Paxl \) (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Fan et al., 1995); in line with this, notochord grafts have been shown to induce differentiation of sclerotome at the expense of myotome, actually repressing \( MyoD \) expression (Goulding et al., 1994). Thus, it appears that the outcome of \( shh \) signalling on somite patterning differs between lower and higher vertebrates. The different organisation of the somites in amniotes and teleosts may thus be accounted for, at least in part, by a modification of the inductive signals that control their specification. Interestingly, recent studies have revealed that, in chick, \( MyoD \) expression can be induced by Shh in presomitic mesoderm when presented in combination with signals from the dorsal neural tube (Munsterberg et al., 1995), an activity that may reflect its original role in the evolution of the vertebrate body plan.

**The role of PKA in \( shh \) signalling**

Intriguingly, as in \textit{Drosophila}, we have found that the effects...
of ectopic shh activity on ptc1 expression can be mimicked by reducing or eliminating the activity of PKA. Although PKA of ectopic the brain and somites of rats and chicks, respectively (Fan et al., 1995; Hynes et al., 1995), our finding that transcription of a ptc homologue in both the neuroectoderm and paraxial mesoderm is repressed by PKA provides the most direct indication to date of the extent to which the elements of the hh signalling pathway have been conserved. Similar effects are seen on the transcription of other targets of shh activity, both in the mesoderm, where myoD transcription is induced, and in the neural tube, where nk2.2 is inappropriately expressed. In addition, we consistently observe the ectopic activation of pax6b in the developing eye, an effect that presages eye abnormalities similar to, though less extreme than, those induced by ectopic shh expression (unpublished observations). Analogous effects of a dnPKA on zebrafish eye development have also recently been described by Hammerschmidt et al. (1996).

The implication of PKA in the regulation of ptc1 is further supported by our finding that constitutive activity of PKA suppresses ptc1 transcription throughout the brain and neural tube. This effect is accompanied by a similar suppression of nk2.2 expression and by cyclopia, a condition closely associated with a loss of midline signalling (Krauss et al., 1993; Hatta et al., 1994; Ekker et al., 1995; MacDonald et al., 1995). Together, all of these effects strongly support the notion that the shh signal acts by antagonising the repressive activity of PKA on various target genes, just as in Drosophila, hh counteracts the repressive effect of PKA on wg, dpp and ptc transcription. In Drosophila, it has been argued that PKA acts in parallel to the repressive effect of PKA on sonic hedgehog, a member of a family of putative signalling molecules is implicated in the regulation of CNS and limb polarity. Cell 75, 1417-1430.


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