THE UNIVERSITY *of* EDINBURGH

Title	Molecular analysis of early vertebrate eye development
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Declaration

I declare that:

1) This thesis was composed by myself
2) Unless otherwise acknowledged this work is my own

Katherine Lucy Hammond
December 1999

"Any system of its own accord will always undergo change in such a way as to increase the disorder."

2nd Law of ~~thermodynamics~~ Lab space

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Abstract

A group of four interacting *Drosophila* genes, *eyeless (ey), eyes-absent (eya), sine-oculis (so)* and *dachshund (dac)* are essential for eye development. In addition all except *so* can induce ectopic eye formation when ectopically expressed in the fly. *Pax6, Eya1-3* and *Six3* are vertebrate homologues of *ey*, *eya* and *so* respectively and are all potentially involved in vertebrate eye development. *Pax6* in particular is essential for vertebrate eye development and the mouse small eye *(Sey)* phenotype, which in its homozygous form has no eyes, is caused by the complete absence of functional *Pax6*. Although *Pax6* is known to be important for development of multiple eye tissues (Quinn et al., 1996) little is known of its targets or method of action in the eye. Using chimeras between small eye and wildtype mouse embryos this study has preliminarily shown that retinal pigment epithelium (RPE) can be specified in mutant cells of the chimera although these cells do not differentiate to produce pigment. *Trp2* and *micropthalmia (Mi),* both RPE specific genes, are expressed in part of this mutant tissue. These preliminary data indicate a possible function for *Pax6* in RPE differentiation.

Secondly this thesis reports the identification of *Dach1*, a murine homologue of *dac*. Two domains of high sequence conservation exist, the more C-terminal of which appears to represent a novel zipper motif. Similarity to the *Ski* family of genes is also seen within these regions suggesting that *Dach1* belongs to a super-family including the *Ski* genes. *Dach1* is expressed in the eye and the limb, structures affected by the *Drosophila* loss of function mutant, and also in the CNS, ear, nasal mesenchyme, lung, gut, genital eminence and dermomyotome. *Pax6* expression overlaps but is not identical to *Dach1* expression and the data presented here suggest that *Dach1* expression is not affected in the small eye mouse brain. In addition *Dach1* maps to 14E3 in the mouse.

Three zebrafish *dac* homologues were also isolated; *zfDachA, zfDachB* and *zfDachC*. *zfDachA* is expressed in the eye, CNS, otic vesicle, lateral mesoderm and somites. *zfDachB* is found in a restricted pattern in the CNS while *zfDachC* is expressed in the CNS, pronephric ducts, and neural crest. At the sequence level *zfDachC* is closest to *Dach1* while *zfDachB* is the least similar. Injection of *zfDachA*

RNA into 2-16 cell zebrafish embryos lead to the formation of ectopic tissue in the midbrain/hindbrain region and to somite defects. Injection of a C-terminally truncated *zfDachA* also lead to somite defects.

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Abbreviations

A	adenine
Bf1	brain factor 1 gene
β-gal	β-galactosidase
BMP	bone morphogenetic protein
Bmp	bone morphogenetic protein gene
BOR	branchio-Oto-Renal syndrome
bp	base pair
BSA	bovine serum albumin
C	cytosine
CAT	congenital cateractous mutant
cDNA	complementary deoxyribonucleic acid
CHAPS	3-3-Cholamidopropyl dimethyl-ammonio 1-propanesulfonate
ci	*cubitus interuptus* gene
CIP	calf alkaline phosphatase
CMV	cytomegalovirus
CNS	central nervous system
C-terminal	carboxyl-terminal
dac	*dachshund* gene (*Drosophila*)
Dach	*Dachshund* gene (mouse)
DACH	*Dachshund* gene (human)
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddNTP	dideoxynucleotide
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
dH_2O	distilled water
DIG	digoxygenin
Dll	*distaless* gene
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxnucleotide triphosphate
dpc	days post coitum
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E	embryonic day
EDTA	ethylenediamine tetra-acetic acid disodium salt
EMBL	European Molecular Biology Laboratory
Eng	*engrailed* gene
ENU	Ethylnitrosurea
ES cells	Embryonic stem cells
EST	expressed sequence tag
ey	*eyeless* gene
eya	*eyes absent* gene
FGF	fibroblast growth factor
FISH	fluorescence *in-situ* hybridisation

FITC	fluorescein isothiocyanate
g	gravity
G	guanine
GPI	glucose phosphate isomerase
HCL	hydrochloric acid
hCG	human chorionic gonadotrophin
HPE-2	holoprosencephaly type 2
hpf	hours post fertilisation
Hx	hemimelic extra toes mutant
INL	inner nuclear layer
IPTG	isopropylthio-β-D-galactosidase
kb	kilobase pairs
lx	luxate mutant
MeOH	methanol
Mi	micropthalmia mutant
nt	nucleotide
N-terminal	amino-terminal
OD	optical density
Oligo	oligonucleotide
ONL	outer nuclear layer
or	ocular retardation
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pfu	plaque forming unit
PMSG	pregnant mares serum gonadotrophin
PTU	1-phenyl-2-thiourea
R	rhombomere
RINS	radioactive *in-situ* hybridisation
RNA	ribonucleic acid
rNTP	ribonucleotide triphosphate
RPE	retinal pigment epithelium
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
Shh	*sonic hedgehog* gene
Sey	small eye
so	*sine oculis* gene
T	thymine
TCA	trichloroacetic acid
TEA	triethanolamine
TEMED	N,N,N',N',-Tetramethylethylenediamine
TESPA	3-aminopropyltriethoxysilane
tll	*tailless* gene
Tm	melting temperature
toy	*twin of eyeless* gene

Trp1-2	tyrosinase related protein 1-2 gene
tRNA	transfer ribonucleic acid
twhh	*tiggy winkle hedgehog* gene
UTR	untranslated region
UV	ultra-violet light
v:v	volume:volume
X-GAL	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
Xt	extra toes mutant

Chapter 1 Introduction

1.1 Overview and aims

During eye development a variety of cell fate decisions must be made. Initially the position of the eyes must be decided, retinal and lens fate being imposed upon relevant regions of the forebrain and surface ectoderm respectively. The timing of this decision is of critical importance. Once eye fate has been determined the component tissues must be specified, differentiate correctly and maintain that differentiated state. Interactions between the developing tissues of the eye as well as intrinsic factors seem to be essential for these processes.

Pax6 has been implicated in the acquisition of both lens and retinal fate and in further development of the optic vesicle; however, comparatively little is known about its method of action and targets within the eye. One aim of this study was to utilise chimeras between small eye, the mouse *Pax6* mutant, and wildtype mouse embryos to elucidate functions for *Pax6* within the developing layers of the mouse eye. The work presented here builds on previous work by Quinn et al. (1996) and focuses primarily on the developing retinal pigment epithelium (RPE).

Recently much attention has focused on a group of genes, *Pax6*, *Six3* and *Eya 1-3* which are expressed in the early eye and are the vertebrate homologues of three of a group of four interacting *Drosophila* genes which are essential to eye development; *eyeless (ey), sine oculis (so)* and *eyes absent (eya)* respectively. The vertebrate genes are all potentially involved in the early specification steps of eye development and may interact in a similar way to the fly genes. These genes are hence of interest from an evolutionary perspective as well as from a developmental point of view. The second aim of this study was to utilise homology with *Drosophila* to isolate and characterise murine and zebrafish homologues of *dachshund*, the fourth member of the above group of *Drosophila* eye development genes. Functional analysis of vertebrate *Dachshund* could then be carried out, including the use of zebrafish as a model system for over-expression assays.

1.2 Anatomy of eye development

The tissues of the mammalian eye have two separate origins. The optic vesicle, and hence later the retina, originate from an evagination of the forebrain neuroectoderm whilst the lens develops from a thickening of the head surface ectoderm. The stages of mammalian eye development are illustrated in figure 1. By approximately E8.0 in the mouse a region of neuroectoderm has acquired retinal fate and forms the optic sulcus (figure 1a). This region evaginates further as shown in figure1b to form the optic vesicle. By E9.5 the vesicle contacts the surface ectoderm and by E10.0 invaginates to form the bi-layered optic cup as shown in figure 1c. The inner layer, the presumptive neuroretina, now begins to thicken relative to the outer layer, the presumptive retinal pigment epithelium (RPE). Also, by E9.5 the lens placode has appeared as a thickening of the surface ectoderm directly adjacent to the region of optic vesicle contact (figure 1b). The placode begins to invaginate at about E10.0 to form the lens vesicle as in figure 1c. By E12.5 the lens vesicle has pinched off from the surface ectoderm, whilst the optic cup has developed two distinct layers and the optic stalk has formed linking the retina to the forebrain (figure 1d). By this stage the outer retinal pigment epithelium (RPE) has become epithelial in character and pigmentation is present. The inner, neuroretinal layer meanwhile, begins to

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Figure 1.1 : Eye development in the mouse.

(A) At E8.5 the optic pits have formed from the neural ectoderm which then evaginate to form the optic vesicles. (B) By E9.5 the optic vesicles contact the surface ectoderm which thickens to form the lens placode. (C) At E10.5 the optic vesicle has invaginated to form the bi-layered optic cup the outer layer of which will become the Retinal pigment epithelium (RPE) and the inner will become the neuroretina. The lens placode has begun to invaginate forming the lens pit. (D) By E12.5 the lens vesicle has formed, the RPE has developed pigment and the layers of the neuroretina begin to differentiate. (E) Shows the adult eye and (F) shows a cross section through the retina of the adult eye. The neuroretina is arranged into a series of layers. The external nuclear layer, closest to the RPE, contains the rod and cone photoreceptor cells, the inner nuclear layer contains the cell bodies of the bipolar, horizontal and amacrine cells and the inner most layer, the ganglion cell layer, contains the cell bodies of the ganglion cells which connect to the optic nerve. Connections between the cells are made in the two plexiform layers. Muller cells stretch throughout the width of the neuroretina.

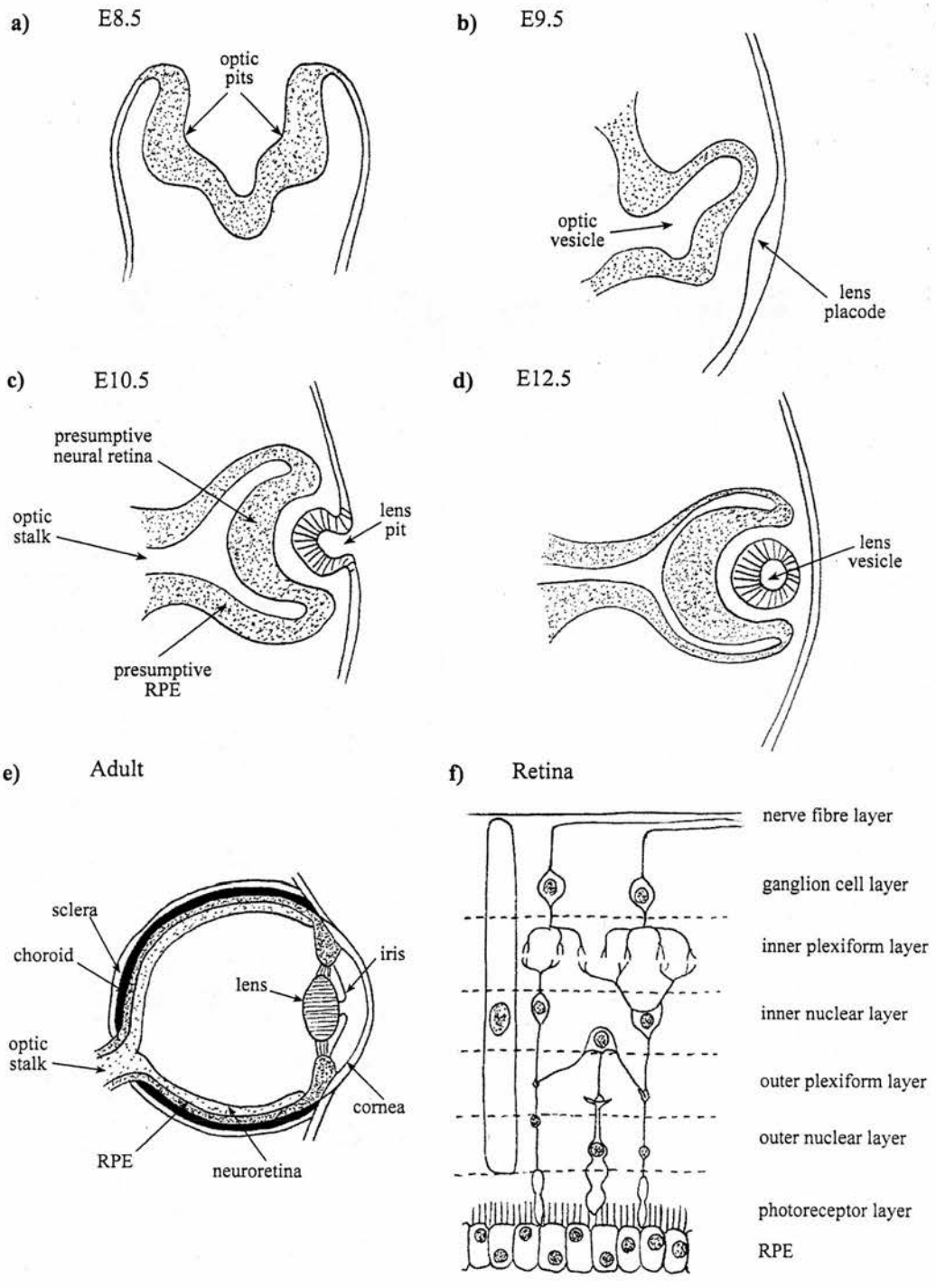

a)
E8.5
optic pits
b)
E9.5
optic vesicle
lens placode
c)
E10.5
presumptive neural retina
optic stalk
lens pit
presumptive RPE
d)
E12.5
lens vesicle
e)
Adult
sclera
choroid
lens
iris
optic stalk
cornea
RPE
neuroretina
f)
Retina
nerve fibre layer
ganglion cell layer
inner plexiform layer
inner nuclear layer
outer plexiform layer
outer nuclear layer
photoreceptor layer
RPE

Figure 1.1

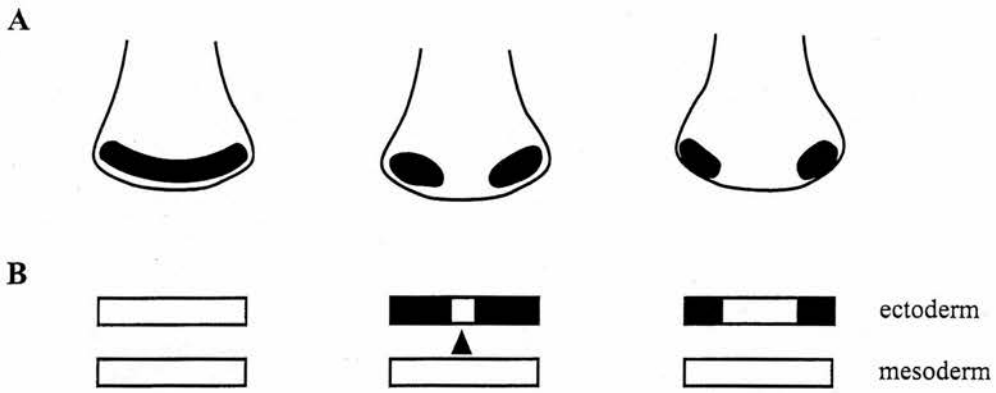


Figure 1.2 : Resolution of the retinal field in vertebrates (After Li et al., 1997)

The model proposed by Li et al., suggests that initially there is a single retinal field in the anterior neural plate. Retinal fate is then suppressed in the centre by signals from the underlying prechordal mesoderm.

(A) shows a dorsal view of the anterior neural plate.

(B) represents a transverse section through the anterior neural plate and the underlying prechordal mesoderm.

differentiate clearly defined layers. The ganglion cell layer will contain ganglion cell bodies and is located closest to the lens, the inner nuclear layer (INL) contains cell bodies of bipolar, amacrine, horizontal and Muller cells, and the outer nuclear layer (ONL), closest to the RPE, contains the rod and cone photoreceptor cells (figure 1f).

These layers are however, not clearly identifiable at E12.5 and regions of the neuroretina remain proliferative. As the lens matures, elongated fibres develop which form the refractive material of the adult eye, only the anterior lens epithelium retaining the ability to divide throughout life.

After lens invagination, the surface ectoderm covering the eye begins to develop into the corneal epithelium, the remainder of the cornea deriving from neural crest derived mesenchyme. The perioptic mesenchyme develops into the vascularised, pigmented choroid and the sclera (see figure 1e) (Kaufman, 1992; Graw, 1996)

Zebrafish eye development is similar to mouse eye development but development occurs in a much shorter space of time. Optic lobes can first be seen at the four somite stage, approximately 11.5 hours post fertilisation (hpf). At the 20 somite stage (19hpf) the lens placode forms and at the prim-5 stage (24hpf) pigmentation begins in the RPE (Westerfield et al., 1993). The first post-mitotic cells in the retina can be seen by about 29 hpf. The major difference in the mechanics of eye development is that the lens forms by internalisation of the lens placode rather than invagination. This means that at no stage is there a lens vesicle. On differentiation lens fibres are also seen to be wrapped around the lens core rather than being linearly arranged perpendicular to the cornea as occurs in mammals (Easter and Nicola, 1996).

The *Drosophila* eye develops completely differently and is a compound eye composed of an array of approximately 750 facets or ommatidia, which combine to interpret the image. It develops from an ectodermal imaginal disc within the larva, and photoreceptor cells develop in a wave of morphogenesis generated by the morphogenetic furrow spreading across the presumptive eye from posterior to anterior (reviewed in Hill and Davidson 1994; Treisman et al., 1999).

1.3 Induction in eye formation

1.3.1 Resolution of a single retinal field produces two retinal primordia.

Initially the field with the potential to form retina is large, extending throughout a significant region of the anterior neural plate. This region later resolves into two smaller lateral domains which give rise to the optic vesicles. Both *Pax6*, essential for mammalian eye development, and *ET*, a novel T domain gene (Li et al., 1997), are initially expressed in regions corresponding to the retinal field, their expression domains then reducing until they are only present in the optic vesicles.

Lineage analysis using a DiI tracer has demonstrated that resolution of the eye field is caused by changes within cells rather than cell movement. Labelled medial cells retain their position after they have ceased to express retinal markers (Li et al., 1997)(Figure 1.2). The prechordal mesoderm was implicated in this process because on its removal from both chick and *Xenopus* the retinal field was not resolved and a single medial eye formed. The entire region also continued to express *Pax6* and *ET*. Confirming the importance of prechordal mesoderm, *Pax6* expression in the retina was reduced when the prechordal mesoderm was transplanted to a region beneath it.

1.3.2 Signals involved in resolution of the retinal field.

Sonic hedgehog (Shh), and a related gene *tiggy winkle hedgehog (twhh)* in the zebrafish, have been implicated as signals which cause the eye field to resolve. *Shh* is expressed in ventral regions of the brain including the prechordal mesoderm, and when *Shh* is absent a single medial eye forms in place of the usual lateral two. This cyclopic phenotype has been observed in $Shh^{-/-}$ mice (Chiang et al.,1996), human fetuses with a homozygous *Shh* mutation which also exhibit holoprosencephaly (Belloni et al., 1996; Roessler et al., 1996) and in *cyclops*. This is a zebrafish mutant encoding a nodal related factor, in which *Shh*and *twhh* are missing from the forebrain including the prechordal mesoderm, but present in their usual position elsewhere (Ekker et al.,1995, Macdonald et al., 1995; Rebagliati et al., 1998).

In the cyclopic eye of both mouse and fish, *Pax6*, normally found in the retina, is expressed in all cells. *Pax2* which is normally expressed in the optic stalk is entirely absent (Chiang et al., 1996, Ekker et al., 1995). Conversely, in the zebrafish,

Pax2 expression increases when *Shh* is overexpressed, leading to hypertrophy of the optic stalks, while *Pax6* decreases and the retina becomes reduced (Macdonald et al., 1995, Ekker et al., 1995). This suggests that the resolution of the retinal field could be mediated by the down-regulation of *Pax6* by *Shh*.

Further evidence comes from the study of two zebrafish mutants, *trilobite* and *krypek,* which exhibit cyclopia to varying degrees. In both mutants the domain of *Shh/twhh* expression in the forebrain is shifted posteriorly with respect to the retinal field, lengthening the distance over which *Shh/twhh* must act. Indeed distance shifted corresponds to the degree of cyclopia in all cases; those fish with *Shh/twhh* furthest from the eye field also have the greatest degree of cyclopia. As it was also shown that both mutants can respond to *Shh/twhh*, the distance over which *Shh/twhh* acts is likely to be critical for eye formation (Marlow et al., 1998).

Ectopic induction of *Bmp4* and *Bmp5* in the chick forebrain also leads to cyclopia. *Pax6* is expressed throughout the medial eye and *Pax2* expression is lost, matching the phenotype caused by a lack of *Shh* (Golden et al., 1999). *Bmp4* and *Bmp5* are highly expressed in dorsal regions, *Bmp4* in the dorsal eye and *Bmp5* in the dorsal midline of the forebrain, and are implicated in providing dorsal cell fate cues just as *Shh* provides ventral signals. The cyclopic phenotype caused by ectopic *Bmp* may be indirect due to increased *Bmp* levels reducing the *Shh* expression domain. The retinal field resolving activity of *Shh* may therefore be independent of its dorso-ventral patterning function, alternatively perturbations of dorso-ventral patterning lead directly to cyclopia.

1.3.3 Dorso-ventral patterning within the optic cup also involves *Shh*.

During the early stages of development the optic vesicle is aligned so that prospective retina is in a dorsal position and prospective optic stalk is ventral. *Pax6,* which marks retina (dorsal), decreases in response to *Shh,* and *Pax2,* which marks optic stalk (ventral), increases, the reverse happening in response to *Bmp*. It is therefore likely that dorso-ventral patterning, mediated by *Shh* and *Bmp* has an important role in distinguishing retina from stalk.

In *brain factor1 (Bf1)* deficient mice the optic stalk is transformed into retina concomitant with increased *Pax6* expression and reduced *Pax2* expression.

It transpires that *Shh* is missing from the telencephalon of these mice. As *Bf1* is only expressed in the anterior region of the optic cup and both anterior and posterior regions of the mutant are affected, a non-autonomous reason for the defects is likely. It is hence probable that the phenotype is caused by the absence of *Shh* from the telencephalon (Huh et al., 1999). It appears that *Shh* can indeed mediate dorso-ventral patterning of the optic cup.

1.3.4 Are splitting of the retinal field and dorso-ventral patterning of the optic vesicle achieved in the same way?

Shh seems to function to suppress *Pax6* expression and hence retinal fate, probably in co-operation with other dorso-ventral patterning molecules such as the BMPs. In the anterior neural plate this splits the retinal field and in the retina restricts retinal fate to the dorsal regions furthest from the *Shh* source. The two processes are therefore basically identical, involving the same signal, and are probably just the early and late phases of retinal fate restriction.

Interestingly however, in the mouse $Shh^{-/-}$ mutant it was noticed that the medial proboscis which replaced the eyes, contained only pigmented RPE like cells (Chiang et al., 1996). This suggests that although *Pax6* is required for retinal fate, *Shh* may also be needed at a low level for neuroretina development.

1.4 Interactions within the developing eye

1.4.1 Lens induction

Early experiments showed that an ectopic lens could be induced to form by the transplantation of an optic vesicle beneath trunk ectoderm (Spemann 1901; Lewis 1904; reviewed in Saha et al., 1989). This suggested that the optic vesicle was both necessary and sufficient for lens induction, however, the interpretation of these experiments is ambiguous, due to the lack of a host/donor marking system. More recently, transplantation of regions of surface ectoderm to positions above the optic vesicle, has shown that only a gradually restricting region of head surface ectoderm can respond to optic vesicle signals. This period of competence continues to occur in the absence of inducing signals in cultures of *Xenopus* animal ectoderm (reviewed in

Saha et al., 1989 and Grainger 1992) which suggests a mechanism intrinsic to the surface ectoderm itself.

The present model for lens development splits induction into four steps; lens competence, lens forming bias, specification and differentiation (Grainger 1992; Grainger 1996). Current opinion suggests that the optic vesicle has a role in the latter stages of lens induction. In cultures of *Xenopus* prospective lens ectoderm isolated at stage 19, just prior to optic vesicle contact, lenses frequently formed but did not develop fibres. In the presence of optic vesicle however, fibrous lenses did form which suggests that the optic vesicle is unnecessary for lens formation but is important for its further development (Henry and Grainger, 1990).

The role of the anterior neural plate was demonstrated by the induction of small lenses from *Xenopus* stage 14 ectoderm co-cultured with anterior neural plate of the same age. Culturing the two tissues in a single congruent sheet, as they are found *in-vivo*, produced lens more effectively than culture of two juxtaposed explants. This suggests that this signal is normally transmitted through the plane of the tissue from the neural plate to the prospective lens (Henry and Grainger, 1990). Lens inducing ability was also enhanced by the addition of dorso-lateral mesoderm to the culture although this mesoderm could not induce lens formation in the absence of anterior neural plate. Dorso-lateral mesoderm is thus also likely to have a role in lens induction (Henry and Grainger, 1990)

Xenopus culture experiments also demonstrated that the signals produced by stage 14 anterior neural plate are not sufficient to induce lens from the ectoderm of the late gastrula (Henry and Grainger, 1990). This suggests that some other signal is necessary at this earlier stage and that anterior neural plate is only capable of inducing lens from tissue which has previously received some biasing signal. The optic vesicle itself is then involved at the later stages of specification and differentiation.

Genetics of lens induction

From its expression pattern and the small eye ($Pax6^{-/-}$) phenotype, *Pax6* has been implicated in the process of lens induction. The regions of lens competence correlate well with the regions of *Pax6* expression which is initially found in a wide

region of the head ectoderm, expression gradually restricting until only the lens and nasal placodes express. The presence of *Pax6* in the ectoderm is therefore likely to be important for lens formation, especially when coupled with the observation by Grindley et al. that *Pax6* expressing tissues are generally those which can trans-differentiate into lens (Grindley et al., 1995). This is reinforced by the fact that small eye mice do not form a lens, a wildtype optic cup cannot induce lens formation from small eye head ectoderm and mutant cells are excluded from the lens in small eye chimeras (Fujiwara et al., 1994; Quinn et al., 1996). *Pax6* expressing cells may therefore be predisposed to form lens.

Other genes thought to be involved include *Otx2* and the *Sox* genes, both of which are expressed in the anterior neural plate and placodal tissues at a time when lens induction is likely to be occurring (reviewed in Grainger 1996). *Sox* genes are expressed in the lens placode region and are known to be crystallin enhancer interacting factors (crystallins are lens proteins). *Sox2* and *3* are expressed just after *Pax6* expression but prior to placode formation and *Sox1* is expressed just after placode formation. If the prospective retinal area of the neural plate is ablated then *Sox2* and *3* expression is lost on that side although *Pax6* expression is not affected (Kamachi et al., 1998) which suggests that induction from the retina is necessary for *Sox* expression. Further circumstantial evidence for the importance of *Sox2* and *3* in lens development comes from the fact that ectopic sites of δ-crystallin expression invariably occur in regions such as Rathke's pouch which co-express both *Sox2/3* and *Pax6* (Kamachi et al., 1998).

The *Bmp7* knockout mouse, which has a reduced or absent lens placode, has provided evidence that *Bmp7* is also important to lens development. In the *Bmp7* mutant, development prior to placode formation appears relatively normal in that *Pax6* and sFRP2 (an early placodal marker and a putative *Pax6* target [Leimeister et al., 1998]) are initially expressed. *Pax6* is however, lost in the placode by E9.5 and sFRP2 by E10 and *Sox2* (a later placodal marker than sFRP2) is never detected. In the small eye mutant neither sFRP2 nor *Sox2* are ever found and neither is *Bmp7* which is normally present at E10. This suggested a model whereby *Pax6* on receipt of some inducing signal from the optic vesicle activates sFRP2 which in

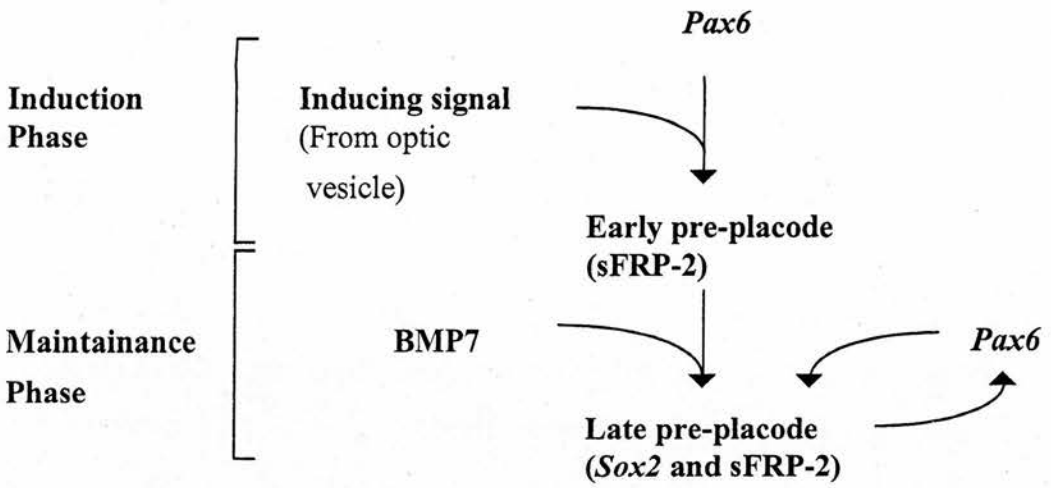


Figure 1.3 : A model for the role of BMP7 in lens placode development (After Wawersik et al., 1999).
During lens induction *Pax6* and an unknown inducing signal combine to initiate pre-placode formation, characterised by sFRP-2 expression. This is independent of BMP7 which is later needed to maintain placode development. The loss of *Pax6* and sFRP-2 expression in BMP7-/- embryos after their initial expression is due to the failure of late placode formation. Feedback signals would normally maintain both *Pax6* and sFRP-2.

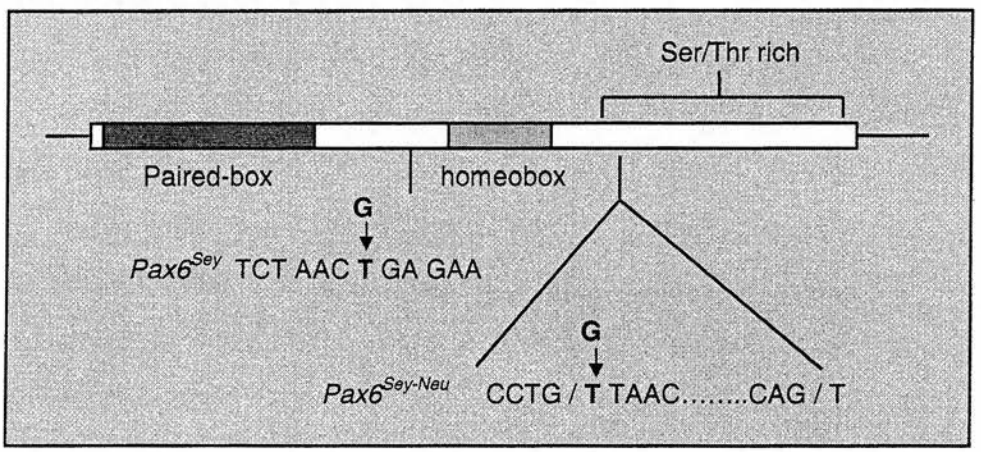


Figure 1.4 : Schemetic view of the *Pax6*^{Sey} and *Pax6*$^{Sey\text{-}Neu}$ mutations (after Hill et al., 1991).
Pax6^{Sey} substitutes a T for a G and truncates the protein by the creation of an in-frame stop codon. *Pax6*$^{Sey\text{-}Neu}$ also substitutes a T for a G abolishing a splice site at exon 10. This intron which is not removed contains a stop codon thus truncating the protein.

collaboration with *Bmp7* activates later placodal markers such as *Sox2*. It appears that *Bmp7*, or some factor activated by it, is necessary to maintain *Pax6* and sFRP2 in the developing lens (Wawersik et al., 1999) (figure 1.3).

Bmp4, which is expressed in the distal optic vesicle, is also implicated in lens induction as no lens induction occurs in *Bmp4* null mutant mice (Faruta and Hogan, 1998). Significantly BMP4 applied to the optic vesicle in explant culture can rescue lens induction, marked by the rescue of *Sox2* ectodermal expression. Other factors produced by the optic vesicle must also be involved however, as the substitution of BMP4 beads for the optic vesicle did not lead to rescue. In contrast to *Bmp7*, *Bmp4* is not affected in small eye mice, nor is *Pax6* affected in *Bmp4*$^{-/-}$ mice. These two genes are hence acting independently of one another.

1.4.2 lens to retina signalling

There is strong evidence that the lens or surface ectoderm signals to the retina during optic cup development. If the optic vesicle is rotated through 180°C so that the presumptive RPE faces the surface ectoderm a second neuroretina develops instead of RPE (Dragomirov, 1937). Further evidence is provided by small eye chimeras where a lens is sometimes present and sometimes absent at a threshold percentage (60%) of mutant cells. Those chimeras without a lens have an extremely malformed optic cup whilst those with a lens are much more normal, even when the proportion of mutant cells is virtually identical (Quinn et al., 1996). There is also convincing evidence from experiments using the two varieties of the fish *Astyanax mexicanus*. The blind cave variety initially develops an optic cup and lens which later degenerate. The sighted surface form has a normal eye. When a lens from the surface fish was transplanted to the cave fish optic cup, development of the eye was rescued. The reciprocal experiment, transplanting the cave fish lens to a surface fish optic cup, resulted in the degeneration of the optic cup (Yamamoto and Jeffery, 1998; Yamamoto and Jeffery, 1999). This demonstrates that a signal which is not present in the lens of the cave form emanates from the lens of the surface variant and is important for retinal differentiation.

This signal is likely to be, or include FGFs, as demonstrated by a variety of *in-vitro* and *in-vivo* experiments. If chicken optic cup culture up to E4.5 is treated

with FGF1 or FGF2 the prospective RPE becomes neuroretina (Pittack et al., 1997). In addition neuroretina does not differentiate correctly when anti-FGF2 antibodies are applied, although the RPE is not affected by this treatment (Pittack et al., 1997). In-vivo experiments corroborate these results, retroviral application of FGF1 into the RPE or the surrounding mesenchyme transforms the RPE to neuroretina (Hyer et al., 1998). Most conclusively ablation of the eye surface ectoderm results in a retina consisting of intermingled cells with a phenotype intermediate between RPE and neuroretina. On replacing FGF1 with a bead the phenotype was rescued with neuroretinal cells developing closest to the bead (Hyer et al., 1998). This suggests that FGF1 is necessary for neuroretinal fate.

1.4.3 Mesenchyme to RPE interactions

Evidence also exists for interactions between the periocular mesenchyme and the RPE although as yet there is no clue as to what the signalling factor may be. If the optic vesicle is flattened and surrounded by mesenchyme only RPE develops (Lopashov & Stroeva 1964) and if the optic vesicle is moved to a location devoid of periocular mesenchyme the presumptive RPE trans-differentiates and becomes neuroretina. (Detwiler & VanDyke 1953, Lopashov & Stroeva 1964). Although the mechanisms are not known it has been suggested that activin could be involved as it is expressed in the RPE and surrounding mesenchyme. Activin receptors are also found in the RPE. Other genes expressed in the periocular mesenchyme such as *Msx1* and *Eya3* may have a role (see section 1.7 and 1.8). It is also thought that there is a factor in the neuroretina which maintains RPE fate as when RPE and neuroretina are prevented from contacting one another the RPE becomes neuroretina (in Hyer et al., 1998).

1.5 *Pax6* in Eye Development

1.5.1 *Pax6* across the phyla and evolutionary considerations.

Pax6 encodes a transcription factor with two alpha helix containing DNA binding motifs, a conserved paired domain and a conserved paired like homeodomain, which are 94% identical at the amino acid level between mammals

and flies. Splice sites within the conserved regions of the gene are also conserved (Quiring et al.,1994). Mutations in *Pax6* most obviously affect the eyes. In *Drosophila* this produces the *eyeless (ey)* phenotype (Quiring et al.,1994), and in rats and mice the small eye phenotype (Hill et al.,1991, Matsuo et al.,1993) both of which cause complete loss of eye structures. In humans various ocular diseases including aniridia (Ton et al.,1991) and Peters' anomaly (Hanson et al.,1994) are attributable to mutations in *Pax6.*

Pax6 has been isolated from a wide variety of organisms underlining its significance across the phyla (Calleaerts et al., 1997). So far in all organisms examined which have them, *Pax6* is found in the eyes. There is also a high degree of identity between the *Pax6* genes of the different species, for instance zebrafish *Pax6* has 97% identity to the mouse gene, even having the same two alternative splice forms (Puschel et al., 1992), and, as mentioned above, the fly *eyeless* gene has 94% homology to the mouse gene. This suggests that *Pax6* was present in the primitive metazoa before the vertebrates and arthropods diverged, retaining an essential function throughout evolution.

Species in which *Pax6* has been studied include a nemertean (the ribbonworm *Lineus sanguineus* [Loosli et al., 1996]), a cephalopod (the squid *Loligo opalescens*[Tomarov et al., 1997]), a roundworm (the nematode *Caenorhabditis elegans* [Chrisholm and Horvitz., 1995]), a flatworm (the planarian *Dugesia tigrina*) two species of chordates (a urochordate *Phallusia mammillata* and a cephalochordate *Branchiostoma floridae* [amphioxus] [Glardon et al., 1998]) and representatives from each class of vertebrates; mammals, (rats, mice and humans [Matsuo et al., 1993; Hill et al., 1991; Ton et al., 1991), teleosts (zebrafish, fugu [Puschel et al., 1992]) and birds (chick and quail [Carriere et al., 1993]). In all these mammalian species *Pax6* is expressed in a similar pattern to the mouse gene (discussed below) and even in *C.elegans*, which has no eyes, *Vab-3*, a *Pax6* family member, appears to be involved in patterning the head region.

In the ribbonworm and the flatworm *D.tigrina* both of which have considerable regenerative capacity, *Pax6* is expressed in the central nervous system (CNS) and eye region of the regenerating head, and is found in the pigmented spots

and photoreceptor cells which represent the earliest sign of regenerating eyes. *Amphioxus* (Glardon et al.,1998) one of the invertebrate chordates closest to vertebrates has *Pax6* expression in the lamellar organ and the frontal eye, the presumed homologues of the vertebrate pineal eye and paired eyes respectively. The underlying plan for mammalian eye development may therefore have been in place since the lower Cambrian period and the evidence suggests a fundamental role for *Pax6* within the developing eye originating earlier than this.

1.5.2 The small eye mutation

Mutations in *Pax6* cause the small eye phenotype in both rats and mice. In mice four alleles have been investigated, each causing a slightly different phenotype. The four mutations are; $Pax6^{Sey}$ (small eye), $Pax6^{Sey\text{-}Neu}$ (small eye - Neuherberg), $Pax6^{Sey\text{-}H}$ (small eye - Harwell) and $Pax6^{Sey\text{-}Dey}$(Dickies small eye). $Pax6^{Sey}$ is the original mutation which arose spontaneously, and is a single base pair change at codon 194, creating an in frame stop codon which truncates the protein before the homeodomain. (Hill et al.,1991) $Pax6^{Sey\text{-}Neu}$ (an ENU induced mutant) abolishes a splice site at exon 10, by changing G to T. The intron, which contains a stop codon, is therefore not removed. This again truncates the protein but this time after the homeodomain (Figure 1.4) (Hill et al.,1991). $Pax6^{Sey\text{-}H}$ (a radiation induced mutant) has a more severe phenotype than $Pax6^{Sey}$ or $Pax6^{Sey\text{-}Neu}$, causing death before or soon after implantation. This is due to a chromosome deletion covering a large region including the Wilms tumour region, linked to *Pax6* on chromosome 2 (Hogan et al., 1986; Kent et al., 1997). $Pax6^{Sey\text{-}Dey}$ (Theiler et al., 1978) has also been shown to be allelic to $Pax6^{Sey}$ (Hogan et al., 1987). It is thought that $Pax6^{Sey\text{-}Dey}$ is also a deletion, based on its severe phenotype including pre-natal death (Hogan et al.,1987).

The work in this thesis utilises only the two truncation mutants, $Pax6^{Sey}$ and $Pax6^{Sey\text{-}Neu}$, as the phenotype of these mutants can be attributed to the effects of the *Pax6* mutation alone. As the phenotypes of these two mutants are virtually identical (Quinn-PhD thesis) the phenotype of $Pax6^{Sey}$, which is described below, can also be taken to be a description of $Pax6^{Sey\text{-}Neu}$.

1.5.3 The small eye Phenotype

The homozygous *Pax6*^{sey} mouse completely lacks eyes and nasal cavities at birth and has significant brain abnormalities, including a complete loss of the olfactory bulbs. Death occurs shortly after birth, possibly due to an impaired ability to breathe; however brain abnormalities may be a contributing factor and the pancreas, where *Pax6* expression is also detectable, may be defective (Hogan et al., 1988).In the rat the phenotype is very similar (Matuso et al., 1993).

During early development there appears to be little difference between the small eye and wildtype mice. By E12.5 however it can be seen that lens induction and nasal pit formation have not occurred, and the optic vesicles have become distorted and degenerate, never coming into contact with the overlying surface ectoderm. There is also an increase in mesodermal cells found between the optic epithelium and the overlying ectoderm. This could cause, or be a result of, the lack of contact between the optic vesicle and lens placode. In the small eye rat it has been shown that midbrain neural crest cells, which normally migrate to the nasal region, only travel as far as the eye (Matuso et al.- 1993). The abnormal positioning of these cells may be involved in abolishing lens and nasal cavity formation given that they would normally be in place before these structures begin to form.

The most obvious feature of the heterozygous phenotype is the smallness of the eyes. The lens is also small and vacuolated (Hogan et al.- 1988) and the anterior edges of the optic cup fold inwards. Cataracts generally form by about three weeks and some mice develop hydrocephaly by about eight weeks on certain genetic backgrounds. Here too there is a small amount of infiltration of mesodermal cells into the eye cup.

The heterozygous phenotype is reminiscent of the human diseases associated with loss of a single copy of *Pax6* although in all of the human conditions eye size is normal compared to the mouse phenotype which has obvious micropthalmia. The human diseases include aniridia (Ton et al., 1991), where the iris is reduced or absent, and Peters' anomaly (Hanson et al., 1994), characterised by central corneal opacity and iris-lens adhesions. The small eye mouse is therefore likely to be a good model for these human conditions especially in the light of a single reported case of a

human homozygous mutant which died at birth due to severe craniofacial and brain abnormalities very similar to the murine homozygous phenotype (Glaser et al., 1994a). *Pax6* mutations are involved in a variety of other ocular defects; isolated foveal hypoplasia (Azuma et al., 1996), anopthalmia (Glaser et al.,1994b), autosomal dominant keratitis (Mirzayens et al.,1995) and a unique ocular syndrome (Epstein et al.,1994) among these.

1.5.4 *Pax6* Expression

The expression pattern of *Pax6* correlates well with the mutant phenotypes, and appears to be similar in all organisms where *Pax6* has been found. In the mouse, expression is found within the spinal chord, brain, eye, nasal epithelium, pineal gland and pancreas (Walther and Gruss, 1991; Grindley et al., 1995).

In the brain *Pax6* is expressed in the presumptive forebrain, including the olfactory bulbs, from E8.0, expression extending to a sharp boundary defining the diencephalon/mesencephalon junction. Hindbrain expression is also found from E8.0 and is maintained throughout development. Later, at E15.5, expression also appears in the metencephalon (Walther and Gruss, 1991).

In the spinal chord *Pax6* expression is detected from neural tube closure, at E8.5, throughout the chord. The dorsal side then begins to down-regulate expression.

In the eye *Pax6* is detectable in all layers, from the earliest stages of development. The optic vesicle, which will form the retina, and the ectodermal placode, which will form the lens, both express *Pax6* strongly from the point of their formation around day E9.5. Even before this, at E8.5, the presumptive optic vesicle and a wide region of the surface ectoderm of the head, which gradually restricts to just the lens and nasal placodes, express *Pax6.* As development proceeds both layers of the optic cup, the inner neuroretina and the outer retinal pigmented epithelium, (RPE) express *Pax6* (Grindley et al., 1995). RPE expression begins to down regulate by E12.5, becoming undetectable by E14.5 and neuroretina expression is higher at the rim of the optic cup and lower at the back from E9.5 onwards. Neuroretina and lens expression, however, remain throughout development, neuroretinal expression becoming localised to the ganglion cell layer and the outer layer of densely packed, mitotically active cells by E15.5. In the adult eye this neuroretinal expression pattern

is maintained, along with lens and cornea expression, suggesting an ongoing need for *Pax6* as well as an early determinative role.

In the human fetus *Pax6* expression is similar to the mouse pattern (Ton et al.,1991) and in the adult teleost expression appears to mimic the mammalian pattern (Puschel et al., 1992). In the chick also expression occurs in the same ocular tissues as in the mouse but appears to occur at earlier stages (Li et al.,1994). Interestingly *Pax6* was not detected by Li in the nasal placodes of the chick where mouse *Pax6* is found (Walther and Gruss, 1991; Grindley et al., 1995). In *Drosophila, eyeless (ey)* can be detected in the embryonic eye anlagen and later, during larval stages, in undifferentiated cells of the eye disc anterior to the morphogenetic furrow. *Drosophila twin of eyeless (toy)*, a second *Pax6* homologue, is found in a very similar pattern but is expressed in the eye from an earlier stage (Czerny et al., 1999). This indicates an early role for *Pax6* in patterning the eye across the phyla and also at later stages at least in the higher vertebrates.

1.5.5 Chimeras reveal multiple functions for *Pax6*

Functional evidence for the multiple roles of *Pax6* within the eye has come from the analysis of chimeric mouse embryos containing a mixture of wildtype and $Pax6^{Sey}$ mutant cells (Quinn et al., 1996). This clearly showed the necessity for *Pax6* within the lens, neuroretina and retinal pigmented epithelium (RPE). No mutant cells were ever present in the lens, mutant and wildtype cells did not mix in the retina and pigmentation never formed in the RPE (for detailed discussion of these results see section 2.1). The work presented in chapter 2 of this thesis aimed to further characterise the role which *Pax6* is playing in each tissue by using this chimeric system.

1.5.6 *Pax6* targets

Exact functions and downstream targets for *Pax6* are still unclear. Based on their possession of *Pax6* binding sites, and the fact that they are affected by the presence of *Pax6* various target have been suggested. Crystallins (lens proteins) are stimulated by *Pax6* in a variety of species. In chickens (Cvekl et al 1994) *Pax6* binds to two sites on alphaA-crystallin which stimulate promoter activity. *Pax6* also

activates a mouse alphaA-crystallin promoter (Cvekl et al.- 1995) and a lens specific promoter on the guinea pig gamma-crystallin gene binds *Pax6* (Richardson et al.- 1995). The acquisition of these binding sites may have been evolutionarily significant as it would have allowed these proteins, which were already present in the body, to be tried out as lens proteins and subsequently maintained.

LI-CAM may also be a *Pax6* target as it contains *Pax6* binding sites and will bind *Pax6 in-vitro* (Chalepakis et al., 1994). Apart from this however very little is know about downstream targets. One possible further target, microopthalmia (*Mi*), is discussed in chapter 2.

1.6 Master Gene(s) for eye development?

1.6.1 *Pax6*-a master gene?

It has been suggested that *Pax6* is a master control gene for eye development as a result of work carried out in flies with *eyeless (ey)*, the *Drosophila Pax6* homologue. In *Drosophila, eyeless* can be detected in the embryonic eye anlagen before morphogenetic furrow movement and later in the undifferentiated cells of the eye disc anterior to the furrrow. Investigation of *eyeless* function has focused mainly on the early role in eye specification and it has been shown to be both necessary and sufficient for eye formation (Halder et al., 1995). *Ey* was ectopically expressed throughout the developing fly, resulting in the production of eyes from the wing, leg and antennal discs in positions corresponding to the sites of *ey* expression. These eyes appeared morphologically normal containing fully differentiated ommatidia and photoreceptor cells, however, it is not known whether they are neuronally connected to the brain. Halder et al. (1995) suggested that *ey* is a master control gene for eye formation, providing a switch which activates the cascade of events needed for eye production. Interestingly mouse *Pax6* can substitute for *ey* in this assay and induce ectopic eyes in *Drosophila* demonstrating the homology of these genes (Halder et al., 1995).

Recently a further *Drosophila Pax6* homologue, *twin of eyeless (toy)*, has been isolated (Czerny et al., 1999). *Toy* acts upstream of *ey* and appears more similar to *Pax6* than *ey* is, at both the sequence level and in its expression pattern. *Toy* is

found in the prosencepalon including the optic lobe area of the early embryo. This domain later gives rise to the brain and to most of the visual system. Later, *toy* expression is also found in the ventral nerve chord in a segmentally repeated pattern. *Ey* is found in a similar pattern in the ventral nerve chord from a slightly earlier stage but is expressed in a different subset of the cells. In the brain *ey* is found in a slightly more restricted pattern and is not expressed until much later than *toy*. During the development of the adult eye *toy* and *ey* are however found in identical patterns.

Toy, like *ey*, can produce ectopic retina when over-expressed in fly imaginal discs and activates *ey* in doing so. This along with the fact that *ey* does not affect *toy* when it is over-expressed suggests that *toy* acts upstream of *ey*. It seems likely that both genes arose from a duplication of a single *Pax6* gene in the fly lineage (Czerny et al., 1999) although it is possible that an as yet unidentified *Pax6* family member also exists in the mammal.

Adding weight to the argument that *Pax6* represents a highly conserved 'master gene' for eye development, overexpression of *Pax6* in *Xenopus* also leads to ectopic retina and lens formation (Chow et al., 1999). The ectopic tissue expressed eye markers including *Rx, Six3* and endogenous *Pax6* and differentiated to produce lens fibres, ganglion cells, Muller cells, photoreceptors and RPE in a similar spatial arrangement to that found in endogenous eyes.

1.6.2 Other "master genes" in *Drosophila*

Ey is one of a group of interacting *Drosophila* genes which also includes *dachshund (dac)*, *eyes absent (eya)* and *sine oculis (so)*. All are expressed in the eye primordium and are essential to *Drosophila* eye development, with loss of function mutants having absent or severely reduced eyes (Mardon et al., 1994; Bonini et al., 1993; Cheyette et al., 1994; Serikaku and O'Tousa, 1994). In the early eye anlagen *eya* and *so* are expressed and involved in events anterior to the morphogenetic furrow, while *dac* is expressed at the posterior of the disc, prior to furrow initiation. Mosaic analysis has suggested that it has an important role in furrow initiation (Mardon et al., 1994).

Dac and *eya,* like *ey,* have the ability to induce the formation of eye structures when ectopically expressed in the fly, though at a lower frequency than *ey*. *Ey*

produces ectopic retina in 100% of cases while *eya* and *dac* have a penetrance of approximately 20%. In addition *eya* and *dac* only produce ectopic retina from the antennal disc while *ey* acts in antennal, wing and leg discs (Bonini et al.,1997, Shen and Mardon., 1997).

Initially it was suggested that *Drosophila ey* was at the top of a regulatory hierarchy of genes which together produce the eye (Halder et al.,1995). Although the existence of further ectopic eye producing genes initially laid doubt on this theory, the differences in penetrance still suggest that *Pax6* is the first acting gene in the series. Indeed *ey* cannot induce ectopic retinal development in the absence of either *dac* or *eya*, and misexpression of *ey* strongly induces *dac* expression (Shen and Mardon, 1997), indicating that *ey* is upstream of *dac* and *eya*

Conflicting evidence however argues for a network of interactions, in which *dac* and *eya* participate, along with *so*, and is inconsistent with a simple linear hierarchy. For example if *dac* and *eya* are overexpressed together they act synergistically, producing ectopic eyes with a much higher incidence than either would alone (Chen et al 1997). The same is true *if eyes absent* and *sine oculis* are overexpressed together despite the fact that *sine oculis* alone cannot produce ectopic retina. Furthermore overexpression of either *eya* or *dac* induces ectopic *ey*, each genes also inducing the other. Hence a network of interacting factors has been postulated, involving a variety of positive feedback loops (Chen et al., 1997). See figure 1.5

In-vitro evidence, using the yeast two hybrid system, has also suggested that *eya* and *dac* form a physical complex as do *so* and *eya* (Chen et al., 1997, Pignoni et al., 1997), leading to the suggestion that *so*, the only one of the three with DNA binding properties, binds DNA, whilst *eya* binds to, and hence links, both *so* and *dac*. The model proposed by Chen et al. (See figure 1.5) suggests that for morphogenetic furrow initiation *dac* would complex with a further unidentified DNA binding factor, whilst for furrow migration only the *so/eya* complex would be necessary. This conclusion was drawn in part from mosaic analysis of *dac* function (Mardon et al., 1994) where *dac* was shown to be necessary for furrow initiation although furrow migration was unaffected in $dac^{-/-}$ patches of eye disc. This suggests that in regions

other than the eye the functions of these genes will be mediated by interactions with other, possibly related, factors.

Recently there have been reports of further genes, *eyegone* and *teashirt*, which can induce ectopic eyes when overexpressed. These genes are hence also likely to have important functions for the early steps of *Drosophila* eye development (Hazelett et al., 1998; Pan and Rubin 1998)

1.7 Vertebrate homologues of *Drosophila* early eye development genes.

Several mammalian genes; *Pax6* and members of the *Eya* and *Six* gene families, have been shown to be homologous to *ey, eya* and *so* respectively. *Pax6, Eya1-3* and *Six3* are expressed during eye development (Walther and Gruss, 1991; Walther et al., 1991; Xu et al., 1997a; Oliver et al., 1995.) At a functional level *Pax6* and *Eya2* have also been shown to be equivalent to their *Drosophila* counterparts; *Pax6* like *ey* can induce ectopic eyes in *Drosophila* and *eya2* rescues the eya^2 loss of function mutant (Halder et al.,1995; Bonini et al.,1997).

In some cases it is known that these vertebrate genes have a function in eye development. *Pax6* is known to be fundamental to eye development, mutations disrupting eye formation in small eye mice and causing aniridia in humans (Hill et al., 1991; Ton et al.,1991; Glaser et al., 1992, Jordon et al.,1992). The *Six* and *Eya* family genes are discussed in detail below.

1.7.2 *Six* family genes

1.7.2.1 Phylogeny

Five murine *Six* genes have been isolated by homology to *so*. All share a conserved homeodomain and a conserved Six domain. This domain appears to mediate both protein-protein and protein-DNA interactions and is the region through which *sine oculis* interacts with *eyes absent* (Pignoni et al., 1997). Initially *Six3*, though not the most similar in sequence, was considered the homologue of *so* based on its expression in the eye and brain. As further vertebrate and *Drosophila Six* family genes have been identified however, phylogenetic analysis has revealed three

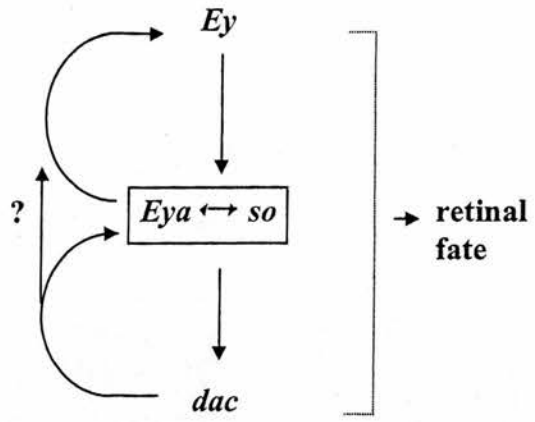


Figure 1.5a : Model for retinal development in *Drosophila* (after Chen et al., 1997; Pignoni et al., 1997)
A network of interacting genes controls *Drosophila* retinal specification. The arrows represent positive transcriptional regulation. It is not known whether *dac* induces expression of *Pax6* directly or via *eya* or another method hence "?". All four genes function together in one or more complexes to direct cell fate specification. Details are in section 1.6.2.

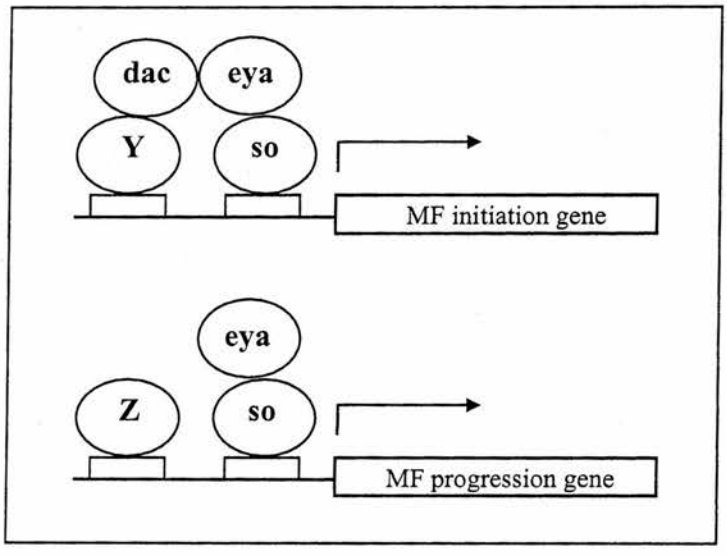


Figure 1.5b : Dac provides specificity to an eya-so complex for morphogenetic furrow (MF) initiation (after Chen et al., 1997).
Dac and eya form a physical complex as do eya and so (Chen et al., 1997; Pignoni et al., 1997). The eya -so complex is necessary for both MF initiation and progression whilst dac is only required for initiation. Dac provides specificity for the eya-so complex during furrow initiation. Neither dac nor eya contain DNA binding sites so interactions with DNA are likely to be via so which does bind DNA and probably further unspecified factor(s) (**Y**) in the case of dac. For furrow progression further unidentified factors (**Z**) may also be involved

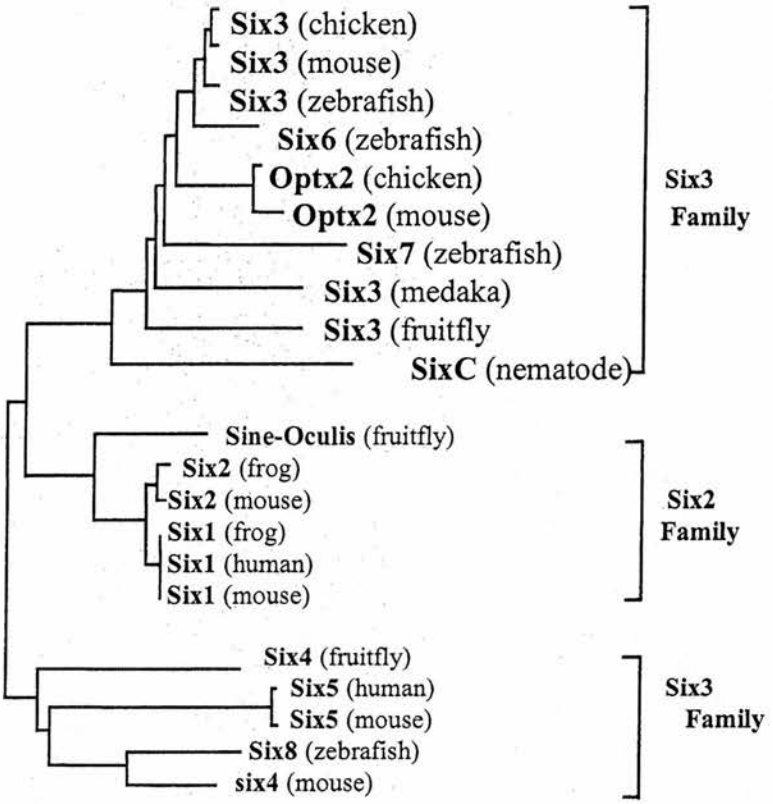


Figure 1.6 : Phylogeny of the Six gene family (After Seo et al., 1999)
The Six domain and the homeodomain, the two highly conserved regions of the Six gene family, were used to construct this phylogenetic tree. The above figure indicates approximate genetic distance but is not exactly to scale.

distinct *Six* family subgroups, each containing one *Drosophila* gene (see figure 1.6). It seems that mouse *Six3* is most similar to the *Drosophila optix* gene rather than *so*, this subfamily also including the vertebrate *Optx2* genes. *Optix* (also known as *D-six3*) is, like *so*, found in the eye/antennal disc of 3rd instar larvae and previously in two bi-lateral foci in the brain which probably develop into the eye/antenal disc (Seo et al., 1999; Toy et al., 1998). *Optx2* is also found in the developing eye of mouse, chick and *Xenopus* (Toy et al., 1999; Jean et al., 1999; Lopez-Rios et al., 1999). *Six1* and *Six2* which are most homologous to *so* are not seen in the developing eye (Seo et al 1999).

Linkage analysis has shown that *optix* and *so* are linked in the fly. In the mouse *Six3* and *Six2* are linked as are *Optx2* and *Six1*. It therefore seems that the fly genes have undergone duplication in the mammalian line and for some reason only the homologues of *optix* have retained their eye function (Seo et al., 1999).

1.7.2.2 The *Six3* subfamily - expression

In the mouse, human, chick and *Xenopus* only a single *Six3* gene and a single *Optx2* gene are known. Murine *Six3* is expressed in the anterior neural plate from E6.5 and is expressed throughout the optic vesicle and the optic stalk by E9.5. Later, at E12.5 expression is found in the neuroretina, lens, optic stalk and, according to some authors, the RPE. As development proceeds expression becomes restricted to the inner nuclear layer of the neuroretina and the anterior epithelium of the lens (Jean et al., 1999; Oliver et al., 1995). Expression in the chick is virtually identical to this, including expression in the RPE at early stages (Bovolenta et al., 1998). As far as reported the pattern is also very similar in *Xenopus* and in humans (Zuber et al., 1997; Granadino et al., 1998; Wallis et al., 1999).

In the zebrafish the situation is more complex as 3 genes exist; *zfSix3*, *zfSix6* and *zfSix7*. *zfSix6* has a similar expression pattern to murine *Six3* although it is restricted to the ventral half of the eye by 24 hours whereas the mouse gene is expressed uniformly throughout the retina. *zfSix3* and *zfSix7* both turn off early in development, by 15 hours, although initial expression in the anterior neural plate suggests that they could have an early eye development function (Seo et al., 1998a; Seo et al 1998b).

The *Optx2* genes (also known as *Six6* in mouse [Jean et al., 1999] and *Six9* in chick [Lopez-Rios et al., 1999]) are also expressed in the eye. In both the chick and the mouse expression is seen in the anterior neural plate, then later in the optic vesicles and ventral forebrain. Expression is then seen in the ventral neural retina and ventral optic stalk, similar to *zfSix6* expression, and according to some authors in the lens placode. In the mouse Toy et al. (1999) detect expression in lens precursors although not in the placode itself. In the chick however, Lopez-Rioz et al. see no lens expression at all and Toy et al. (1998) see placodal expression. Later still neuroretinal expression restricts to the inner nuclear layer (Jean et al., 1999; Lopez-Rios et al., 1999; Toy et al., 1998; Toy et al., 1999).

1.7.2.3 The *Six3* subfamily- functional analysis

Six3 subfamily members may have a role in retinal specification. Expression of *Optx2* in chick RPE culture caused the expression of the neuroretinal markers *Chx10* and visinin, which suggests a change to neuroretinal fate. *Six3* however had no effect (Toy et al., 1998). Intriguingly, when mouse or medaka *Six3* RNA was injected into two cell stage killifish medaka embryos (Loosli et al., 1998), ectopic tissue expressing the retinal marker *Rx2* was induced at the midbrain/hindbrain boundary. *Pax6* and endogenous *Six3* were also induced in this ectopic tissue suggesting the existence of feedback control. Previously, mouse *Six3* cDNA had been overexpressed in medaka under the control of a CMV promoter resulting in the production of ectopic lenses from the otic vesicle (Oliver et al., 1996). The *Six3* expressing cells themselves were found outside the ectopic lens itself and did not express *Pax6* or retinal markers. This suggests that the induction of *Pax6* by *Six3* is essential for the formation of ectopic retina and that this can only occur in the midbrain/hindbrain boundary region. *Six3* probably also has an important role in lens induction, but other factors found in the otic vesicle must be important too, as ectopic lens was only found at this site.

In *Xenopus* the overexpression of *Six3* RNA causes an increase in retinal size (P.C. in Loosli et al., 1998) as does ectopic *XOptx2* RNA (Zuber et al.,1999). *XOptx2* increases cellular proliferation in the retina and retinal enlargement is dependent

upon this. *Pax6* acts synergistically with *XOptx2* suggesting that the two genes interact (Zuber et al., 1999).

In the zebrafish ectopic expression of *Six6* RNA (referred to in this paper as *Six3*, Kobayashi et al., 1998) caused an enlargement of the forebrain and optic stalks. Although no ectopic retina was identified Kobayashi et al. suggest that ectopic retina/lens could have been masked by severe brain dysmorphology. From the expression pattern of *Six6* (Seo et al., 1998a) it was previously suggested that *Six6* may have a role in optic stalk formation which has also been suggested by the expression of murine *Optx2* (Jean et al., 1999). so^{mda} , a fly *sine oculis* mutant, also has no optic stalk (Serikaku and O'Tousa, 1994)

Interestingly, in humans *Six3* has been mapped to the holoprosencephaly type two (HPE-2) region on chromosome 2p12-p16 and mutations in the *Six3* gene, predicted to interfere with transcriptional activation have been identified in patients (Granadino et al., 1998; Wallis et al., 1999). This suggests that in humans as well as zebrafish, *Six3* family genes have an important role in forebrain development.

1.7.3 The *Eya* family.

There are four known mammalian homologues of *Drosophila eya* and all share a highly conserved C-terminal domain, the Eya domain (also known as ED1) which mediates protein-protein interactions with both *so* and *dac* (Chen et al., 1997). The Eya protein has no DNA binding potential but is nuclear and an N-terminal weakly conserved domain (ED2) exhibits transactivation potential. Three of the mammalian homologues, *Eya 1,2* and *3*, are expressed in the eye (Xu et al., 1997a) and *Eya4* is found in craniofacial mesenchyme, dermamyotome and the limb (Borsani et al., 1999). In the eye *Eya1* is found in the lens and optic stalk and later in the peripheral neuroretina, the optic nerve and the surface ectoderm which gives rise to the cornea. *Eya2* is expressed in the prospective sclera and the neuroretina, restricting to the inner nuclear layer by E14.5. *Eya3* is found in the optic vesicle and the perioptic mesenchyme, later being found in the neuroretina and in the lens, although expression is not seen in the lens placode (Xu et al., 1997a; Zimmerman et al., 1997).

Eya3 is never seen in placodal structures although it is concentrated in the regions surrounding them. *Eya1* and *Eya2* on the other hand are expressed in the cranial placodes including the nasal placode, in an overlapping but non-identical pattern. Only *Eya1* is found in the otic and lens placodes and Rathkes pouch while only *Eya2* is found in the trigeminal placode.

In small eye mice, *Eya1* in the lens and nasal placodes disappears, as does *Eya2* from the nasal placode. *Eya1* decreases in Rathke's pouch but increases in the perinasal mesenchyme. *Eya2* is also now found in the perinasal and perioptic mesenchmye where it is not normally expressed. The remainder of the expression pattern is unaffected (Xu et al., 1997a). This does suggest that *Pax6* has some effect on *Eya* genes.

Human *Eya1* has been isolated and implicated as the causative factor in Branchio-Oto-Renal (BOR) syndrome. This syndrome is characterised by hearing loss, renal defects and branchial abnormalities. This correlates with the mouse expression pattern reported in this paper, where *Eya1* is never seen in the eye, but conflicts with the data of Xu et al. discussed above (Abdelhak et al.,1997). Perhaps the eye function of *Eya1* is secondary to its function in other regions. Zebrafish *Eya1* (Sahly et al.,1999) was also undetectable in the eye. In addition *Eya1* knockout mice have no obvious eye defects apart from open eyelids (Xu et al., 1999)

1.7.4 Conserved relationships and functions in vertebrates and *Drosophila*?

Based on their expression throughout the eye *Pax6, Eya1-3* and *Six3/Optx2* may all have fundamental roles in early eye development. The fact that *Pax6* and *Six3* can induce ectopic retina and lens to form is consistent with this and may suggest a universal 'master' regulatory role for these genes.

Relationships between the members of this gene group in flies and vertebrates could potentially be conserved. The absence of *Eya1* and *2* from the lens placode area in *Sey* mice suggests that *Pax6* may function upstream of *Eya* just as *ey* is upstream of *eya* in the fly. Care must be taken in the interpretation of this data however as *Eya* may be absent purely as a result of the lack of a lens placode in *Sey* mice (Xu et al., 1997a). The overlapping expression patterns of members of the three gene families may also indicate that interactions between the gene family members occurs in other

regions. For example *Eya4* and *Six1* expression correlates in the dermamyotome, also overlapping with the *Eya1, Eya2* and *Pax3* domains, and *Pax2/8* may interact with *Eya1* in the kidney (Abdelehak et al., 1997; Borsani et al., 1999). Most conclusively in $Eya^{-/-}$ mice *Six1* expression is absent from the ear and *Six2* is absent from the kidney where they are normally be expressed (Xu et al., 1999).

The isolation of *Dach*, the vertebrate homologue of *Drosophila dac*, in this study (see chapter 3 for *Dach1* and chapter 4 for *zfDachA, B* and *C*) adds weight to the possibility that this interacting network of eye development genes is conserved between flies and vertebrates.

1.8 Other genes involved in eye development

1.8.1 Genes involved in the early stages of eye development

Other genes implicated in early eye development (ie. optic vesicle development and the transition to optic cup) include *Rx, Lhx2, Msx1/2, Xtll* and the *Gli* genes. All have been implicated due to their expression patterns and mutant phenotypes. Notably *Rx1* is the only reported gene, apart from those discussed in section 1.7, which can form ectopic retinal structures when overexpressed. *Rx* however produces only ectopic RPE.

1.8.1.1 *Rx* genes

Rx is a paired-like homeobox containing gene and the mouse mutant, $Rx^{-/-}$, has a reduced forebrain from which no optic vesicles form (Mathers et al., 1997). This suggests an extremely early role in eye development. The murine expression pattern correlates well with this phenotype and *Rx* is found in the anterior neural plate from E7.5, then in the neuroretina and ventral forebrain by E10.5. The neuroretinal expression restricts to the photoreceptors and the inner nuclear layer by birth, subsequently disappearing entirely (Mathers et al., 1997). The *Xenopus* expression patterns of both *XRx1* and *2* are identical and basically the same as the mouse (Mathers et al., 1997). Zebrafish on the other hand has three genes; *Rx1* and *2* are found in the retina, whilst *Rx3* is primarily found in the forebrain, although some expression is found in the bipolar cells on day 2 of development (Chuang et al., 1999;

Mathers et al., 1997). The *Drosophila* gene does not appear to be expressed in the eye but is expressed in two dorsolateral spots in the early prosencephalon which may give rise to the eye primordia (Eggert et al., 1998).

When *XRx1* was overexpressed in *Xenopus*, ectopic RPE formed and RPE hyperproliferation was seen. The forebrain also expanded at the expense of the hindbrain and midbrain (Mathers et al., 1997). When *XRx1* was inactivated the forebrain became reduced and cell death increased in this region, similar to the mouse mutant phenotype. Midbrain and hindbrain however were unaffected suggesting that the anterior defects were due to cell death rather than transformation to more posterior fates (Andreazzoli et al., 1999).

Rx is hence implicated in specifying anterior fate and seems to act at an earlier stage than *Pax6*. Expression of *Rx* is seen before *Pax6,* and in small eye mice the optic vesicle develops then degenerates where in $Rx^{-/-}$ mice development arrests prior to vesicle development.

1.8.1.2 *Lhx2*

Lhx2 is expressed throughout the optic vesicle from E8.5. It is subsequently found throughout the neuroretina, restricting postnatally to the inner nuclear layer. The $Lhx2^{-/-}$ mutant is anophthalmic; the optic vesicle initially develops but arrests prior to optic cup formation which suggests that *Lhx2* acts at a similar stage to *Pax6*. In addition the lens placode does not develop in $Lhx2^{-/-}$ mice despite the fact that *Lhx2* is never expressed in the lens or placode. This suggests that *Lhx2* has a role in lens induction.

$Lhx2^{-/-}$ mice do express *Pax6* although not in the ectoderm where the lens placode should form. As *Lhx2* is also expressed in the small eye mutant *Pax6* and *Lhx2* are likely to be involved in parallel pathways (Porter at al., 1997).

1.8.1.3 *Msx* genes

Msx1 and *2* are both implicated in eye development (Monaghan et al., 1991) but appear to have redundant functions. In double knock-outs the phenotype ranges from microphthalmia to arrested development at the optic vesicle stage, individual knockouts having no effect (Satakata and Maas, 1994; Rauchman et al., 1997). The

more severe double mutant phenotypes resemble the small eye and *Lhx2*$^{-/-}$ phenotypes suggesting an early function for *Msx*. The *Msx2*$^{-/-}$/*Pax6*$^{+/-}$ mutant, which has a phenotype similar to both small eye and the *Msx* double mutant, provides further evidence that *Pax6* and *Msx* function in similar processes (Rauchman et al., 1997). *Msx* expression also suggests that an early function is possible.

Expression of *Msx1* is seen in the distal tips of the neuroretina from E12.5 and in the perioptic mesenchyme, while *Msx2* is found in lens placode, lens, optic vesicle and in the neuroretina until E12.5. (Monaghan et al., 1991). A more recent study (R.Holme PhD thesis) also found *Msx1* expression in the anterior lens epithelium at E12.5 and in the dorsal neuroretina from E11.5. This distal expression extended around the entire neuroretinal rim by E12.5 as previously reported. *Msx2* expression was initially seen throughout the posterior optic vesicle at E9.5, from E10.5 being found only in the distal tips of the dorsal/posterior quarter. This pattern was maintained at E13.5, the latest stage analysed.

1.8.1.4 *Gli* genes

Three murine *Gli* genes have been identified and all are expressed in the eye. At E9.5 all three are present in the optic vesicle and subsequently at E14.5 *Gli* is found in the neuroretina while *Gli2* and *3* are expressed in the optic stalk and lens. In the *Gli3* mutant, extra-toes (*Gli3*^{xt}), the eye phenotype varies from virtual normality to the presence of a persistent optic vesicle with no lens. Hui et al. suggest that this variability is due to the action of modifiers (Hui et al., 1994; reviewed in Freund et al., 1996). The presence of all three genes in the optic vesicle at E9.5 coupled with the failure of *Gli3*^{xt} to develop beyond optic vesicle stage does suggest an early role for *Gli*. In the zebrafish *you-too* mutant, which has a truncated *Gli2* gene, ectopic lenses form from the diencephalon which may also suggest a specific role for this gene in lens development (Karlstrom et al., 1999).

1.8.1.5 *Xtll*

Xtll, the *Xenopus* homologue of *Drosophila tailless (tll)* is expressed in the presumptive eye region of the anterior neural plate, overlapping with but not identical to the *Pax6* expression domain. This initial *Xtll* expression occurs later than initial

Pax6 expression. Subseqently, *Xtll* is found in the optic stalk and distal optic nerve and later still is present in the ciliary margin of the neuroretina (Holleman et al., 1998).

Inhibition of *Xtll* function, using a dominant negative engrailed repressor containing construct, inhibited optic vesicle formation in *Xenopus*, indicating an early role for *Xtll* in the eye. In the mouse *mtll*$^{-/-}$ mutant the eye develops normally however (Monaghan et al., 1997). Holleman et al., suggest that this may be due to functional redundancy, as the dominant negative construct could potentially interfere with the action of more than one gene.

1.8.2 Genes involved in the development of specific eye tissues

Various genes have been implicated in the development of specific eye tissues. *Chx10* is important for neuroretinal development, the *Chx10*$^{-/-}$ ocular retardation *(or)* mouse having microphthalmic eyes with a thin hypoproliferative retina which entirely lacks bipolar cells (Burmeister et al., 1996). *Chx10* has hence been implicated in retinal proliferation and bipolar cell specification. Consistent with this phenotype expression in neuroretinal cells begins at E9.5, later becoming restricted to the inner nuclear layer which contains the bipolar cells (McInnes et al., 1997). *Alx*, the zebrafish *Chx10* homologue, is expressed in a similar pattern, and when gene function was removed, by application of anti-sense RNA, the phenotype resembled that of the *or* mouse. *Chx10* is thought to be a possible downstrean target of *Pax6* (Barabino et al., 1997).

MitF is important for RPE development and is responsible for the *microphthalmia (mi)* phenotype in mouse. In this mutant the RPE is hyper-proliferative, does not develop pigment and in some cases regions of the RPE develop neuroretinal character. (Nakayama et al., 1998; Scholtz and Chan 1987) In the B/B quail, which has a truncated *Mi* gene, small regions of neuroretinal character are also found (Mochi et al., 1998a). *MitF* is specifically expressed in the RPE from E10.5 although low levels are initially detected throughout the optic vesicle at E9.5 (Nguyen et al., 1997). *MitF* is discussed further in chapter 2.

Pax2 has been implicated in optic stalk development. It is expressed in optic stalk fated regions from an early stage and persists in optic stalk glial cells. *Pax2*$^{-/-}$

mice exhibit aberrant axonal pathways in the optic stalk, extension of the RPE into the optic stalks, and coloboma as a result of the failure of optic fissure closure. (Torres et al., 1996). As discussed in section 1.3.1 *Pax2* responds to patterning signals, including *Shh*, which position the eye. This also suggests that *Pax2* has an important role in the early specification of the developing eye.

Hes1 appears to have an essential role as a negative regulator of neurogenesis in the neuroretina. It is expressed highly in the neuroretina from E10.5 and only declines after birth when terminal differentiation of the final neural progenitor cells has occurred. The *Hes1*$^{-/-}$ mutant exhibits premature retinal differentiation which causes gross optic cup defects. The mutant also entirely lacks bipolar cells indicating a specific function in their development. Conversely, when *Hes1* is over-expressed the retina remains undifferentiated (reviewed in Freund et al., 1996).

Further genes with probable roles in the developing eye include *Prox1* which is expressed in the lens and *Dlx1* which is found in the neuroretina from E11.5 to E14.5 (Freund et al., 1996; Graw, 1996).

1.8.3 Homologies to *Drosophila* genes

Quite apart from the *Pax6/Eya/Six3* group, many other vertebrate eye development genes are homologous to *Drosophila* genes. *Rx* has a *Drosophila* homologue which may be involved in the initial steps of eye development, being found in two bilateral foci which perhaps represent the presumptive eye anlage (Eggert et al., 1998). *Gli* and *Dlx* are homologous to *cubitus interuptus (ci)* and distal-less *(Dll)* respectively (Hui et al., 1994; reviewed in Graw, 1996 and Freund et al., 1996). Genes involved in the early patterning and positioning of the vertebrate eye, *Shh* and the BMPs are related to *hedgehog (hh)* and *decapentaplegic (dpp)* respectively, both of which are important in *Drosophila* eye morphogenesis in the initiation and movement of the morphogenetic furrow.

Pax2 is homologous to the *Drosophila* gene *sparkling* which like *Pax2* is expressed in non-neuronal cells of the eye, *Pax2* being found in optic stalk glia and *sparkling* in non-photoreceptor accessory cells (Fu and Noll, 1997). In a similar way the mammalian gene *Prox1* is found in the CNS and the developing lens, while its *Drosophila* counterpart, *prospero,* is found in the CNS and in the lens secreting cone

cells (Freund et al., 1996). *Hes1* is homologous to the *Drosophila* genes *enhancer-of-split* and *hairy*. The two fly genes are involved in the suppression of neurogenesis via the proneural genes of the acheate-scute complex, similar to the suppression of neuronal differentiation in the retina by *Hes1*.

The fact that even later acting genes shared by vertebrates and flies appear to be performing analogous functions is somewhat surprising as the eye structures in which they are present are morphologically extremely diverse. It might have been expected that only those genes present in the very earliest steps of eye specification would be shared.

1.9 A common evolutionary origin for eyes?

The diversity of eye types which exist throughout the animal kingdom suggests independent evolution of eye types. Recently however, as evidence has amassed that many regulatory genes are shared between flies and mammals, there has been much discussion of a common eye ancestry. This raises the possibility that as yet undefined associations exist between distant organisms.

As a variety of genes are conserved in several species it seems unlikely that this eye function is merely coincidental and due to their expression within the CNS. Perhaps some property of these genes made them particularly suitable for specific eye functions thus allowing them to be independently acquired for similar functions in different species. The phylogeny of the *Six* gene family may argue for this type of process. The fly gene *so* has an eye function, while its closest mammalian homologues, *Six1* and *Six2*, do not appear in the developing eye. Eye function is instead performed by *Six3* and *Optx* which are *optix* homologues (Seo et al., 1999).

Another possible scenario is that a rudimentary unit existed which was the precursor of all eye types and expressed one or more of the conserved regulatory genes. Other conserved genes could then have been co-opted due to favourable interactions with those already present. This type of retrograde evolution has been proposed by Gehring and Ikeo 1999, based on the assumption that *Pax6* was present and the driving force behind eye evolution. All known visual systems have specialised photoreceptor cells which express light sensitive proteins called opsins. Studies based on the structure of photoreceptor cells, and the homology among the

opsins of at least 12 species, suggest a monophyletic origin of this cell type (Land and Fernald., 1992). *Pax6* is expressed either in photoreceptors or in their precursors in all species examined. This suggests that ancestral *Pax6* could have become associated with the regulation of a target gene fundamental to photodetection and then acted as the cohesive force during evolutionary change.

Nilsson and Pelger (1994) surmised that, beginning with a light sensitive tissue, eyes could have evolved separately more than 1000 times since the first fossil evidence of eyes, about 550 million years ago. Independent evolution of all eye types from an initial photosensitive structure therefore seems at least plausible, however there is no proof that the ancestral gene was in fact *Pax6*. Perhaps further elucidation of functions of conserved genes and the developmental mechanisms by which they act will throw light on how evolution of eyes occurred.

Chapter 2 Analysis of *Pax6* function using chimeras.

2.1 Introduction

2.1.1 Use of chimeras to study *Pax6* function

This study uses mouse aggregation chimeras to study the effects of the *Pax6* mutant, small eye *(Sey),* on eye development. The work follows on from a previous study by Quinn et al.,1996.

Small eye homozygotes have an extremely degenerate eye with no lens or placode and virtually no optic cup structures by E12.5 (Grindley et al., 1995). *Pax6* is expressed in several eye tissues, the RPE, the neuroretina and the lens, and probably has more than one function. In the small eye mutant however, the phenotype observed is the general failure of early eye development which precludes the study of later effects of *Pax6*. The disruption is also too great to allow early function within specific tissues to be analysed. The chimeric system overcomes this problem by partially rescuing the phenotype. The study of mutant cell behaviour in a wildtype background and vice versa is also made possible.

2.1.2 Occurrence and production of chimeras

A chimera is the term used to describe an animal which is derived from two or more zygotes. Chimeras can form spontaneously and perhaps the best known example of this is the blood chimerism found in cattle twins which shared a single fused placenta. The blood of each fetus circulates through both twins, so that two genotypically different populations of blood cells are present in each calf. As development of antibodies to the foreign blood would kill the other twin, tolerance is developed and the blood remains chimeric after birth (Vigier et al., 1984). Human examples of chimerism have also been identified based on blood grouping anomalies or sex chromosome aberrations, for example the possession of both XX cells and XY cells in a single individual. This type of spontaneous chimerism may occur by the aggregation of two fertilised eggs in utero or the independent fertilisation of both the egg and polar body (Stain et al., 1998a; Stain et al., 1998b).

Experimental chimeras can be split into two categories. Primary chimeras are produced early in development so that all or most tissues are chimeric, while secondary chimeras are produced later, by methods such as transplantation, so that only a subset of tissues are chimeric. Both approaches provide useful tools for the analysis of development and organogenesis. This study utilises primary mouse chimeras, which can be made in two basic ways (although a number of variations on these techniques have been used), by aggregation of two pre-implantation embryos (Tarkowski, 1961; Mintz, 1962) or by injection of cells into a blastocyst (Gardner, 1968). (The cells injected are now usually embryonic stem (ES) cell lines rather than the disaggregated embryo cells which were originally used.) There are advantages to both methods, aggregation chimeras being technically less demanding to produce and giving good mixing of cell types, whilst injection of ES cells allows manipulation of the cell line before injection and means that every chimera produced is of the requisite genotype (this is a major advantage). ES cell technology has also allowed the development of sophisticated techniques, such as embryos derived entirely of ES cells, produced by aggregating ES cells and tetraploid embryos (Nagy et al.,1993) Chimeras produced from targeted embryonic stem (ES) cells injected into wildtype blastocysts are now also an important intermediate in the production of targeted mouse mutants.

2.1.3 Some uses of chimeras and their role in the study of eye development

Chimeras are an extremely powerful technique for the analysis of mutant phenotypes and can often be used to identify the primary sites of activity of a gene with multiple effects. Lethal mutant phenotypes may also be rescued in a chimera which allows any later effects of the gene to be studied. Chimeras can also yield much information regarding cell mixing, cell lineage, patterns of tissue growth and interactions between cell types.

In particular, autonomy of gene action can be recognised in a chimera, as a lack of mutant cells in a tissue or by behavioural changes of mutant cells. In small eye chimeras the exclusion of mutant cells from the lens suggests that *Pax6* is required autonomously (Quinn et al., 1996). In chimeras derived from the congenital cataractous mouse (CAT), on the other hand, both wildtype and mutant cells were

found to be present in the lens, which had a wildtype phenotype. In the CAT mutant, the lens is abnormal and develops cataracts. The rescue of the lens phenotype in the chimera demonstrates the non-autonomy of gene action (Muggleton-Harris et al., 1987).

Cell lineage and growth patterns in the cornea have been investigated by the aggregation of mouse embryos containing a LacZ marker with a strain lacking the marker. The resulting pattern of LacZ staining, in stripes radiating from the centre of the cornea, was shown by computer modelling to be consistent with a situation where growth occurs entirely at the periphery of the cornea. This type of analysis has also been used successfully to investigate cell origins and patterns of growth in other organs. (West, 1999).

Elucidation of interactions between tissues is also often facilitated by the use of chimeras. For instance in the small eye chimeras (Quinn et al., 1996) the optic cup was far more convoluted in the absence of a lens than when a lens was present, even when the percentage of mutant cells in the chimeras was virtually identical. This clearly demonstrates the existence of a lens to optic cup signal. Experiments with retinal dystrophy (rdy) rat chimeras have also shown an interaction between the RPE and the neuroretina. Patches of degenerate neuroretina were always found juxtaposed to mutant RPE suggesting that the RPE is the site of gene action in this case (Mullen and LaVail, 1976).

2.1.4 Markers in chimeric systems

Chimeras can only be utilised to the full if the two cell populations which constitute the chimera can be distinguished. Various cell markers have been developed. Some allow determination of the proportion of each cell type within the chimera as a whole while others allow determination of the cell specific distribution of the two populations.

Pigmentation was one of the earliest markers to be used, however this method can only be used in selected tissues such as the RPE or the skin/coat where pigment is expressed (Muggleton-Harris et al., 1987; Mintz, 1967; Tarkowski, 1964). RPE pigmentation can however, also be used as an estimator of the contribution of the two cell types to the embryo as a whole (West et al., 1997).

A commonly used biochemical marker for estimating cell contribution to tissues is the enzyme glucose phosphate isomerase (GPI). Three allozymes, GPI1-A, B and C can be separated by their electrophoretic mobility. The relative contributions of each can then be determined by band intensity (Carter and Parr, 1967; Chapman et al., 1971; Chapman et al., 1972).

In order to ascertain the spatial distribution of each cell type in the chimera, cell specific markers are required. One such marker is the β-globin transgene present in strain 83 mice (Lo et al., 1986). This can be detected by DNA-DNA in situ hybridisation and is developmentally neutral, at least in the hemizygous state (West et al., 1996).This transgenic marker is cell autonomous and ubiquitously present in all tissues. The transgene only marks the cell nucleus however, which means that transgene containing cells will not be labelled if the nucleus is not contained in the plane of a section.

Transgenic lacZ expressing lines such as TgR(ROSA26)26Sor (Friedrich and Soriano, 1991) are also widely used, lacZ being detected by β-galactosidase staining. This approach has the advantage that cytoplasm is marked so that all lacZ containing cells in a section are obvious. It is also possible to stain whole tissues without sectioning which is not possible with the β-globin transgene. This technique does however rely on the tissue in question expressing the transgene which is not an issue with the β-globin marker.

It should also be possible to use the recently developed green fluorescent protein as a cell specific marker. This would have similar advantages to the lacZ method but should also allow living tissue to be examined (Hadjantonakis et al., 1998; Cubitt et al., 1995).

2.1.5 The small eye chimeric system

Using small eye chimeras, Quinn et al. showed a need for *Pax6* in all layers of the developing eye; the lens, the neuroretina and the RPE. Firstly, at E12.5, mutant cells were excluded from the lens. Further investigation (Collinson et al., 1999) has shown that at E9.5, before the lens placode invaginates, very few mutant cells are present in the presumptive lens or in the area immediately surrounding it. This suggests that *Pax6* has an early cell autonomous role crucial to the recruitment of

cells to the lens. As the area of exclusion extends outside of the placode itself, *Pax6* is likely to be important for pre-placodal lens competence.

Quinn also found that there was a threshold of 40% wildtype cells below which no lens formed, suggesting that there is a critical mass for lens formation. However as placodes are observed in higher percentage chimeras at E9.5 (Collinson et al., 1999) this mass must become critical during invagination or differentiation of the lens.

Secondly the study of Quinn et al., 1996, showed that mutant cells are never found in morphologically normal regions of the RPE. It was noted that although the mutant cells are genotypically pigmented *(C/C)*, the chimeric eyes never contain pigment. However *Trp2*, a gene involved in the pathway leading to pigment formation, is expressed in the RPE at E9.5 in both the mutant and wildtype regions and is also found in the degenerating optic cup of E12.5 small eye mutants.(Quinn, PhD thesis; Grindley et al., 1995). This suggests that initially the RPE is specified but in the mutant areas does not differentiate far enough to produce pigment, which normally occurs at E11.5. *Pax6* presumably has a role in RPE differentiation between these time points.

Thirdly, within the neuroretina there are distinct regions of mutant and wildtype cells which do not mix, suggesting that *Pax6* may be affecting cell surface proteins. It is known that *Pax* genes can interact with the promoters of cell adhesion molecules, *Pax6* interacting with LI-CAM (Chalepakis et al., 1994). N-CAM and cadherins are further possible effectors of *Pax6* function in the neuroretina.

The optic cup of E12.5 chimeras is often disrupted. Abnormality increases with increasing percentage of mutant cells. The wildtype regions are morphologically normal, consisting of two distinct layers, whilst the mutant regions are severely abnormal. There is however some overlap of phenotype at the boundaries, wildtype cells showing an abnormal phenotype. This suggests that the action of *Pax6* is not entirely autonomous.

Quinn also showed that the optic cup dysmorphology is more extreme in the absence of a lens, suggesting that an interaction occurs between the lens and the presumptive neuroretina. In chimeras which do have a lens there is exclusion of

mutant cells from the retinal region directly surrounding the lens which also hints at an organising signal emanating from the lens.

2.1.6 Further use of small eye chimeras in relation to this thesis.

The above study (Quinn et al., 1996; Quinn PhD thesis) raises many further questions which could be addressed using small eye chimeras. In particular, it is important to address the question of whether tissues of the eye (ie. the neuroretina and RPE) are specified in mutant regions, and if so how far they differentiate. This relates to, and should also provide insights into, the question of which genes *Pax6* targets and at what stage it acts in each tissue. Other related questions, such as what is causing wildtype and mutant cells to segregate in the retina and when and why mutant cells are excluded from the lens could also be addressed.

Many of these questions are related, for instance knowledge of the timing of action of *Pax6* will help to identify downstream targets. The answers to these questions may also be related. *Pax6* may actually have only one or two roles at a functional level although it appears to have multiple effects at first sight. The exclusion of mutant cells from the lens could, for instance, be brought about by the same mechanism as the segregation of mutant and wildtype cells in the retina, *Pax6* perhaps affecting cell adhesion properties.

Quite apart from the study of *Pax6* function, this chimeric system provides an opportunity to investigate retinal development in the absence of a lens. If the lens has a role in organising differentiation of the retina there may be differences between wildtype regions of the chimera in the absence or presence of a lens.

The preliminary results presented in this thesis relate to the role of *Pax6* in the RPE. To elucidate the timing of action of *Pax6* and to investigate the extent to which the RPE differentiates, RPE specific markers were used. The first, the *Trp2* gene, is part of the pigmentation pathway, acting at E9.5. *Micropthalmia (Mi)* expression is specific to the RPE by E10.5 although it is expressed throughout the optic vesicle from E9.5 (Nguyen et al., 1997), and is particularly interesting as *Mi* loss of function mutants have similarities to the small eye phenotype. In *Mi* mutants there is retinal degeneration, regions of hyperproliferation, and loss of RPE

pigmentation, the extent dependent on the allele in question (reviewed in Moore 1995, Mochii et al., 1998a/b). In many ways this parallels the abnormalities and lack of pigmentation found in mutant regions of small eye chimeras (Quinn et al., 1996). *Mi* is hence a potential target for *Pax6* within the RPE.

2.2 Production and analysis of small eye chimeras

2.2.1 Production of small eye chimeras.

Chimeras were produced (according to Quinn et al., 1996) by aggregating a wildtype 8 cell embryo (BALB/c or AAF_2 ie.[BALB/c x A/J]F_2) with a second 8 cell embryo which was the product of a mating between a $Pax6^{Sey/+}$ male and a $Pax6^{Sey\text{-}Neu/+}$ female (see below for strain information), hence only one in four chimeras was of the required genotype, $Pax6^{Sey}/Pax6^{Sey\text{-}Neu} \leftrightarrow +/+$. The compound heterozygote was used, rather than a single small eye allele, so that homozygous and heterozygous chimeras could be distinguished. The PCR genotyping strategy used is discussed below. The experimental scheme used to produce the chimeras is shown in figure 2.1 and described in section 6.2. The following is a brief outline of the procedure:

Embryos were collected at the 8-cell stage, aggregated in pairs and cultured overnight before transferring into a pseudopregnant host on day 2.5 of pseudopregnancy. Aggregation was accomplished by first removing the zona pellucida which surrounds the embryo then pushing the embryos together. The following day embryos which had developed to late morula or blastocyst stage were transferred surgically into the uterus of the host mother. Abnormal embryos were discarded. Pregnancy was then allowed to continue until day E12.5, taking the day of transfer as E2.5. At this point embryos were removed for analysis of phenotype, percentage chimerism, histology and genotype.

Markers of cell type

In any chimeric system it is necessary to include markers to distinguish between the two cell types. In this case three marker systems were used, a pigmentation locus, allozymes of Glucose phosphate isomerase 1 (GPI1), and a β-globin transgene as in table 2.1

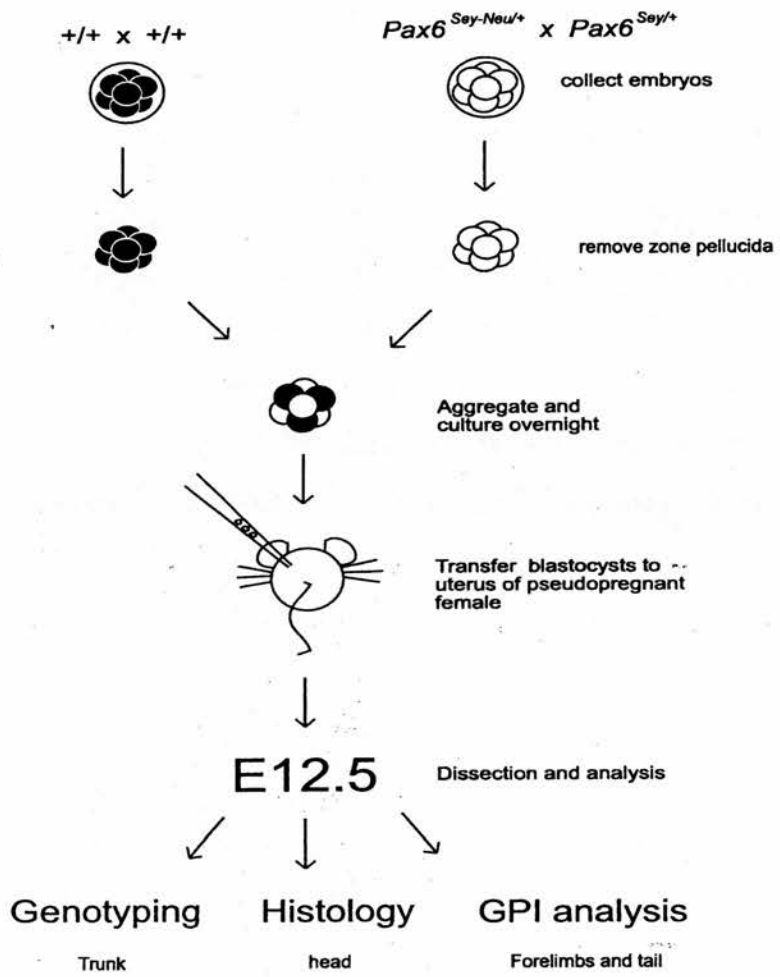


Figure 2.1 : Schematic diagram of small eye chimera production.

Pigmentation is a commonly used marker system, chimerism then being obvious at a glance in pigmented tissue. In this case the wildtype embryo was albino *(c/c)* whilst the small eye embryo was genotypically pigmented *(C/C)*. However, it transpired that pigmentation does not occur in homozygous small eye RPE cells, rendering this marker useless, in this instance, for determining chimerism. Since heterozygous and wildtype embryos from the small eye cross do express pigment however, a non-pigmented chimeric eye is a useful indicator of null homozygosity.

The GPI1 allozymes allow the relative contribution of the mutant and wildtype embryos to a specific tissue to be elucidated. The percentage of GPI1-B (*Sey*) in each tissue indicates the proportion of cells which are derived from the small eye cross. As it has been shown previously (Falconer et al., 1981) that chimerism in one tissue correlates with contribution in the rest of the embryo the average of these results was taken as an estimate of chimerism within the embryo as a whole and hence the eye.

A β-globin transgene, *Tg*, was homozygous in the $Pax6^{sey/+}$; $Tg^{+/+}$ males which were used to produce the small eye embryos; these embryos are hence $Tg^{+/-}$ (see strains information below). The transgene can be visualised histologically by DNA *in-situ* hybridisation using a digoxygenin labelled β-globin probe. This gives cell specific localisation to the mutant cells.

Table 2.1 : Genotype of embryos used for chimera production (genotypes of mouse strains are shown in section 6.2)

Embryo type	**Pigmentation**	**Gpi 1 allele**	**β-globin transgene**
Small eye embryo ($Pax6^{Sey/+}$ x $Pax6^{Sey\text{-}Neu/+}$)	C / C	b / b	$Tg^{+/-}$
Wildtype embryo (AAF_1 or BALB/c	c / c	a / a	$Tg^{-/-}$

Mouse Strains

Wildtype embryos: Initially BALB/c embryos were used; this is an albino strain *(c/c)* homozygous for $Gpi1^a$. 18 chimeras were produced with this strain, however contribution to the chimeras was low with small eye cells predominating (see table 2.2). Mean GPI1-A was only 25.4% and the range 10-40%. This gave a very biased set of chimeras which was not ideal. It was therefore decided to use AAF_1 mice to produce the embryos instead. AAF_1 mice are an F1 hybrid between BALB/c and A/J. These are also albino and $Gpi1^{a/a}$, and should compete better with the small eye embryo (West et al.,1995). This was indeed the case and the remaining 36 chimeras were made using this strain. Mean GPI1A was 48.7% and the range 10 - 90% for these embryos.

Small eye embryos: Throughout, the mice used for the cross were:

Female- $Pax6^{Sey\text{-}Neu/+}$

Male - $Pax6^{Sey/+}$; $Tg^{+/+}$

The Pax^{Sey} animals were derived by Quinn (Quinn et al.,1996, Quinn - PhD thesis) from $Pax6^{sey/+}$ mice on a CBA/Ca background crossed to TGB stock (Keighren and West, 1994) . The TGB stock was derived from crosses between strain 83 (Lo et al.,1986, 1987) and (C57BL x CBA/Ca)F_1 and carries the β-globin transgene, TgN(Hbb-bl)83clo β-globin. It was shown (West et al., 1996) that the inclusion of this transgene does not in any way compromise the ability of the cells to colonise the chimera.

The $Pax6^{Sey\text{-}Neu}$ strain is also on a CBA/Ca background and is derived from the original Neuherberg stock.

Both small eye stocks are pigmented *(C/C)* and $Gpi1^{b/b}$

Host Mother strain: The strain used for the pseudopregnant female was an F1 hybrid strain, CF_1 derived from a cross between two congenic strains based on C57BL and BALB/c. These mice were albino and $Gpi1^{c/c}$ which was important in recognising any contaminating host tissue in chimeric samples.

2.2.2 Analysis of the chimeras produced

Chimeric embryos were analysed at day E12.5. On dissection the weight and crown-rump length of the fetus were measured and developmental stage according to hind limb development recorded (Mclaren and Buehr, 1990; Palmer and Burgoyne, 1991) (see table 2.2). Taken together these three measurements gave an indication that development was occurring normally. Statistical analysis has shown that there is no significant difference between the homozygous chimeras and the wildtype and heterozygous chimeras with regard to fetus weight ($T = 0.809$, $p > 0.2 < 0.3$, $df. = 49$), crown-rump length ($T = 0.618$, $p > 0.2 < 0.3$, $df. = 49$) or developmental stage by hind limb development ($\chi^2 = 1.822$, $p > 0.3 < 0.5$, $df. = 4$).

In each case the appearance of the embryo was recorded, taking special note of eye pigmentation and craniofacial morphology. Homozygous mutant chimeras have no pigment in the eye and when a high proportion of cells are mutant, have abnormal eyes and craniofacial defects like homozygous small eye embryos. At this stage some non-chimeric embryos were also albino. Heterozygous and wildtype chimeras are obvious from the pigmentation pattern in the eye, a mixture of pigmented and albino cells in the RPE. Percentage chimerism could hence be estimated from the percentage of pigmentation in these cases.

Subsequently dissection was carried out, the tail and forelimbs being taken for GPI analysis, the head fixed overnight in 4% paraformaldehyde for histological analysis and the remaining trunk region taken for PCR genotyping.

GPI analysis

GPI analysis allowed the percentage of each cell type in the chimera to be ascertained (Eicher and Washburn, 1978; Peterson et al., 1978; West and Flockhart, 1994). GPI1A represents the wildtype portion of the chimera and GPI1B the portion from the small eye mating. The presence of GPI1C would indicate contaminating tissue from the host mother, this was never seen in this study.

GPI was extracted from each tissue in turn, that is the forelimbs and the tail, then these extracts subjected to electrophoreses (section 6.2). GPI 1B runs faster towards the cathode than GPI1A with GPI1C running still faster. After staining,

scanning densitometry allowed the percentage contribution of each isomer to be elucidated. Non-chimeric embryos could now be identified and removed from further analysis as they contain only one GPI1 isomer. Non-chimeric fetuses occurred at this stage with a frequency of approximately 1 in 2.

In combination with the appearance of the chimeric eye it was possible to identify homozygous chimeras as these contained no pigment. The pigmented chimeras were either heterozygous or wildtype. However, to separate the heterozygotes and the wildtypes PCR genotyping was necessary. It was also necessary to confirm the genotype of the homozygotes.

PCR genotyping

The genotyping strategy is dependent on the presence of two different small eye alleles in the homozygous chimeras, $Pax6^{Sey}/Pax6^{Sey\text{-}Neu} \leftrightarrow +/+$. Each allele was amplified separately using a specific set of PCR primers and then digested to distinguish wildtype from mutant. (figure 2.2 and section 6.2). The $Pax6^{Sey}$ allele is a single base pair substitution which creates a novel DdeI site, so that the 2 wildtype bands of 83 and 74bp become 3 bands of 83, 55 and 19bp in the mutant. The $Pax6^{Sey\text{-}Neu}$ allele, also a single base pair substitution, adds a HindII site to the wildtype band of 220bp producing two bands of 140 and 80 bp in the mutant. If both alleles are present then the chimera is a mixture of homozygous mutant and wildtype cells, heterozygotes having only one allele and wildtypes none.

Table 2.2 : Chimera analysis data
PIG: pigment, Neu: $Pax6^{Sey\text{-}Neu}$, Ed: $Pax6^{Sey}$, c: conceptus, f: fetus, c-r: crown-rump

Chim. No.	GPI1A %	GPI1B %	PIG?	Neu?	Ed?	Wt strain	c-weight	f-weight	c-r length	stage
KH3	30	70	Y	Y	N	BALBc	0.327	0.119	9.68	L7
KH5	20	80	Y	N	N	BALBc	0.313	0.116	9.82	L7
KH6	10	90	Y	N	N	BALBc	0.363	0.112	9.55	L7
KH7	30	70	N	Y	Y	BALBc	0.371	0.119	9.55	L7
KH8	40	60	N	Y	Y	BALBc	0.432	0.115	10.09	L7
KH9	10	90	Y	N	N	BALBc	0.346	0.13	9.82	L7
KH11	20	80	Y	N	Y	BALBc	0.375	0.129	9.68	L7
KH14	40	60	Y	N	N	BALBc	0.33	0.096	9.27	L7
KH15	40	60	N	Y	Y	BALBc	0.306	0.112	9.41	L7
KH16	10	90	Y	Y	N	BALBc	0.32	0.099	9.27	L7

KH18	20	80	Y	Y	N	BALBc	0.305	0.103	9.27	L7
KH19	20	80	Y	N	N	BALBc	0.363	0.13	9.82	L7
KH20	20	80	Y	-	N	BALBc	0.302	0.098	8.59	E7
KH21	13	87	Y	Y		BALBc	0.339	0.109	9.14	L7
KH22	30	70	Y	Y		BALBc	0.339	0.106	9.55	L7
KH24	40	60	Y	Y		BALBc	0.346	0.12	9.55	L7
KH30	40	60	Y	Y		BALBc	0.305	0.104	9.55	L7
KH34	-	-	-	Y		BALBc	0.358	0.134	9.55	L7
KH38	70	30	Y	Y	N	AAF1	0.27	0.084	8.59	L6
KH39	50	50	Y	N	N	AAF1	0.264	0.09	9.14	L7
KH42	30	70	Y	Y	N	AAF1	0.369	0.102	9.55	L7
KH43	25	75	N	Y	Y	AAF1	0.284	0.109	9.55	E8
KH44	85	15	Y	N	N	AAF1	0.278	0.089	9	L7
KH46	40	60	N	Y	Y	AAF1	0.2697	0.083	9	E7
KH47	50	50	N	Y	Y	AAF1	0.356	0.127	10.23	L7
KH48	15	85	Y	-	Y	AAF1	0.305	0.113	10.09	L7
KH49	80	20	Y	Y	N	AAF1	0.321	0.098	9.27	L7
KH50	15	85	Y	N	Y	AAF1	0.358	0.135	9.82	L7
KH51	90	10	N	-	N	AAF1	0.266	0.081	9.27	L7
KH57	75	25	Y	Y	N	AAF1	0.229	0.063	7.64	5
KH58	15	85	Y	-	Y	AAF1	0.3115	0.065	8.45	5
KH59	85	15	Y	Y	N	AAF1	0.281	0.09	9.07	L7
KH60	50	50	Y	-	N	AAF1	0.394	0.123	9.95	E8
KH61	50	50	Y	-	Y	AAF1	0.442	0.091	7.09	5
KH63	65	35	N	Y	Y	AAF1	0.3316	0.109	9.55	L7
KH67	-	-	N	Y	Y	AAF1	0.4663	0.072	7.64	5
KH68	-	-	Y	-	N	AAF1	0.4624	0.1095	8.86	E6
KH70	-	-	Y	-	N	AAF1	-	0.0426	6.14	5
KH71	70	30	Y	-	-	AAF1	0.269	0.089	8.59	L7
KH72	60	40	N	-	-	AAF1	0.293	0.092	9	E7
KH73	35	65	Y	-	-	AAF1	0.333	0.108	9.41	-
KH75	40	60	Y	-	-	AAF1	0.304	0.095	9.82	L7
KH76	10	90	N	-	-	AAF1	0.334	0.11	9.41	L7
KH77	20	80	N	-	-	AAF1	0.315	0.097	9.27	L7
KH78	30	70	Y	-	-	AAF1	0.304	0.107	9	L7
KH79	-	-	N	-	-	AAF1	0.292	0.078	8.73	L7
KH82	15	85	N	-	-	AAF1	0.351	0.107	9.55	L7
KH83	60	40	Y	-	-	AAF1	0.373	0.123	9.68	L7
KH84	80	20	N	-	-	AAF1	0.308	0.098	8.86	L7
KH86	60	40	N	-	-	AAF1	0.277	0.094	8.86	L7
KH88	20	80	Y	-	-	AAF1	0.33	0.106	9.41	L7
KH94	70	30	Y	-	-	AAF1	0.247	0.083	8.73	E7

2.2.3 Histological analysis of small eye chimeras.

Selection of chimeras

Selection of suitable chimeras for histological examination, based on genotyping and GPI data, was now possible (data for each chimera is shown in table 2.2). The ideal situation for the proposed experiments was to investigate mutant

regions of RPE in an environment where wildtype areas were still present. Those chimeras selected were therefore between 50% and 70% small eye, as from previous work (Quinn et al., 1996) this range appeared to fulfil the above criteria. Below this range the eye was entirely rescued and above this range extremely disorganised (Quinn et al., 1996).

Those chimeras thought to be suitable were therefore; KH15 (60% GPI1-B), KH46 (60% GPI1-B) and KH47 (50% GPI1-B). Several others were potentially useful; KH43 (75% GPI1-B) and KH77 (80% GPI1-B). KH7 (70% GPI1-B) and KH8 (60% GPI1-B) were also possibilities, however, in both cases good mixing of the two cell populations has not occurred. Although GPI correlations are never perfect between the

tissues of a single aggregation chimera, the extent of the differences seen in these two cases is unusual. KH7 has a right forelimb with 70% mutant cells and a left forelimb and tail with virtually 100% GPI1-B. KH8 is similar having a left forelimb with 60% mutant cells and a right forelimb and tail with 100% mutant cells. In both cases the eye phenotype seemed to correlate with this pattern, the side which was 100% mutant having no eye, whilst the chimeric side had a small eye rudiment.

For the present analysis KH47 and KH46 were sectioned. KH47 was analysed as described below but KH46 had no regions of mutant tissue within the eye so was not analysed further.

Analysis of chimera KH47

To investigate the effect of *Pax6* on development of the RPE, KH47 was serially sectioned and radioactive in-situ hybridisation (RINS) carried out. Adjacent eye sections were hybridised with antisense *Trp2*, *Pax6* and *Mi* RNA probes. The protocol used is shown in section 6.2. The probes were labelled with ^{35}S-UTP and slides exposed for three weeks and three days before autoradiography.

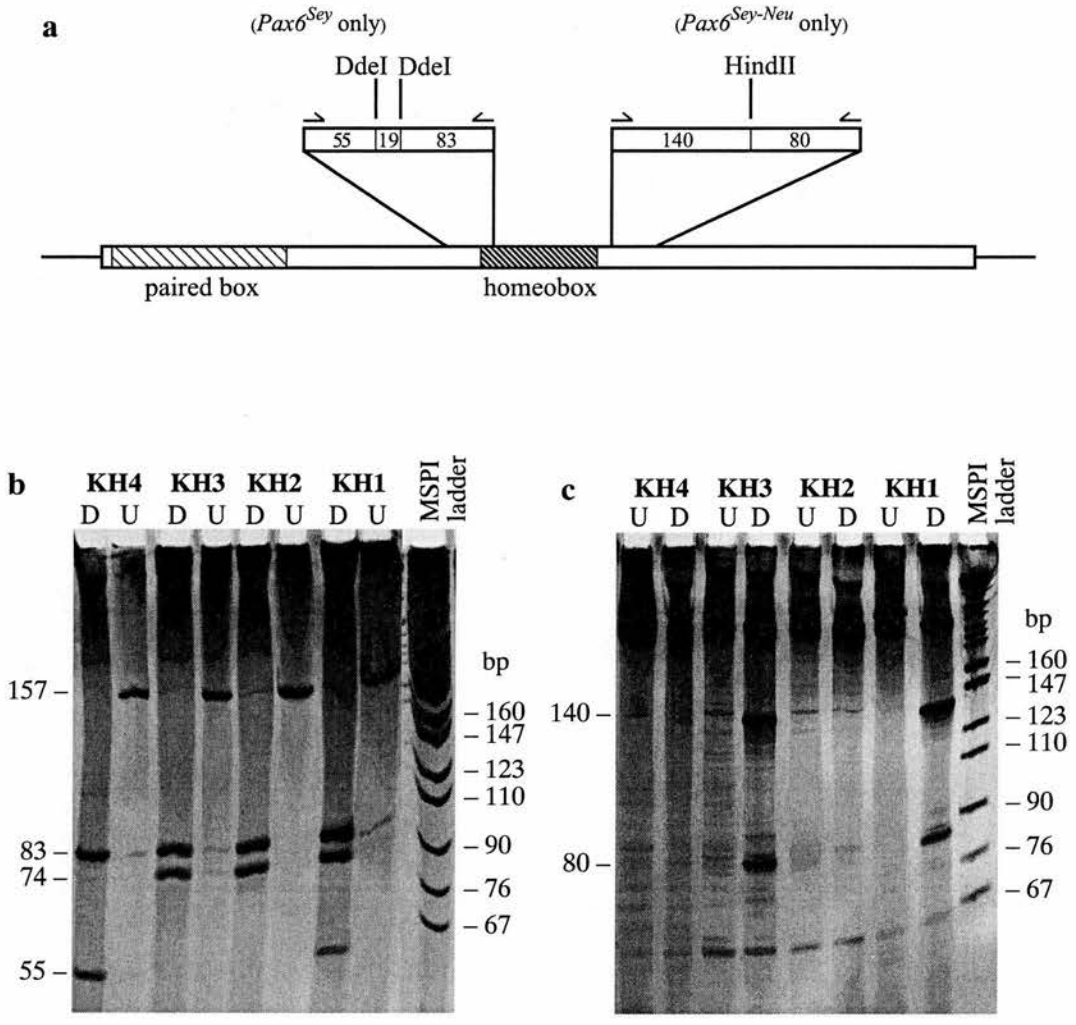


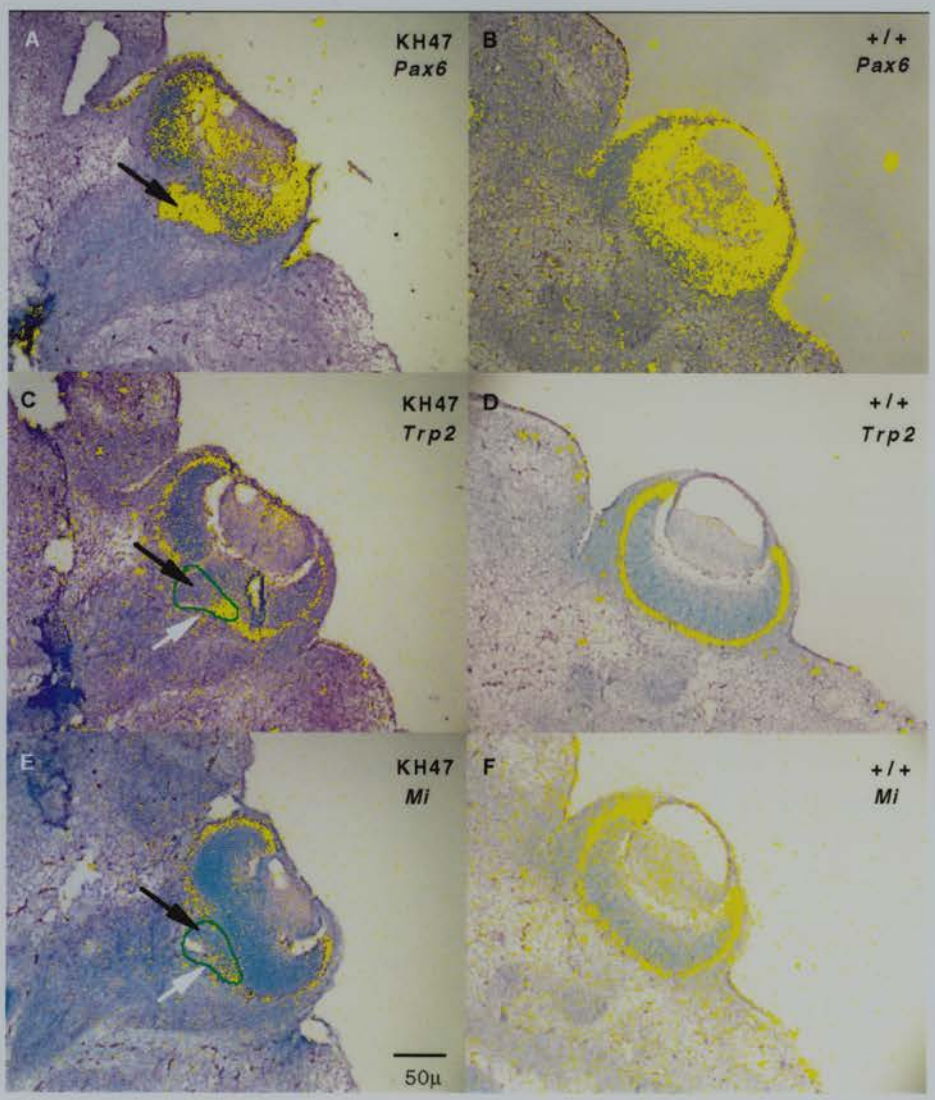
Figure 2.2 Chimera genotyping by PCR

Both the *Pax6*^{Sey} and *Pax6*$^{Sey\text{-}Neu}$ mutants have single base pair changes (illustrated in fig 1.4) which give rise to novel DdeI and HindII restriction sites respectively.

(a) A schematic diagram of the *Pax6* gene showing the primers used for PCR amplification, the restriction sites and the size of fragments produced.

(b) shows analysis of the *Pax6*^{Sey} region. The undigested PCR product is 157bp, the wildtype digested fragments are 83bp and 74bp in length and the mutant product digests to 83bp, 55bp and 19bp. Sample KH1 and KH4 therefore contain the mutant allele.

(c) shows analysis of the *Pax6*$^{Sey\text{-}Neu}$ region. The undigested PCR product is 220bp. The wildtype product does not cut with HindII while the mutant digests to two fragments of 140bp and 80bp. Sample KH1 and KH3 therefore contain the mutant allele.


A
KH47
Pax6
B
+/+
Pax6
C
KH47
Trp2
D
+/+
Trp2
E
KH47
Mi
F
+/+
Mi
50μ

MARKERS

Trp2 and *Mi* were chosen as markers because they are specifically expressed in the RPE between E9.5 and E11.5. The wildtype expression patterns of all three genes at E12.5 are shown in figure 2.3.

Trp2 is expressed at E9.5 in the developing RPE and in developing melanocytes in other regions of the body (Steel et al., 1992). This gene is an essential part of the pathway leading to RPE pigmentation and was previously shown to be present, at E14.5 and E12.5, in both mutant and wildtype regions of small eye chimeras (Quinn - PhD thesis). This suggested that RPE had been specified in mutant areas although pigment formation had not occurred. In this study *Trp2* was used to confirm that RPE had been specified in mutant regions by E12.5. Assuming that this was the case the presence of *Trp2* would confirm that the region of mutant tissue under examination was indeed RPE.

Mi is expressed specifically in the RPE by approximately E10.5. The mutant *Mi* mouse has a phenotype which is strikingly similar to the mutant regions of RPE in the small eye chimeras, having retinal degeneration and an absence of pigmentation in the RPE. The degree of abnormality in the eye is dependant on the particular allele of *Mi* (reviewed in Moore, 1995). This, together with its expression in the RPE between E9.5 and E11.5, the putative window of *Pax6* action, make *Mi* a potential downstream target of *Pax6*.

The *Pax6* probe used hybridises to the mutant form of the gene as well as the wildtype form because the two alleles used are point mutations rather than deletions.

Figure 2.3 : Radioactive *in-situ* hybridisation analysis of the eye of chimera KH47 and a wildtype control.
a, c and **e** show sections 1, 2 and 5 of a congruent series, hybridised with *Pax6*, *Trp2* and *Mi* respectively. The **black arrow** points to a region of dysmorphology thought to represent $Pax6^{-/-}$ mutant tissue in an otherwise wildtype eye. This region is encircled in green in **c** and **e**
b, d and **f** show congruent sections of a wildtype eye also hybridised with *Pax6*, *Trp2* and *Mi* respectively.
(a) *Pax6* expression is normal but raised within the abnormal region.
(c) *Trp2* expression is normal throughout the wildtype RPE. It is absent from the majority of the abnormal tissue but expressed in a small region adjacent to the wildtype tissue (**white arrow**).
(e) *Mi* expression is also normal within the wildtype RPE and is absent from the majority of the abnormal tissue. Like *Trp2* it is expressed in a small region adjacent to the wildtype tissue (**white arrow**).

It has also previously been shown that *Pax6* expression is higher in mutant regions of the RPE than in wildtype regions (Quinn - PhD thesis). This probe also identifies the tissue of the mutant eye as at E12.5 *Pax6* is expressed in all eye layers.

RADIOACTIVE *IN-SITU* HYBRIDISATION (RINS) RESULTS

KH47 was 50% $Pax6^{Sey/Sey\text{-}Neu}$ with one region of morphologically abnormal tissue in the retina which was presumed to be mutant. This could not however, be confirmed, via in-situ hybridisation to the β-globin transgene, for technical reasons. The RINS sections are shown in figure 2.3, the sections are 1,4 and 5 of a series and show *Trp2, Mi* and *Pax6* expression respectively. The sections show, that in wildtype regions all three genes are expressed, whilst in the bulk of the mutant region only *Pax6* is expressed while *Trp2* and *Mi* are not. In the region of mutant tissue nearest to the neighbouring wildtype region however, both *Trp2* and *Mi* expression are observed.

The level of *Pax6* expression within the presumed mutant region is higher than in the adjacent wildtype tissue. This tends to confirm, in lieu of other confirmatory evidence, that this region is indeed mutant tissue, as it was previously shown (Quinn - PhD thesis) that mutant regions of small eye chimeras do express *Pax6* more highly.

Mi and Trp2 are therefore not expressed in the majority of a region lacking functional *Pax6* in a small eye chimera. Both genes are however expressed in adjacent regions of wildtype tissue and in a small region of the presumed mutant tissue.

2.3 Discussion

2.3.1 Limitations of this study

The data presented in this chapter is preliminary due to the limited sample size and the absence of a marker with which to confirm that the morphologically abnormal region is indeed mutant. From previous work (Quinn - PhD thesis) it was noted that *Pax6* expression is raised in mutant regions and in the absence of another marker this suggests that this abnormal region is indeed mutant tissue.

In other respects the data in this study does not agree with the results reported by Quinn. Quinn (PhD thesis) observed that *Trp2* is present in mutant areas at both E12.5 and E14.5. On closer examination of the data however, it seems that at E12.5 the sections obtained by Quinn appear to parallel the data obtained in this study: *Trp2* is not present in the majority of the mutant tissue but is expressed in a small region adjacent to the wildtype cells. At E14.5 interpretation of Quinn's sections is inconclusive as the dysmorphology in the optic cup is great.

2.3.2 Does *Pax6* act upstream of *Mi* and *Trp2*?

In the wildtype situation, at day E9.5, *Trp2* is expressed strongly in the proximal optic vesicle which will become the RPE (Steel et al., 1992). At this stage *Mi* is expressed at a low level throughout the optic vesicle. As the vesicle comes into contact with the surface ectoderm expression of *Mi* disappears from the distal layer of the optic cup which will become the neuroretina, becoming specific to the presumptive RPE by E10.5. At this point levels of expression are also significantly raised (Nguyen et al., 1997). Expression of both genes can still be found specifically in the neuroretina at E12.5 and beyond.

In this study expression of *Trp2* and *Mi* was absent from the majority of a morphologically abnormal region of optic cup. Both genes were, however, present in a region of this abnormal tissue adjacent to the wildtype RPE to which it is joined. This raises the immediate question, is this morphologically abnormal tissue entirely composed of mutant cells and if so, why are *Trp2* and *Mi* expressed in only a small region?

Is the morphologically abnormal region mutant?

The abnormal region is thought to be mutant in spite of the absence of an independent marker with which to verify this. The histological appearance alone suggests that this region is not wildtype, and the raised *Pax6* level observed is characteristic of mutant regions in small eye chimeras (Quinn-PhD thesis). In addition the *Trp2* expression data obtained here corresponds to that seen by Quinn in an experiment where the presence of mutant cells was confirmed with the β-globin transgenic marker. It is possible however, that wildtype cells are present at the edge

of the abnormal tissue. The non-automomous inclusion of wildtype cells at the edges of morphologically abnormal regions of retina has been previously reported (Quinn et al., 1996).

What is the difference between the regions of mutant tissue which do express *Trp2* and *Mi* and those which do not?

A simple explanation is that the region which expresses *Trp2* and *Mi* actually contains wildtype cells while the remainder of non-*Trp2/Mi* expressing tissue is mutant. Alternatively the presence of adjoining wildtype tissue may be sufficient to rescue the expression of these genes in the adjacent region of mutant tissue. Both of these explanations suggests that *Pax6* is normally responsible for activating *Trp2* and *Mi* which are thus not expressed in the absence of functional *Pax6*. As expression of *Trp2* has been reported in the optic vesicle of homozygous small eye fetuses however, these explanations are not likely (Grindley et al., 1995). Tissue containing no functional *Pax6* obviously can express *Trp2* at least.

While this implies that *Trp2* and *Mi* expression is independent of *Pax6* it does not explain why some regions of the mutant tissue express these genes but others do not. *Trp2* expression (and hence *Mi*, or vice versa) must therefore be restricted to certain regions of the developing eye by some other factor. It is possible that *Trp2* activation is due to interactions with another eye tissue which is present in a restricted pattern. For instance the peri-ocular mesenchyme has been implicated in determining RPE fate and could perhaps be achieving this through regulation of *Trp2/Mi*. If this is the case it may be that the non-*Trp2/Mi* expressing mutant tissue is present in an abnormal position such that this external influence cannot act upon it. Indeed the mutant regions observed do seem to be positioned towards the back of the optic cup, perhaps outside the domain where RPE normally forms.

In the study of Grindley et al. (1995) the region of *Trp2* expression in homozygous small eye fetuses was restricted to the tip of the degenerating optic cup (see figure 2.4) perhaps in the prospective retinal region. *Trp2* however, appears to be expressed throughout the optic cup rather than being restricted to the outer layer. It is known that ectopic mesenchyme collects between the optic vesicle and surface

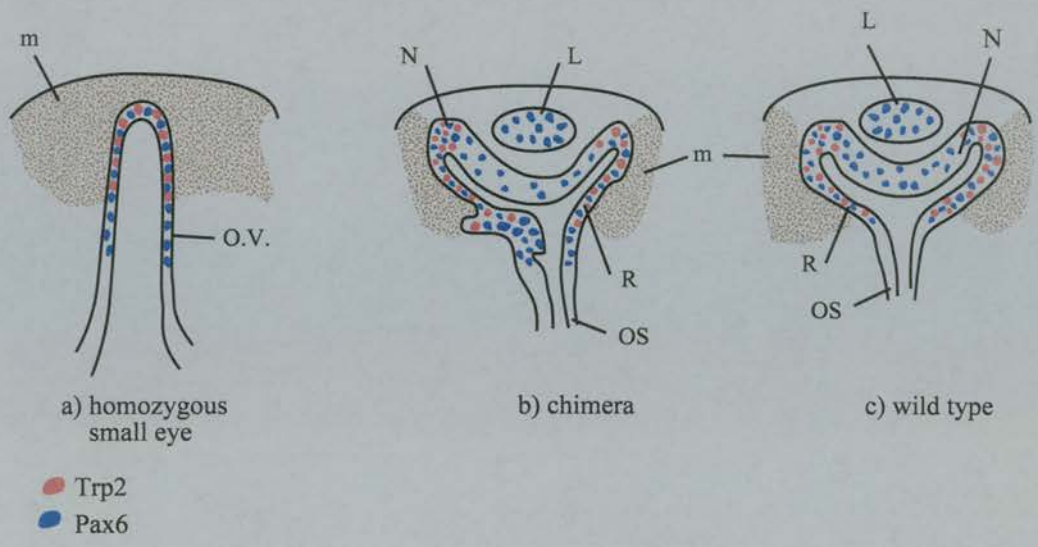


Figure 2.4 : Schematic of *Trp2* and *Pax6* expression in small eye, small eye chimera and wildtype eyes all at E12.5.
m: mesenchyme, N : neuroretina, L ; lens, R : RPE, O.V. : optic vesicle, O.S. : optic stalk

ectoderm in small eye mice so perhaps this also suggests that mesenchyme is indeed regulating *Trp2*.

2.3.3 What is this tissue which expresses *Pax6* highly but does not express *Trp2* or *Mi*?

In wildtype E12.5 eyes, *Pax6* expression is found within the neuroretina and RPE but it barely extends beyond the region of *Trp2* and *Mi* expression (ie the RPE) into the optic stalk. In *Sey* mice *Pax6* expression extends well beyond the *Trp2* expressing region into the presumed optic stalk (Grindley et al., 1995) (figure 2.4). Similarly in the mutant region of the chimera a large region of *Pax6* expression is found outside of the *Trp2/Mi* zone. What is this tissue? The absence of *Trp2* and *Mi* expression suggests that it is not RPE fated. The hypertrophied, non-epithelial morphology of this region agrees with this interpretation.

Could the mutant tissue have adopted a neuroretinal fate? The morphology of the mutant region of the chimera does resemble neuroretina more closely than RPE, being more than the usual mono-layer thick. It is also known that in the absence of *Mi,* RPE can trans-differentiate to neuroretina. In both the $Mi^{-/-}$ mutant mouse and the B/B quail, which has a truncated *Mi* gene, small regions of neuroretinal character are found in the RPE (Scholtz and Chan 1987; Mochii et al., 1998a). It would therefore be interesting to see if neuroretina specific genes, such as *Chx10*, are expressed. The morphology of the *Pax6* expressing/non *Trp2* expressing region in small eye mice however does not have this hypertrophied neuroretina-like appearance. Perhaps this region is optic stalk fated. *Pax6* is normally excluded from the optic stalk so this explanation does not at first seem likely. As *Pax6* is non-functional however it is possible. The use of an optic stalk marker such as *Pax2* could help to resolve this.

Perhaps the most likely explanation for the increased *Pax6* levels is that the tissue is simply immature. Prior to E12.5 *Pax6* expression does extend further back into the optic stalk which would account for the observed *Pax6* expression pattern in small eye mice (Grindley et al., 1995). *Pax6* is also seen at higher levels in the RPE before it is down regulated at E12.5. Whatever it is that down regulates *Pax6* in the RPE is presumably not present in mutant tissue because RPE differentiation stops before it can be activated. It is hence possible that the mutant region is either

immature RPE or immature optic stalk in the chimera. The tissue may of course be too immature to have properly adopted either fate.

2.3.4 The effect of *Pax6* on RPE development.

It is known that the RPE does not develop far enough to form pigment in mutant regions of small eye chimeras (Quinn et al., 1996). In fact a small amount of pigment has been seen in E18.5 chimeras but this is thought to be from the choroid rather than the RPE (D.Caric- personal communication) so it is probable that development of this tissue has ceased rather than being merely retarded. As both *Trp2* and *Mi* can be expressed by mutant regions of the chimera the RPE is obviously initially specified in at least some cells. It appears therefore, that *Pax6* functions in a parallel pathway to *Trp2* and *Mi* and is itself essential to RPE development. Some factor in the developing RPE must then be responsible for the reduction in *Pax6* expression which normally occurs in the RPE. It has been suggested that *Mi* may itself be a down-regulator of *Pax6*. Firstly, *Pax6* levels were abnormally high in quail RPE cultures taken from *Mi* mutant birds (strain B/B) (Mochii et al., 1998a); and secondly, *Pax6* expression increases in the absence of *Mi* and is inhibited when *Mi* is overexpressed in chicken RPE culture (Mochii et al., 1998b). As *Mi* is expressed in a region of abnormal tissue expressing high levels of *Pax6* RNA (this hybridising *Pax6* RNA is non-functional) this cannot however be the whole story.

2.3.5 Further work

Initially the data presented here needs to be confirmed in the presence of an independent marker of mutant cells. It would be informative to investigate slightly higher percentage chimeras as dysmorphology is greater. It may then be possible to investigate regions of mutant tissue away from the optic stalk area to confirm that expression of *Trp2/Mi* can occur in mutant cells.

It would also be of interest to investigate the identity of the mutant non-*Trp2/Mi* expressing cells by the use of neuroretina and optic stalk specific markers such as *Chx10* and *Pax2* respectively. A later marker of RPE development such as *Trp1* (which is activated at approximately E10.5) would help to identify the exact

time of *Pax6* action given that it is known that pigment, which usually develops at E11.5, is not expressed in mutant tissue.

Chapter 3 Cloning and characterisation of murine *Dachshund*

3.1 Introduction

3.1.1 *Drosophila dachshund (dac)*

Drosophila dac is involved in both eye and leg development, as demonstrated by loss of function mutants in which the legs are drastically shortened and the eyes are reduced or absent (Mardon et al.,1994). Shortening of the segmental leg structure is due exclusively to fusion and condensation of the intermediate segments; the proximal and distal segments are unaffected. In the eye, disruption of development occurs at the earliest stages, affecting initiation of the morphogenetic furrow. Expression of *dac* is found in the imaginal discs of both the eye and leg in domains directly associated with the phenotype. In the leg this is in a ring of expression corresponding to the region from which intermediate segments develop. In the eye expression is found initially at the rear of the eye disc in the region of furrow initiation and later anterior to the progressing furrow. Other tissues in which *dac* is expressed are the antennal and wing imaginal discs and the central nervous system, including the optic lobe of the larval brain; however, there is no obvious phenotype in these areas (Mardon et al., 1994).

Dac protein is localised to the nucleus and is thought to interact with other proteins rather than DNA as it contains no known DNA binding motifs. It has been postulated that *dac* has a function in cell proliferation as increased cell death was observed in the mutant fly in the regions of *dac* expression in both the leg and the eye disc. This may however, be as a result of a failure of the tissue to differentiate correctly in the absence of *dac* rather than because of a specific cell proliferation defect of *dac* itself (Mardon et al., 1994).

3.1.2 *Drosophila* eye development genes and their mammalian homologues

dac is one of a group of genes, also including *eyeless (ey)*, *eyes absent (eya)* and *sine oculis (so),* which are essential to *Drosophila* eye development and interact during the early stages of eye formation. All of these genes are expressed in the eye anlage and their loss of function mutants have absent or severely reduced eyes. Much

attention has been paid to three of these genes, *dac*, *ey* and *eya* as potential master genes for eye formation, as when they are ectopically expressed they can induce the formation of ectopic eye structures (discussed in detail in section 1.6: Bonini et al., 1997; Halder et al., 1995; Shen and Mardon, 1997).

Mammalian homologues of *ey, eya* and *so* have been reported and these are *Pax6, Eya1-3* and *Six3* respectively. All are expressed within the eye and are hence potentially involved in mammalian eye development (Walther and Gruss, 1991; Walther et al., 1991; Xu et al., 1997a; Oliver et al., 1995). Functional evidence that these genes do have a role in eye development comes from the *Pax6* mutant, small eye, which is characterised by a complete absence of eye structures (Hill et al., 1991). In addition both *Pax6* and *Six3* can produce ectopic retina and lens when over-expressed in *Xenopus* and the killifish medaka respectively (Chow et al., 1999; Oliver et al., 1996; Loosli et al., 1998). There is no direct evidence that the *Eya* genes have a role during eye development but it seems likely given the conservation of expression domains between the fly and mouse (Xu et al., 1997a), discussed in section 1.7.

The apparent existence of a conserved interacting network of genes involved in both fly and mammalian eye development is unexpected because the morphology and developmental mechanisms of the two eye types are very different. It would hence be interesting from an evolutionary point of view to know whether the fourth member of the group, *dac*, also has a mammalian homologue with a role in eye development. From a developmental perspective the isolation of genes fundamental to mammalian eye development is also an important first step in understanding the mechanisms involved in producing the eye. This group of genes is of particular interest due to their early action and eye inducing capacity in both flies and vertebrates.

This study therefore set out to isolate and characterise a murine homologue of *dac*. The intention was that if a homologue expressed in the eye was identified it might be possible to identify any effects of *Pax6* on this gene by the use of small eye mice and perhaps later the small eye chimeras discussed in chapter 2. The identification of *Dach1*, a murine *dac* homologue, is presented in this chapter. *Dach1*

is expressed in eye, limb and CNS amongst other regions and its expression in the brain does not appear to be affected in small eye mutants. *Dach1* maps to mouse chromosome 14E3.

Alongside this work a human homologue of *dac*, *DACH1*, was identified and characterised by Dr. Isabel Hanson . Unless otherwise stated any human data referred to in this chapter was produced by her.

3.2 Results : Cloning and characterisation of murine *Dachshund*

3.2.1 Cloning and sequence analysis

I identified a human EST (accession number AA059243) in a search of the EMBL EST database using the *Drosophila dachshund* nucleotide sequence. To search the database the tBLASTx algorithm was used, which compares translations of the sequences in all six reading frames. The EST identified was derived from an IMAGE consortium human adult retina cDNA clone, ID 381801. A 1.8kb SmaI/XhoI fragment of this EST was then used to screen approximately $1x10^6$ clones of an embryonic E11.5 mouse cDNA library and hybridisation was carried out at 65°C (see section 6.7 for protocols). Positive clones were then isolated by PCR, using primers to the GT11 vector arms, and cloned into PCR2.1 using a TA cloning kit (Invitrogen). This led to the identification of two clones overlapping by 10bp, the more 5' of which was designated 5A, the 3' clone being named 3A. Further

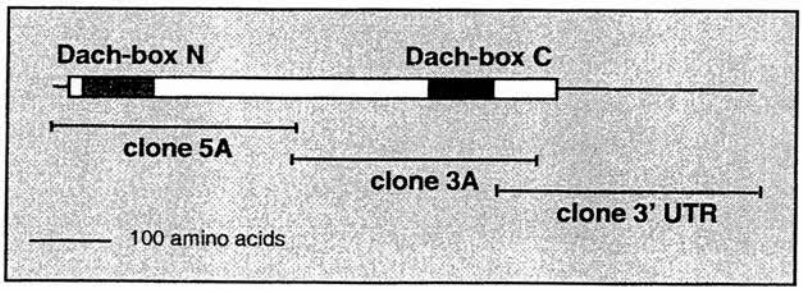


Figure 3.1 : Schematic view of the *Dach1* contig illustrating the position of the 3 cDNA clones.
Clone 3A: nucleotides 0 - 820
Clone 5A: nucleotides 810 - 1674
Clone 3'UTR: nucleotides 1540 - 2469

human ESTs had by now been identified (Dr I.Hanson) and one of these (Image clone 668097) was used to re-screen the library resulting in the isolation of another 3' clone overlapping 134bp with clone 3A (see figure 3.1).

The contig produced stretches 2.5 kb and includes a stop codon at 1773bp. It was initially considered that a methionine at 65bp (see figure 3.2) was likely to be the start site, in spite of the much reduced length of the gene with respect to *Drosophila*, since the similarity between mouse and human *Dachshund* decreases dramatically 5' of the predicted coding region in the cDNAs isolated. However, sequencing from a genomic mouse *Dachshund (Dach1)* clone (see section 3.4) revealed the presence of sequence coresponding to that of the human *(DACH1)* cDNA clone in the mouse gene. This and the lack of a good Kozak consensus sequence close to the methionine residue at 65bp led to the conclusion that this methinine was not in fact likely to be the initiating codon. Indeed Caubit et al. (1999) have recently reported the isolation of a mouse *Dachshund* cDNA clone which extends 549 bp 5' of the sequence reported in this thesis and contains an initiating methionine preceded by stop codons 5' of the methionine reported here. The methionine at 65bp in figure 3.2 is hence not the start codon.

3.2.2 An alternative splice in the *Dach1* gene

The predicted coding region of the mouse *Dachshund* gene (*Dach1*) is highly similar to the predicted human *Dachshund* (*DACH1*) open reading frame (99.3%)

Figure 3.2 : Amino acid alignment of the predicted protein products of *Dach1* (mouse) and *DACH1* (human).

Nunbering begins at the most 5' methionine residue in the sequence isolated (boxed). This residue has subsequently been shown not to be the initiating methionine (Caubit et al., 1999)

The mouse protein has an insertion of 52 amino acids (residues 187-238) compared to the human protein which appears to correspond to the inclusion of an alternatively spliced exon. Two regions of significant homology with *Drosophila* dachshund protein are shown as shaded boxes: Dach-box N, from residues 21-103; and Dach-box C, from residues 427-498 of the mouse protein and 375-446 of the human protein. A strongly predicted α-helical coiled-coil domain is indicated by α.....α. Within this helical region, the residues which comprise the basic-hydrophobic heptad repeat are underlined.

```
DACH  AAASNGSGGG GGGISAGGGV ASSTPINAST GSSSSSSSSS SSSSSSSSS
dach  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                                          1
DACH  SSSSSCGPLP GKPVYSTPSP VENTPQNNEC KMVDLRGAKV ASFTVEGCEL 19
dach  ~~~~~~~~~~ SGAAATAAAA ATAAAAAGEC KMVDLRGAKV ASFTVEGCEL 19

DACH  ICLPQAFDLF LKHLVGGLHT VYTKLKRLEI TPVVCNVEQV RILRGLGAIQ 69
dach  ICLPQAFDLF LKHLVGGLHT VYTKLKRLEI TPVVCNVEQV RILRGLGAIQ 69

DACH  PGVNRCKLIS RKDFETLYND CTNASSRPGR PPKRTQSVTS PENSHIMPHS 119
dach  PGVNRCKLIS RKDFETLYND CTNASSRPGR PPKRTQSVTS PENSHIMPHS 119

DACH  VPGLMSPGII PPTGLTAAAA AAAAATNAAI AEAMKVKKIK LEAMSNYHAS 169
dach  VPGLMSPGII PPTGLTAAAA AAAAATNAAI AEAMKVKKIK LEAMSNYHAS 169

DACH  NNQHGADSEN GDMNSSV... ..........  .......... .......... 186
dach  NNQHGADSEN GDMNSSVGSS GGSWDKETLH SPPSQGSQAP VTHARMPAAF 219

DACH  .......... .........G LELPFMMMPH PLIPVSLPPA SVTMAMSQMN 217
dach  SLPVSHPLNH LQHSHLPPNG LELPFMMMPH PLIPVSLPPA SVTMAMSQMN 269

DACH  HLSTIANMAA AAQVQSPPSR VETSVIKERV PDSPSPAPSL EEGRRPGSHP 267
dach  HLSTIANMAA AAQVQSPPSR VETSVIKERV PDSPSPAPSL EEGRRPGSHP 319

DACH  SSHRSSSVSS SPARTESSSD RIPVHQNGLS MNQMLMGLSP NVLPGPKEGD 317
dach  SSHRSSSVSS SPARTESSSD RIPVHQNGLS MNQMLMGLSP NVLPGPKEGD 369

DACH  LAGHDMGHES KRMHIEKDET PLSTPTARDS LDKLSLTGHG QPLPPGFPSP 367
dach  LAGHDMGHES KRIHIEKDET PLSTPTARDS IDKLSLTGHG QPLPPGFPSP 419

                                      α.....................
DACH  FLFPDGLSSI ETLLTNIQGL LKVAIDNARA QEKQVQLEKT ELKMDFLRER 417
dach  FLFPDGLSSI ETLLTNIQGL LKVAIDNARA QEKQVQLEKT ELKMDFLRER 469

      ..................................................
DACH  ELRETLEKQL AMEQKNRAIV QKRLKKEKKA KRKLQEALEF ETKRREQAEQ 467
dach  ELRETLEKQL AMEQKNRAIV QKRLKKEKKA KRKLQEALEF ETKRREQAEQ 519

      ......α
DACH  TLKQAASTDS LRVLNDSLTP EIEADRSGGR TDAERTIQDG RLYLKTTVMY 517
dach  TLKQAASADS LRVLNDSLTP EIEADRSGGR ADAERTIQDG RLYLKTTVMY 569
```

throughout; however, the mouse sequence, 559 amino acids in length, contains an additional 52 amino acids compared to the human gene. Analysis of this region by RT-PCR (figure 3.3) suggests that this additional coding region is a rare alternative splice form; the major mouse transcript being identical to the human transcript.

To carry out this assay, primers on each side of the dissimilarity were used to amplify from whole mouse genomic cDNA (primers C and D in figure 3.3), producing PCR products of 330bp and 170bp which correspond to the sizes expected with and without the extra 52 amino acid section. Two other bands of 310bp and 500bp are also observed in this lane, which are likely to indicate further splice forms. The 170bp band was much stronger than the larger bands, suggesting that the smaller band is the major transcript although, strictly speaking the PCR protocol used was not quantitative.

To ensure that the extra mouse sequence was indeed present and that these faint larger bands were not simply background contamination, a further assay was carried out using primers overlapping from the shared sequence into the mouse specific region (primers A and B in figure 3.3). These primers would only produce a product if the extra sequence was present in the context of the shared sequence. As can be seen in figure 3.3 a band of 190bp was seen when this RT-PCR reaction was carried out which corresponds to the length of the mouse specific sequence plus the length of the primers. This indicates that the extra sequence is indeed present in a subset of transcripts.

3.2.3 There are two conserved domains in the *Dach1* gene and *Ski*, a proto-oncogene has similarity to *Dach1* in these regions.

Two regions of high sequence conservation exist between *Drosophila dac* and the mammalian genes. The N-terminal domain, referred to as Dach-box N is 83 amino acids in length with an overall similarity of 87% between the *Drosophila* and mammalian proteins. The C-terminal domain, Dach-box C, is 72 amino acids long and the degree of similarity to *Drosophila* is 63% (see figure 3.4). The position of these two regions is shown in figure 3.4, Dach-box N being located between nucleotides 20 and 103 of the mouse protein and Dach-box C between residues 375 and 446. These two regions are also found to be highly conserved in a *C.elegans dac*

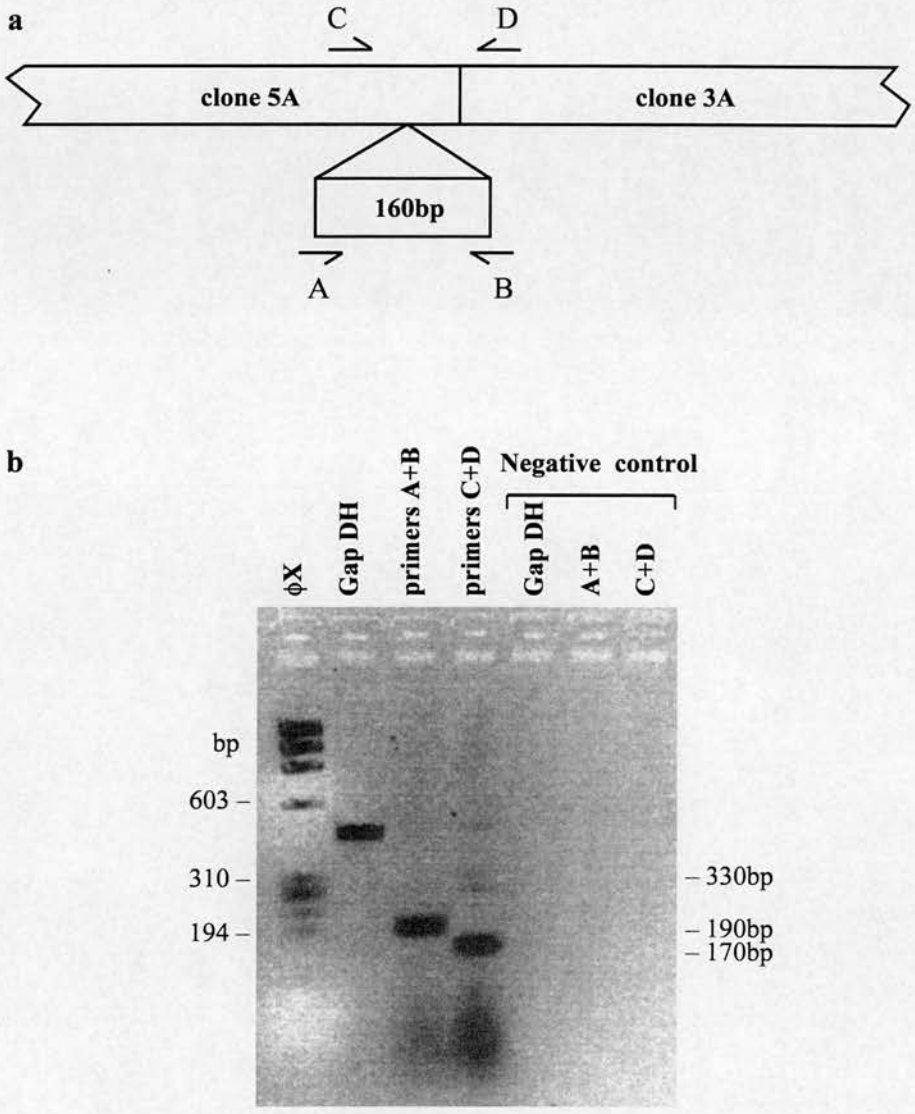


Figure 3.3 : RT-PCR analysis of *Dach1*.

A region of 52 amino acids is present in *Dach1* which is not present in *DACH1*. RT-PCR analysis suggests that these may be alternative splice forms

(a) Primers **C** and **D** amplify across the region containing the extra sequence. Primers **A** and **B** overlap into the mouse specific region from the common sequence and only amplify mouse specific sequence present in the context of the common sequence.

(b) **ϕX;** ϕX174-HaeIII size marker. **GapDH**; primers to the GapDH gene were used as a positive control and amplify a specific band. **Primers A+B**; The 190bp band represents the 160bp mouse specific sequence with the addition of the length of the primers. **Primers C+D**; the 170bp and 330bp bands correspond to the exclusion and inclusion of the mouse specific sequence respectively. Two further bands of 310bp and 500bp perhaps represent further alternative splice forms. Negative controls are reactions carried out with water replacing first strand cDNA.

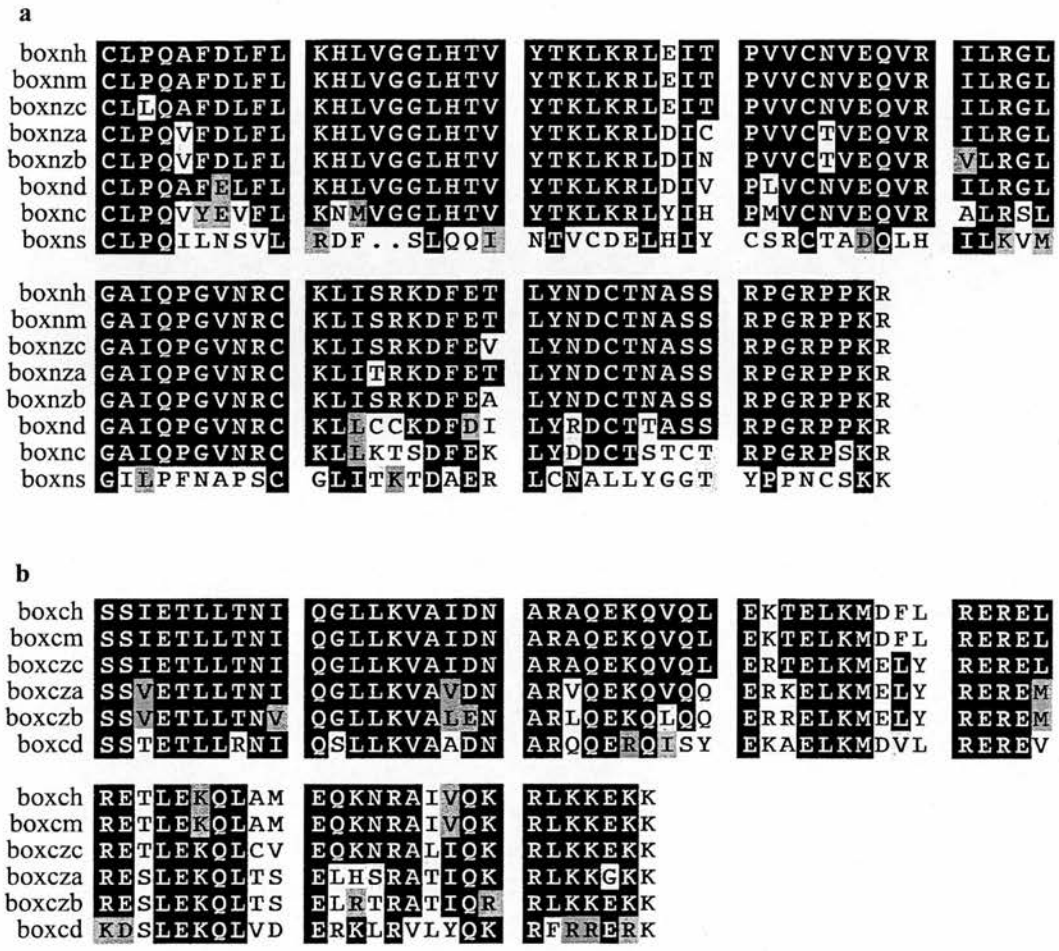


Figure 3.4 : Conserved amino acids in homologues of dachshund.

(**a**) Dach-box N, which spans 83 amino acids near the N-terminus of all members of the Dach/Ski/Sno superfamily. boxnh: human, boxnm: mouse, boxnzc: zfDachC, boxnza: zfDachA, boxnzb: zfDachB, boxnd: *Drosophila* dachshund (splice variant 4, accession no. U19269 [Mardon et al., 1994]), boxnc: C.elegans dachshund homologue (cosmid B401), boxns: consensus amino acid sequence of the vertebrate Ski/Sno protein family (domain ID no. 4362 from ProDom)

(**b**) Dach-box C, which spans 72 amino acids near the C-terminus of *Drosophila* dac and its homologues. boxch: human, boxcm: mouse, boxczc: zfDachC, boxcza: zfDachA, boxczb: zfDachB, boxcd: *Drosophila* Alignments were compiled using pileup and displayed using pretty box (both programs available through GCG)

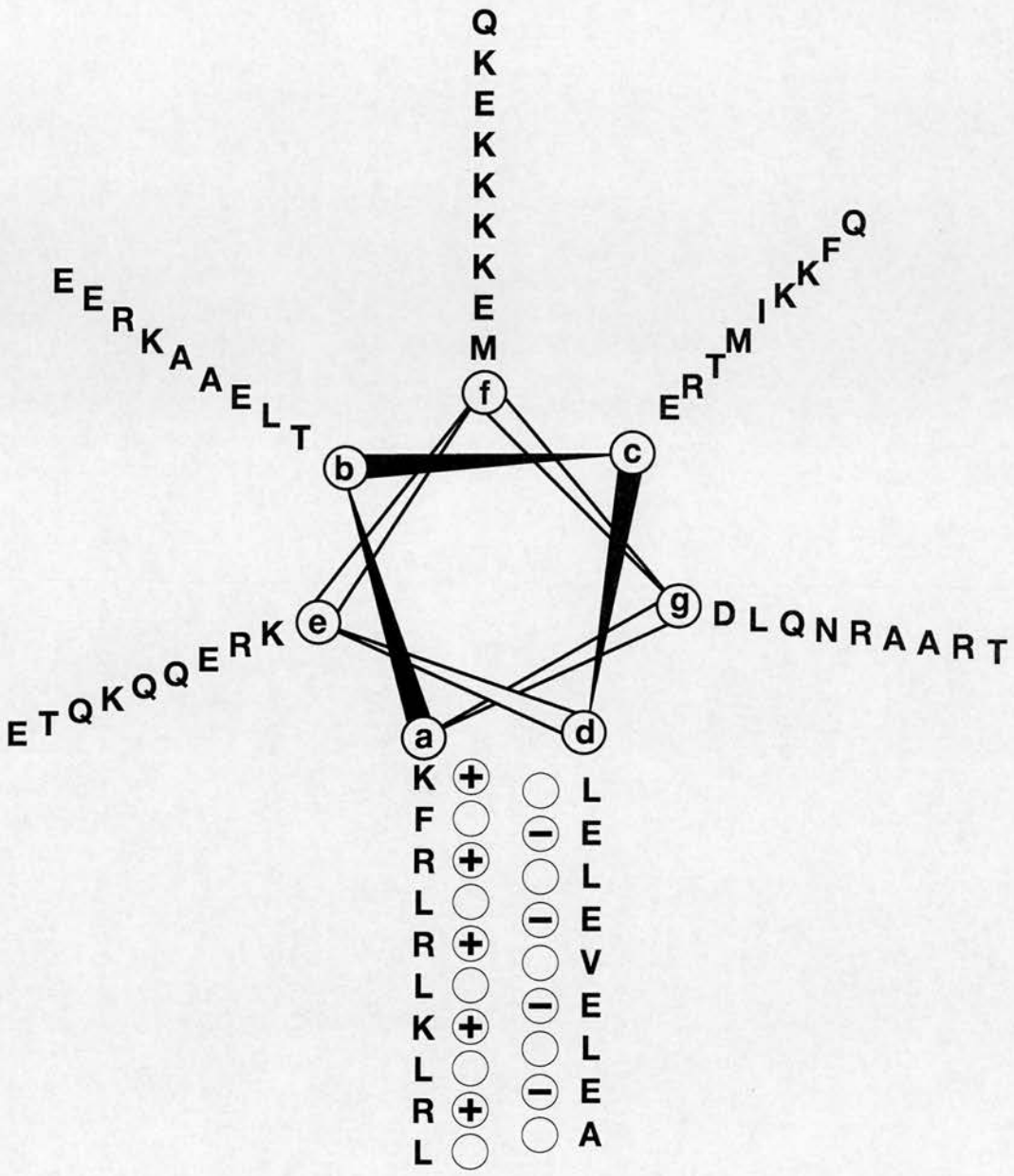


Figure 3.5 : Helical wheel projection of the predicted coiled coil domain of *Dach1*.

Residues 468-521 of *Dach1* are shown as a schematic -helix in which the side chain of every seventh amino acid is aligned. Each spoke represents a face on the helix, which is viewed end on with most N-terminal residues at the centre of the wheel. Spokes (a) and (d) are shown closer together to highlight the novel basic-hydrophobic, acidic-hydrophobic heptad motif.

homologue (cosmid U80953, identified by Dr I.Hanson, Dach-box N only as the cosmid is only part of the gene) and in the three zebrafish *Dachshund* genes (see chapter 4) as shown in figure 3.4. These regions are therefore likely to have important conserved functions.

A degree of similarity to the *Ski* family of proto-oncogenes was also found by searching the EMBL translated sequences (TREMBL) database with the entire Dach1 protein sequence. Dach-box N was found to have similarity to an N-terminal region of Ski and Dach-box C to a C-terminal region. In Dach-box N sequence similarity is significant, 28% between Dach1 and the consensus of the Ski family genes, but in Dach-box C similarity is confined to the occasional alignment of basic and hydrophobic residues which is nevertheless likely to have functional significance. This C-terminal region of the *Ski* family genes forms an α-helical domain with a tendency to form an extended coiled coil structure, the basic and hydrophobic residues forming a heptad repeat along one face of the helix. Dach1 shares this structure, possessing an α-helical region within Dach-box C which begins 20 amino acids into the box, at nucleotide 393 and extends 81 amino acids 3' to this (Garnier-Osguthorpe-Robson algorithm). Like Ski, this domain of Dach1 is predicted to have a tendency to form an extended coiled-coil structure (Coilscan). The basic / hydrophobic repeat shared with Ski forms one face of the helix, but there is also an acidic / hydrophobic heptad repeat forming an adjacent face as shown in a helical wheel projection in figure 3.5. This motif appears to be unique to *Dachshund* and may therefore form a novel zipper motif.

In contrast to this Dach-box N does not contain any recognisable structure or motif. *Dachshund* does not appear to contain any known DNA binding sites either within or without the Dach-boxes.

3.2.4 There is unlikely to be a large family of *Dachshund* genes in mammals.

Southern blot analysis has indicated that *Dachshund* is likely to be a member of a small family of genes or is perhaps unique. The blot was prepared by digesting genomic mouse DNA with rare cutting enzymes: AccI, ApaI, EcoRI, NdeI and PstI. This was hybridised using, initially, a probe consisting of nucleotides 138 to 382, which included Dach-box N, as described in section 6.8 After autoradiography the

blot was stripped and re-hybridised with a probe consisting of nucleotides 1100 to 1516 which included Dach-box C.

When the Dach-box N probe was used the blot showed a single hybridising band in all lanes except for Acc1, which cuts within the known probe sequence (figure 3.6). This indicates that *Dach1* is present in the genome at a low copy number, perhaps as a single gene. When Dach-box C was used as a probe, however, there were multiple bands present in all lanes despite the fact that none of these enzymes cut within the probe. This may suggest the presence of more than one *Dachshund* gene. However the pattern of hybridisation is still relatively simple, with no more than 3 strongly hybridising bands in any lane, again suggesting that *Dach1* is not likely to be part of a large gene family.

3.3 Results : Analysis of expression pattern

3.3.1 *In-Situ* hybridisation probes.

To analyse *Dach1* expression, *in-situ* hybridisation was carried out using both wholemount techniques and radioactive in-situ hybridisation (RINS) to sections. The probe used for the majority of this analysis was made from nucleotides 1674 - 810 (clone 3A) of the mouse *Dach1* gene. This area was chosen as it contains no repetitive sequence and should be large enough to ensure specific hybridisation, despite containing a region of high conservation, Dach-box C. A second probe, produced from clone 5A (nucleotides 820 - 0) and a third probe from nucleotides 1337 to 841 gave identical hybridisation patterns at E11.5. This suggests that the pattern produced was indeed likely to be specific, even in the event of there being further unidentified members of the mammalian *Dach1* family. As probes made to 3' UTR are often more specific than those made from open reading frame, a probe was also made from nucleotides 2469 to 2092, however this probe did not hybridise well so was abandoned. Sense probe (nucleotides 841 - 1337) was also used as a control and produced no specific hybridisation when used in either whole mount or RINS.

Whole mount *in-situ* hybridisation was carried out using DIG labelled probes and radioactive *in-situ* hybridisation (RINS) to 7μm sections was carried out using ^{35}S-UTP labelled probes, as described in section 6.11.

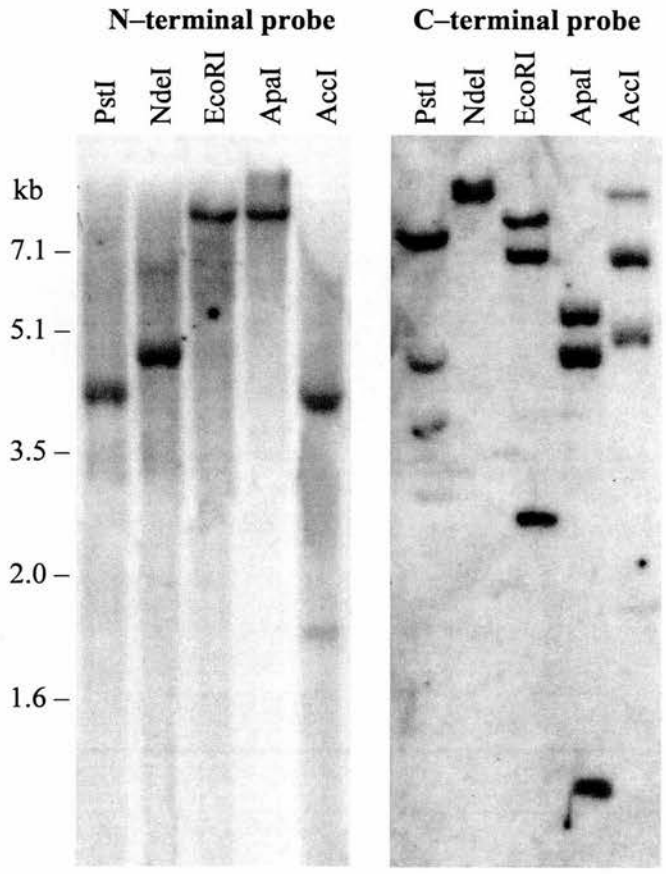


Figure 3.6 : Southern analysis using probes to the highly conserved Dach-box N and Dach-box C regions of *Dach1*.

The probes were hybridised to whole genomic mouse DNA digested with restriction endonucleases which do not cut within the probe, except for Acc1 which cuts once in the Dach-box N probe. The analysis was carried out on two separate DNA preparations from AKR/J and DBA/2J adult mouse livers, with the same result in both cases. The AKR/J blots are shown.

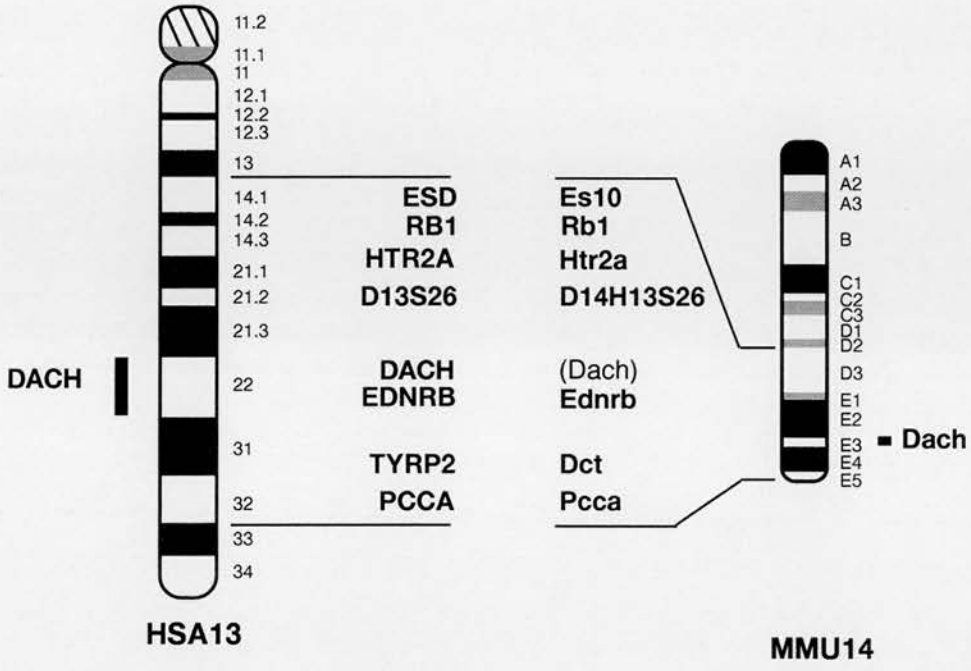


Figure 3.7 : Chromosomal localisation of the *Dach1* and *DACH1* genes.

Solid black bars show the position of *Dach1* and *DACH1* on human chromosome 13 (HSA13) and mouse chromosome 14 (MMU14) as determined by fluorescence *in-situ* hybridisation. Selected markers are shown from the homology region between the human and mouse chromosomes. Radiation hybrid mapping of human *DACH1* places it just above the *EDNRB* gene and the most likely map position of the mouse gene is indicated by (Dach).

3.3.2 Expression of *Dach1* in the wildtype mouse embryo

Expression was analysed in E10.5 - 12.5 whole mouse embryos, in the whole limbs of E13.5 embryos and in sections of E10.5 - E14.5 embryos. Whole embryos were sectioned to 100μm for analysis and *Dach1* transcripts were detected at all stages examined, expression being found in the limb, eye, brain, CNS, genital eminence, dermamyotome and in various ganglia including the trigeminal, glossopharangeal-vagal, and dorsal root ganglia. Expression was also detected in the developing ear, branchial arches, nasal mesenchyme, gut mesoderm, and lung when RINS sections were examined.

3.3.3 Eye expression

Dach1 transcripts are detected in the predominantly neural crest derived perioptic mesenchyme, which surrounds the eye, at all stages from E10.5 to E14.5 (see figure 3.9). Expression is also found in the neuroretina at these stages but not within the lens or the RPE. Some expression in the lens may however, be occurring by E14.5 but this may be trapping of probe. Expression is initially detected throughout all layers of the neuroretina but by E12.5 this expression is higher on the surface adjacent to the lens, this pattern being maintained until E14.5. It can also be seen that although expression is seen adjacent to the optic stalk none is seen within the stalk itself.

Pax6 expression in the eye, shown at E12.5 (figure 2.3), overlaps with *Dach1* expression, both genes being found in the neuroretina. The two patterns are not identical however, *Pax6* being found in the lens and RPE where *Dach1* is not expressed but not in the perioptic mesenchyme where *Dach1* is found.

3.3.4 Limb expression

Dach1 is expressed in both the fore and hind limbs at all stages analysed, from E10.5 to E13.5 (figure 3.8). At E10.5 expression can be seen in both the posterior and anterior of the limb bud, with the anterior region extending centrally into the core. At E11.5 *Dach1* expression becomes increasingly peripheral, extending around the entire handplate, in the mesenchyme beneath the apical ectodermal ridge.

At E12.5 expression is entirely peripheral with no central expression remaining and by E13.5 *Dach1* is localised to the mesenchyme at the distal tips of the digits.

3.3.5 Brain expression

Dach1 is expressed from E10.5 to E14.5 in the telencephalon including the olfactory bulbs. Initially, at E10.5, E11.5 and E12.5 expression is found throughout the thickness of the telencephalic lobes, however by E13.5 expression is seen only in the ventricular zone and in the outermost layer, the marginal zone. This pattern is maintained and becomes increasingly obvious by E14.5 (figure 3.10). The ventricular zone contains highly proliferative, mitotic cells whilst the marginal zone consists of the first born cells of the telencephalon.

Strong expression is also seen in the hindbrain from E10.5 until E14.5 in the walls of the fourth ventricle (figure 3.11), although expression is not present in the median sulcus (floor plate) along the midline of the brain (figure 3.11). In the forebrain too, expression is excluded at all ages examined from the midline structure, the lamina terminalis which is situated between the telencephalic vesicles.

Expression is also observed in regions of the diencephalon from E10.5 to E14.5 (figure 3.13). Expression is seen in the thalamus and hypothalamus some distance from the lumen of the third ventricle at E12.5, specifically not being expressed in the neuroepithelium. This pattern is maintained at E13.5 the expression domain again being restricted to tissue outside the neuroepithelium. By E14.5 expression is maintained in the thalamus, however a sharp expression boundary is seen which may correspond to a boundary between prosomeres. It is not possible to say whether this boundary was present at earlier ages as the sections were taken in slightly different planes.

A very strong, isolated region of expression just anterior to the buccal cavity is also seen at E11.5 (the only stage examined in this exact region). This probably corresponds to the wall of the infundibular recess which is the neural part, the neurohypophysis, of the developing pituitary gland (figure 3.12).

Pax6 too is expressed in the brain, with strong expression in the telencephalon and the anterior diencephalon, again including the olfactory bulbs. These regions all show abnormalities in small eye mice (Caric et al., 1997; Warren

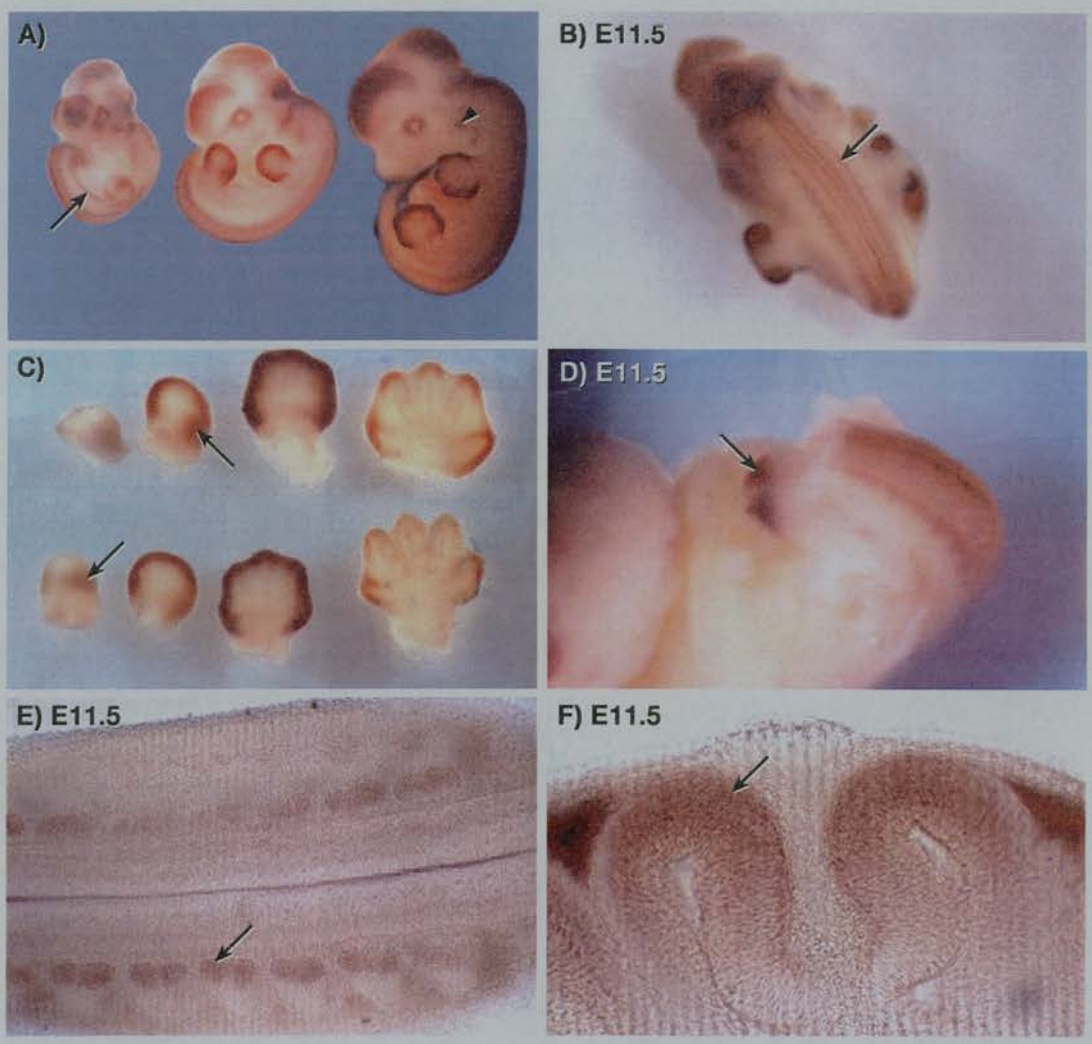


Figure 3.8: *Dach1* expression in midgestation embryos.
Whole mount *in-situ* analysis, sections are 100μm thick.
A) *Dach1* expression in E10.5, E11.5 and E12.5 (left to right) mouse embryos. Expression is seen at all three stages in the eye, limb buds, neural tube, brain and trigeminal and glossopharangeal - vagal ganglion complex (arrowhead). Dermomyotome expression is detected at E11.5 and E10.5 only (arrow)
B) Dorsal *Dach1* expression in the neural tube and dorsal root ganglia (arrow)
C) *Dach1* expression in E10.5 to E13.5 limb buds. (E10.5 left, E11.5 and E12.5 centre and E13.5 right; forelimbs at the bottom). At E10.5 there is an anterior region of expression which extends to the centre of the limb bud and is beginning to disappear by E11.5 (arrows). Expression becomes peripheral by E11.5 to E12.5 becoming localised to the distal tips of the digits by E13.5.
D) *Dach1* expression in the genital eminence (arrow)
E) A coronal section through the back of an E11.5 embryo showing *Dach1* expression in the dorsal root ganglia (arrow)
F) Transverse vibratome section showing *Dach1* expression in the olfactory bulbs (arrow) of an E11.5 forebrain.

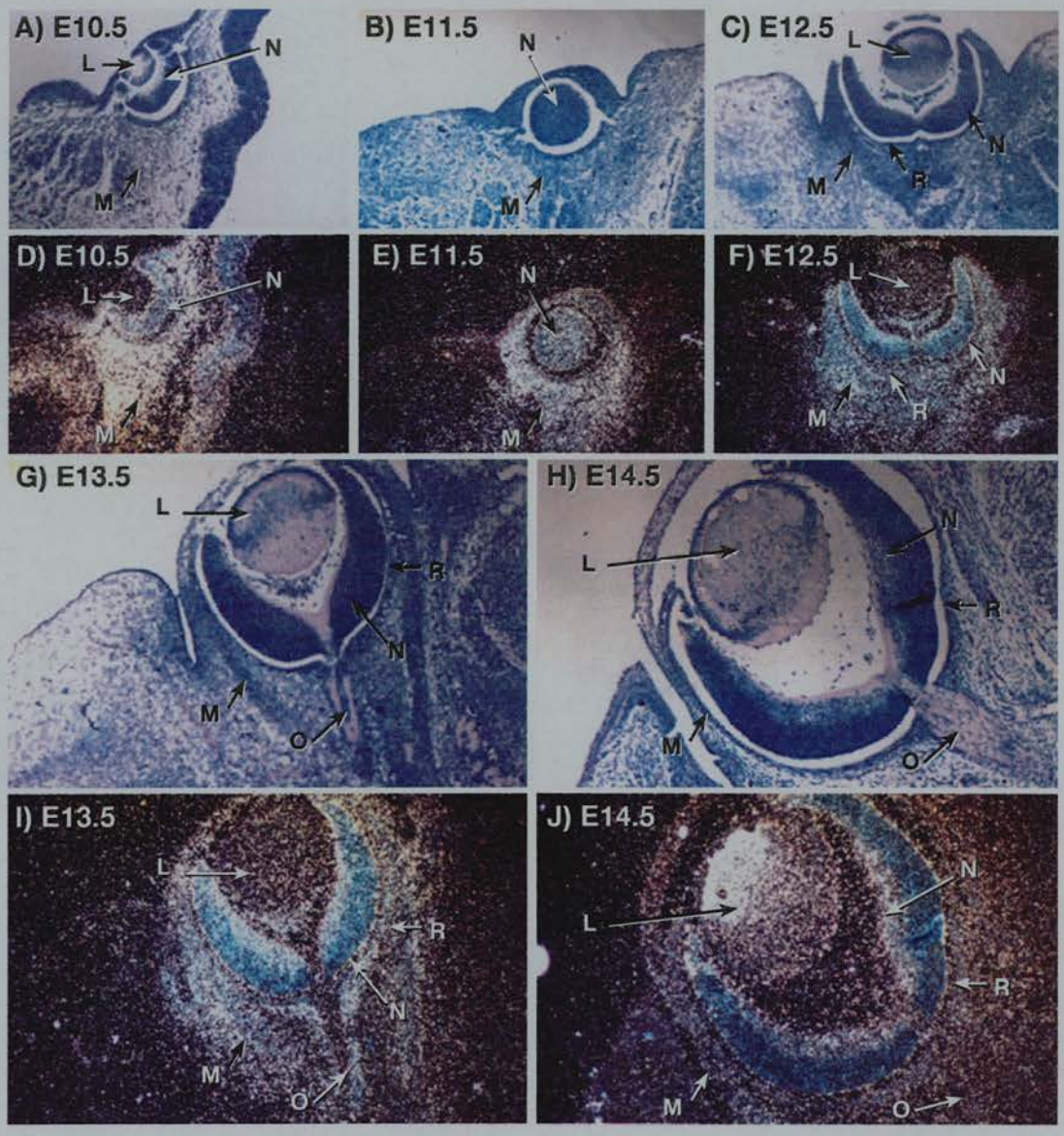


Figure 3.9: *Dach1* expression in the developing mouse eye.

Transverse 7μm sections through the lens and retina except (**B/E**) where the plane of section only transects the retina.

L: lens, **N**: neuroretina, **M**; perioptic mesenchyme, **R**: retinal pigmented epithelium, **O**: optic stalk.

A-J) *Dach1* expression is found in the lens and retina at all stages examined becoming stronger in the inner layer of neuroretina by E12.5 (**C/F**).

C/F, G/I) At E12.5 and E13.5 *Dach1* expression is also found in mesenchymal cells between lens and retina.

H/J) At E14.5 *Dach1* expression is detected in the anterior lens.

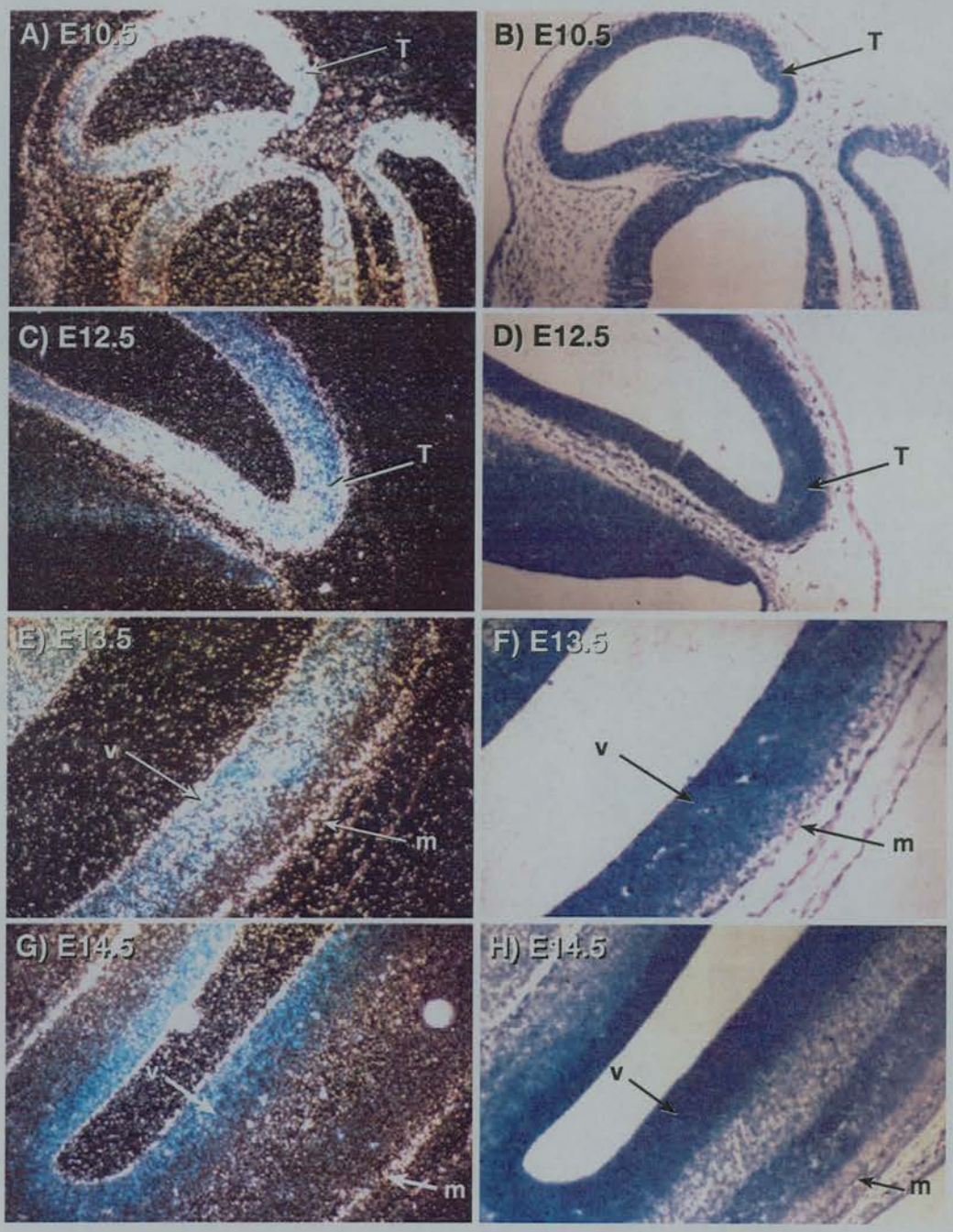


Figure 3.10: *Dach1* expression in the telencephalon of E10.5 to E14.5 mouse embryos.
Transverse 7μm sections.
A-D) Expression is detected throughout the width of the telencephalon from E10.5 to E12.5. **T**; telencephalon.
E-H) *Dach1* expression is detected in the ventricular zone (**v**) and the marginal zone (**m**) of the telencephalon at E13.5 and E14.5. All tissue shown is telencephalic, the external surface of the brain is to the right.

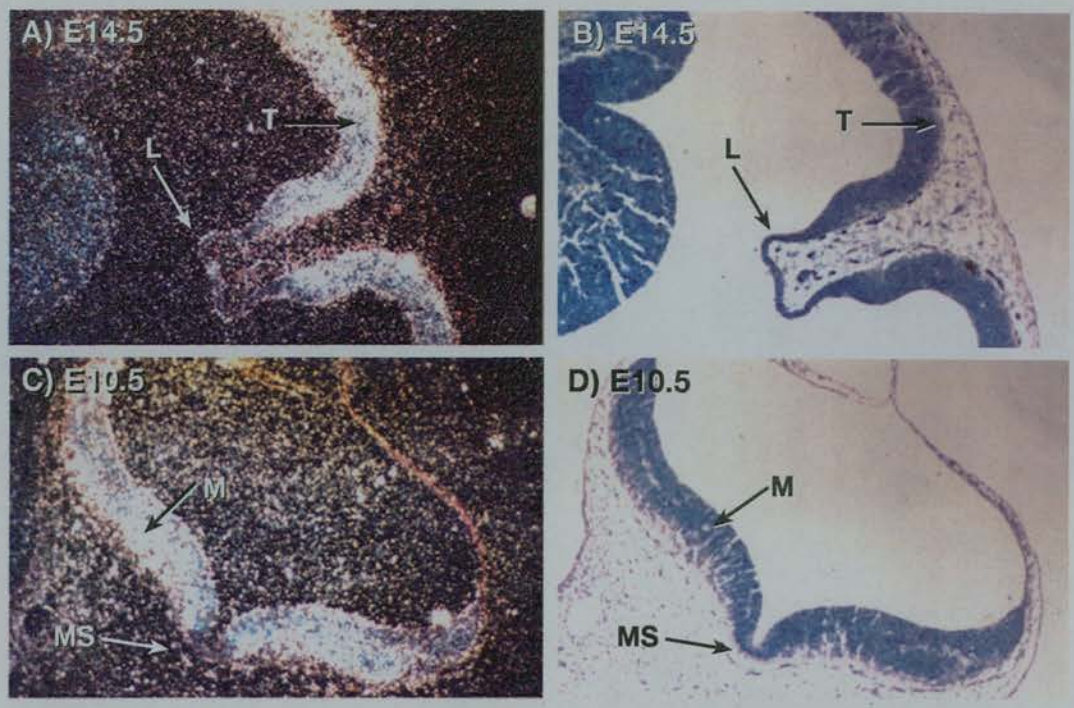


Figure 3.11: *Dach1* is not expressed along the midline of the forebrain and hindbrain of E10.5 to E14.5 mouse embryos. Transverse 7μm sections.

A/B) *Dach1* expression is excluded from the lamina terminalis which is situated in the midline of the forebrain. The sections pictured are from an E14.5 embryo but this pattern is observed from E10.5. **T**; telencephalon. **L**; lamina terminalis (roof of third ventricle)

C/D) *Dach1* expression is excluded from the median sulcus which is situated in the midline of the hindbrain. The sections pictured are from an E10.5 embryo but this pattern is still observed at E14.5. **M**; myelencephalon. **MS**; median sulcus.

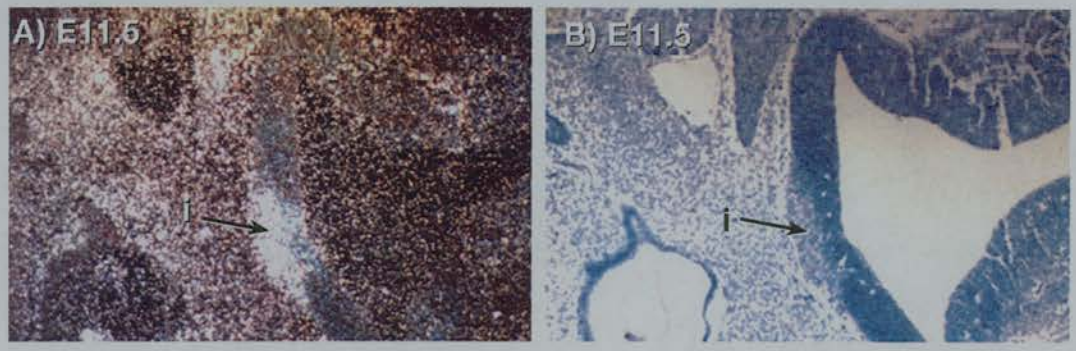


Figure 3.12: *Dach1* expression in the developing neurohypophysis.

Transverse 7μm section showing strong expression in the neurohypophysis (**i**) which originates as an evagination of the diencephalon (right), the infundibular recess, and becomes the neural part of the pituitary gland.

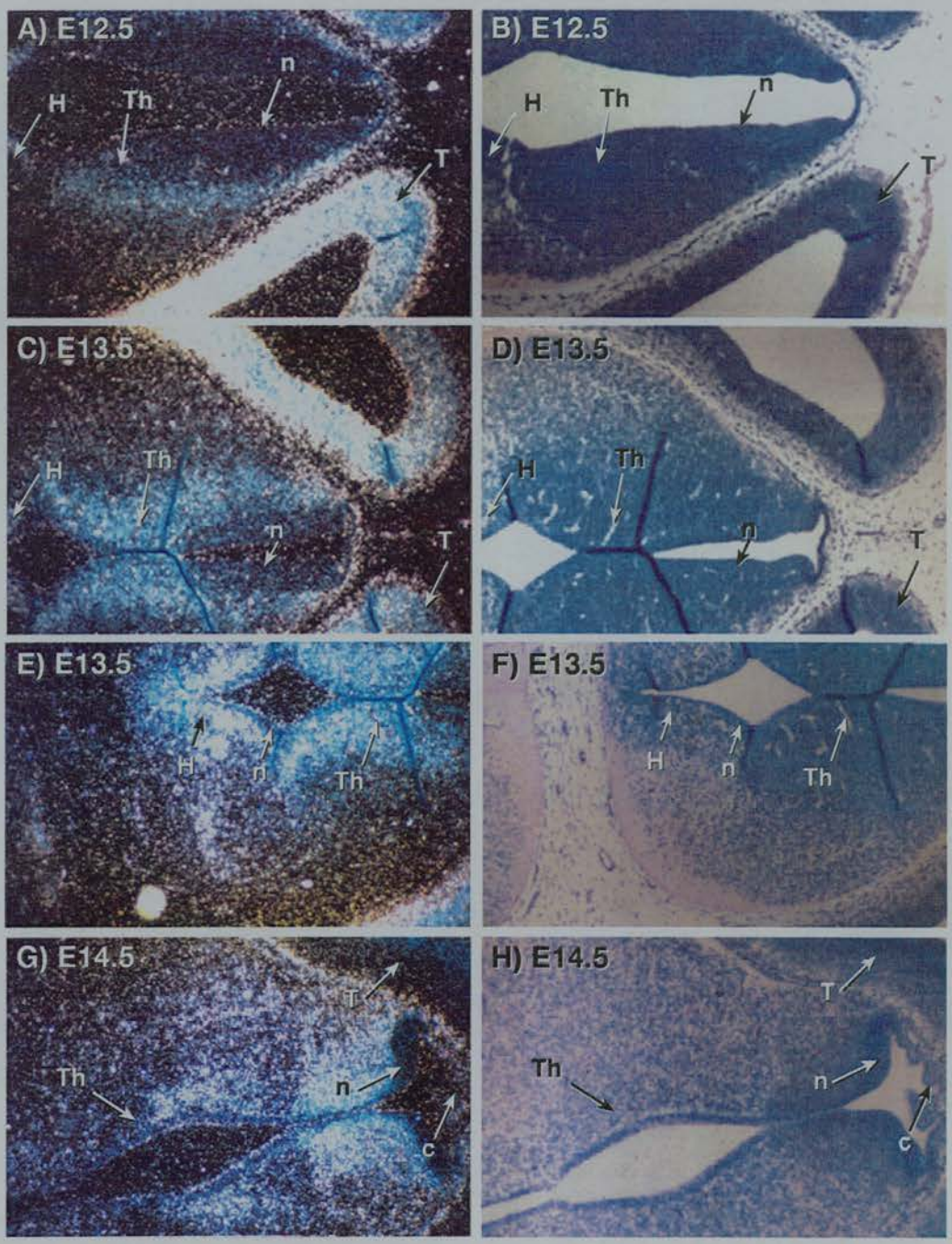


Figure 3.13: *Dach1* expression in the diencephalon.

Transverse 7μm sections. Anterior is to the right.

Th: thalamus, **H**: hypothalamus, **c**: choroid plexus, **t**: telencephalon

A/B) At E12.5 *Dach1* expression is seen in the thalamus and hypothalamus at a distance from the lumen of the third ventricle. No expression is seen in the neuroepithelium.

C/D, E/F) At E13.5 *Dach1* expression is similar to E12.5 expression, in the thalamus and hypothalamus but not in the neuroepithelium.

G/H) At E14.5 *Dach1* expression is seen within the thalamus up to a sharp boundary. This may represent a boundary between prosomeres.

and Price, 1997; Stoykova et al., 1996). As in the eye therefore, *Dach1* expression in the brain overlaps with, but is not identical to that of *Pax6*.

3.3.6 Central Nervous system expression

Dach1 is expressed throughout the length of the neural tube from E10.5 to E12.5 and is still found in the neural tube until E14.5. There is also expression of *Dach1* within the dorsal root ganglia extending to the region where the ganglia join the neural tube. The expression domain of *Dach1* is mainly within the mantle of the neural tube, with expression in dorsal, lateral and ventral regions (figure 3.14). There is also a dorsal region of expression within the ventricular zone and expression is particularly strong in the basal plate region of the ventral neural tube (figure 3.14). Expression is noticeably absent from the floorplate, as in the hindbrain.

Pax6 is expressed in the ventral ventricular zone of the neural tube, and not at all in the dorsal root ganglia, in a complementary pattern to *Dach1* expression. Again *Dach1* expression does not correspond with *Pax6* expression.

Expression can also be seen in cranial ganglia, in the trigeminal ganglion and the glossopharyngeal-vagal ganglion complex. This is found at E10.5 - 14.5 (see figure 3.8 and 3.15).

3.3.7 Further regions of expression in the head

Within the head various other regions of expression are found. The mesoderm surrounding the olfactory epithelium expresses *Dach1* at E14.5; although the nasal epithelium, which expresses *Pax6*, does not express *Dach1*. This is therefore a further region where expression of the two genes is complementary. Within the developing ear the semicircular canals and developing cochlea express *Dach1* at E14.5 and at least one of the branchial arches also expresses at this age (figure 3.15 and 3.16).

3.3.8 Other regions of expression

At E10.5 and E11.5 *Dach1* is expressed in a punctate pattern on the ventral side of the embryo between the fore and hind limbs, we believe this is the dermomyotome. This expression has however disappeared by E12.5. At E11.5 and

E12.5 *Dach1* expression is also found within the genital eminence (figure 3.8). At E14.5 (the only age examined in these regions) expression was also seen in the mesoderm of the gut and the lung. In both cases expression was excluded from the epithelium surrounding the lumen (figure 3.15).

3.3.9 *Dach1* expression in small eye mice

Pax6 is a putative activator of *Dach1* because in the fly *eyeless* is thought to activate *dac*. However, as mentioned above, although the *Pax6* and *Dach1* expression domains overlap they do not correspond exactly and in some cases are complementary to one another. It therefore seemed unlikely that *Pax6* would activate *Dach1* directly, certainly other factors would need to be involved in some way. It remained a possibility, however, that *Pax6* was needed to activate *Dach1* in tissues where their expression domains do overlap. To investigate this, the expression pattern of *Dach1* in small eye mice was analysed.

The expression domains of *Dach1* within the E12.5 small eye mouse looked superficially identical to the wildtype situation in wholemount. Further analysis of *Dach1* forebrain expression adds evidence that *Dach1* does not need to be activated by *Pax6*. In the brain of E12.5 small eye mice *Dach1* expression is found in the same areas as in wildtype embryos (figure 3.17). The level of expression of *Dach1* also appears to remain the same in small eye and wildtype mice, leading to the conclusion that *Pax6* is not necessary for *Dach1* expression within the embryonic forebrain. In sections at E12.5 *Dach1* expression was also observed in the peri-optic mesenchyme surrounding the degenerating optic cup, however *Dach1* was not detected in the degenerating optic cup itself. From E10.5 to E12.5 the optic cup of wildtype mice expresses *Dach1* strongly and Caubit et al., (1999) report the presence of expression from E9.5 in the dorsal region of the optic vesicle. *Pax6* is therefore important in some way for *Dach1* expression. This function may be in the activation or maintenance of *Dach1* or alternatively *Dach1* could be absent in the mutant due to the degenerating optic cup tissue being immature or abnormally fated.

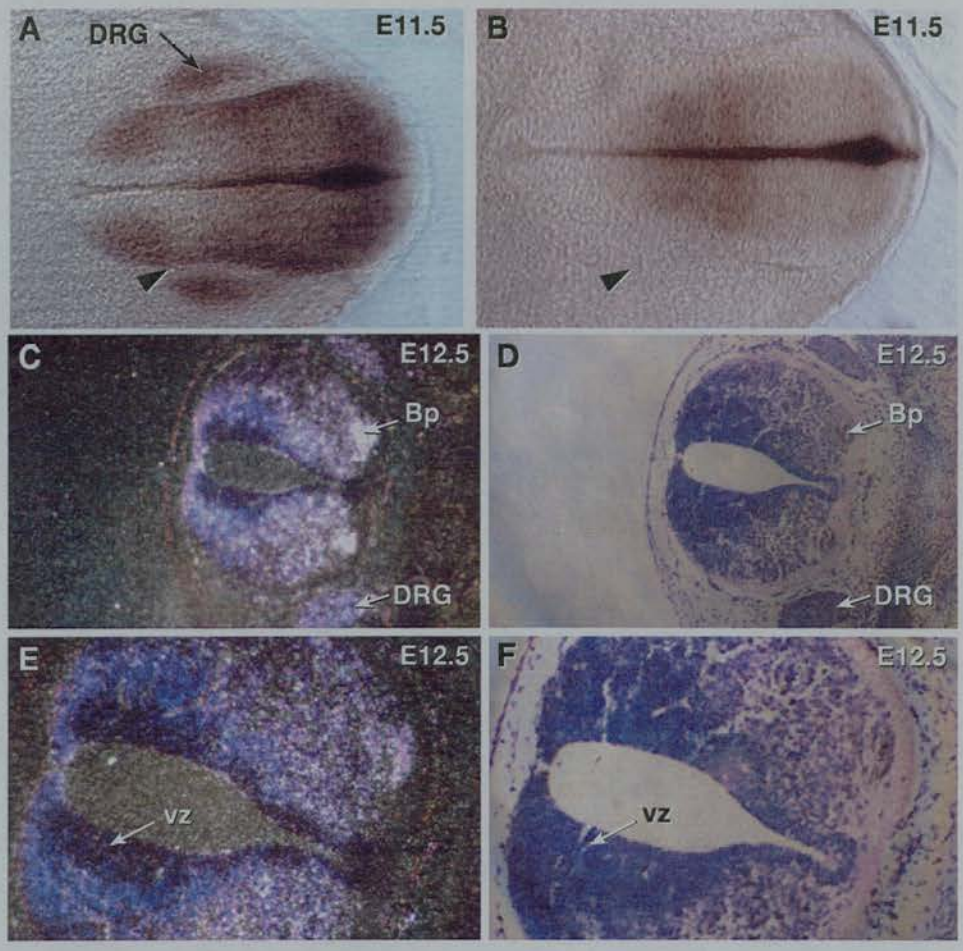


Figure 3.14: *Dach1* expression in the spine.

A/B) Transverse 100μm vibratome sections of whole mount *in-situ* hybridisation analysis. **C-F**) Transverse 7μm radioactive *in-situ* hybridisation sections.

A,C-F) *Dach1* expression is detected in the dorsal root ganglia (**DRG**) at E11.5 and E12.5 and in the mantle of the neural tube in dorsal, lateral and ventral regions. At E11.5 (**A**) expression is also detected in the dorsal ventricular zone however at E12.5 (**C-F**) *Dach1* expression is excluded from the ventricular zone (**vz**). At E12.5 expression is particularly strong in the basal plate region in two nucleii (**Bp**) **B**) *Pax6* expression is detected mainly in the ventral ventricular zone at E11.5 and is not found in the mantle and dorsal root ganglia. The arrowhead shows the same region in (**A**) and (**B**)

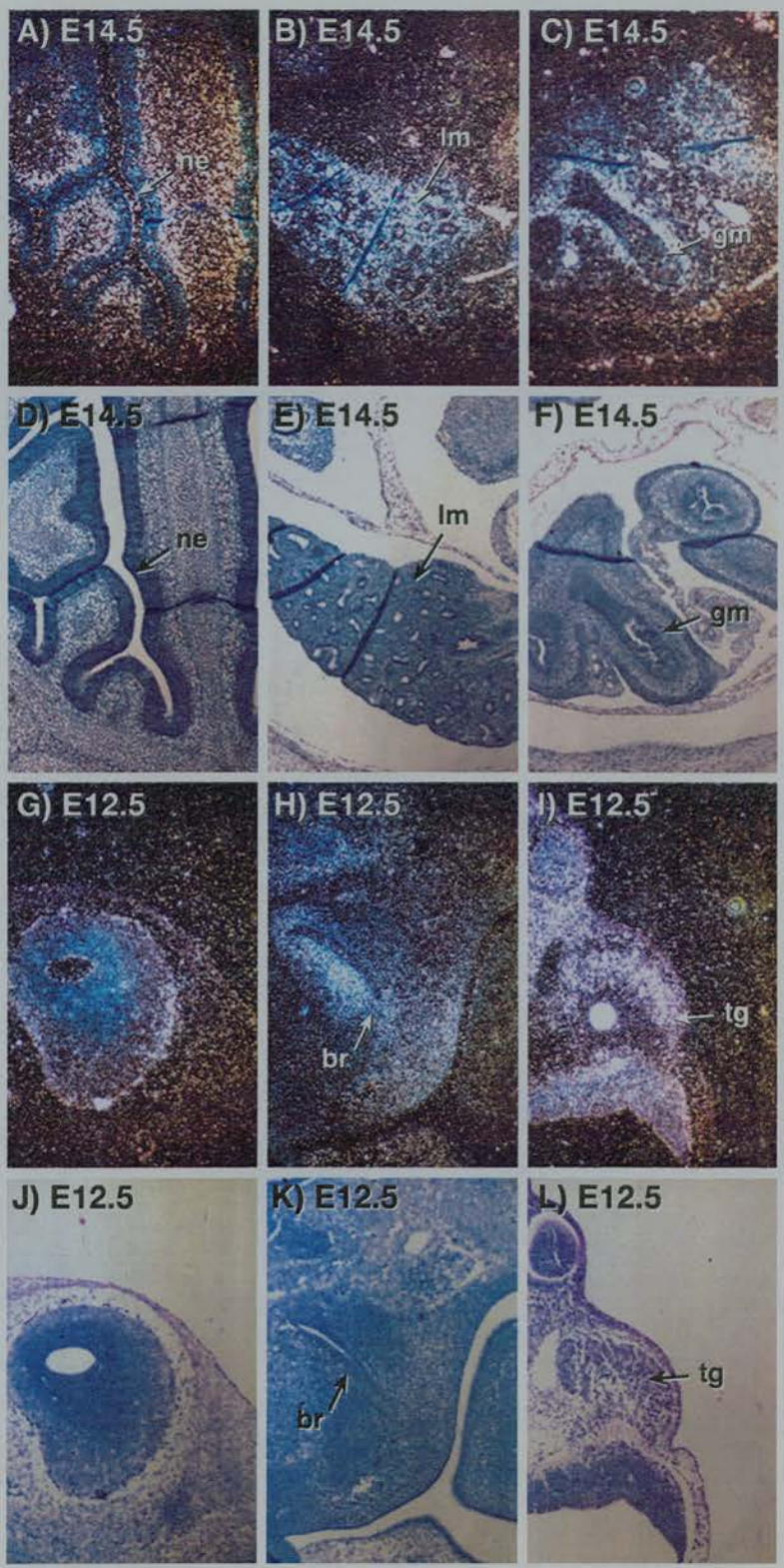


Figure 3.15: *Dach1* expression.
Transverse 7μm sections
A/D) *Dach1* in the mesenchyme surrounding the nasal epithelium (**ne**) at E14.5
B/E) *Dach1* in the lung mesenchyme (**lm**) at E14.5
C/F) *Dach1* in the gut mesenchyme (**gm**) at E14.5
G/J) *Dach1* in the olfactory bulb of the forebrain at E12.5
H/K) Dach1 in the mesenchyme surrounding a branchial arch (**br**) at E12.5
I/L) Dach1 in the trigeminal ganglion (**tg**) at E12.5

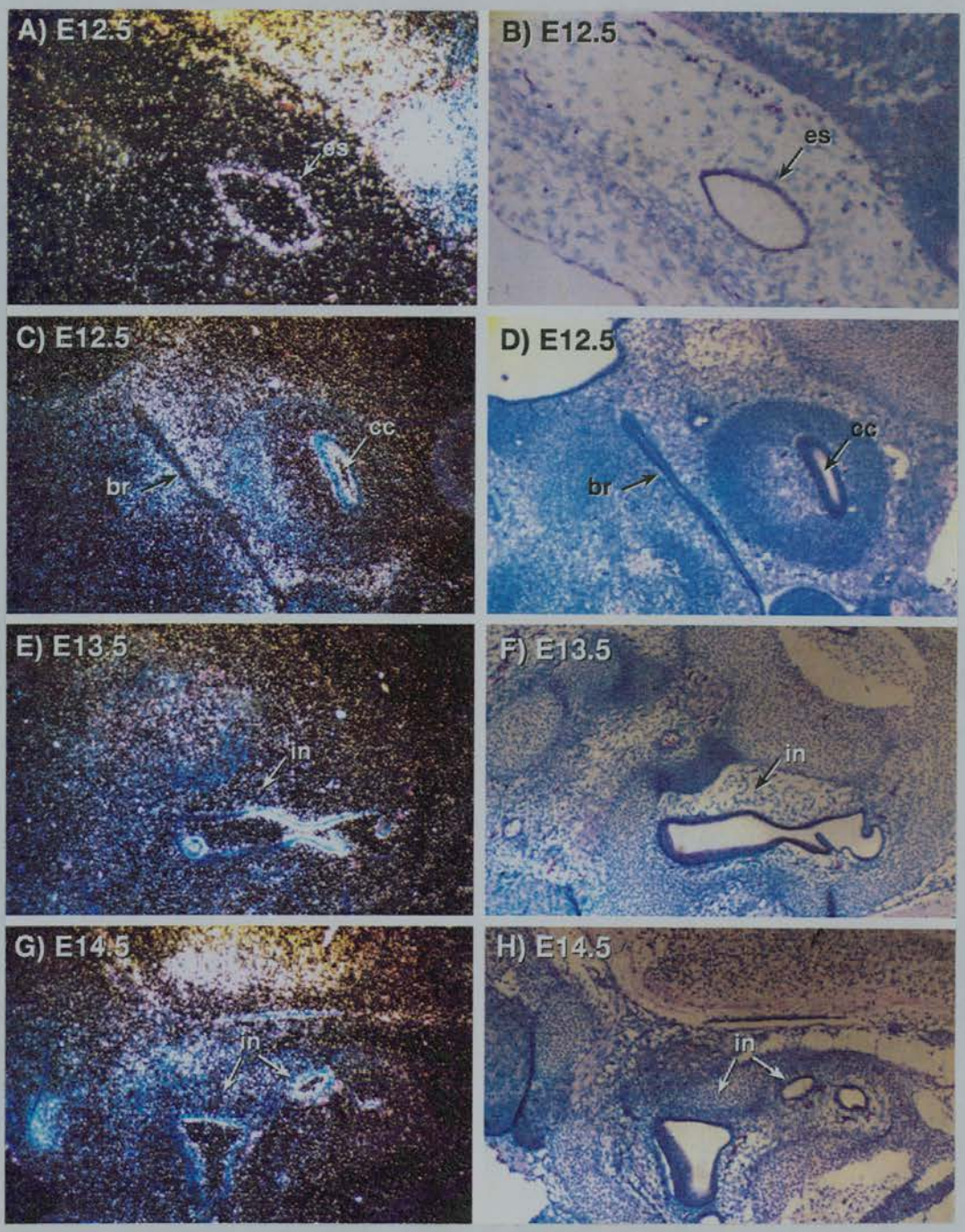


Figure 3.16 *Dach1* expression in the developing ear of midgestation mouse embryos.
Transverse 7μm sections
es: endolymphatic sac, **cc**: cochlear canal, **in**: inner ear, **br**: branchial arch
A/B) *Dach1* expression is detected in the epithelium of the endolymphatic sac at E12.5.
C/D) expression is seen in the cochlea at E12.5.
E/F, G/H) expression in the inner ear region of E13.5 and E14.5 embryos.

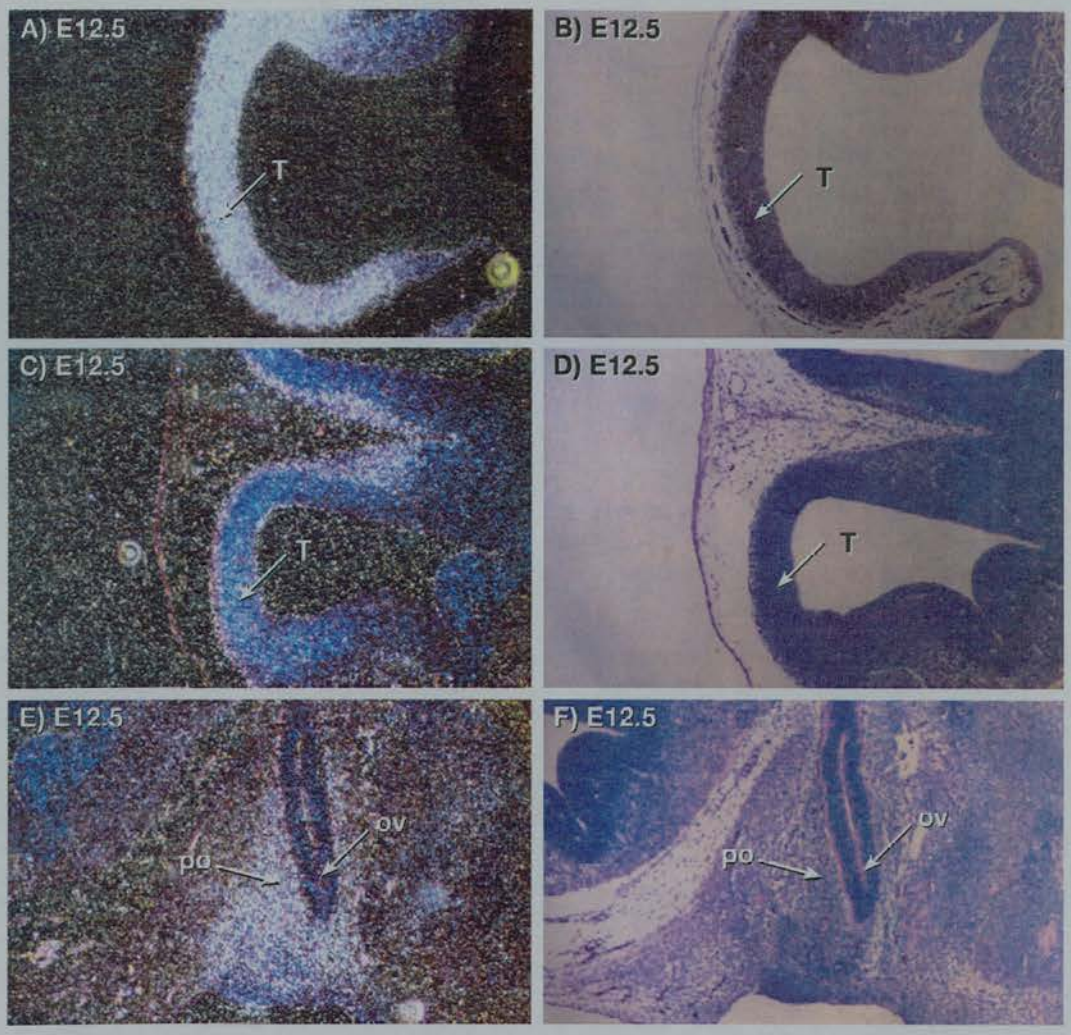


Figure 3.17: Analysis of *Dach1* expression in small eye mouse embryos

Transverse 7μm sections

T: telencephalon, **po**: perioptic mesenchyme, **ov**: optic vesicle

A/B) *Pax6* expression in wildtype E12.5 telencephalon. Like *Dach1* it is found throughout the whole width of the telencephalic vesicles.

C/D) *Dach1* in an E12.5 small eye telencephalon. Expression is detected throughout the whole width of the telencephalic vesicle as in the wildtype situation.

E/D) *Dach1* expression in the optic region of an E12.5 small eye embryo. Expression is detected in the perioptic mesencyhme surrounding the degenerating optic vesicle but not noticeably within the optic vesicle itself.

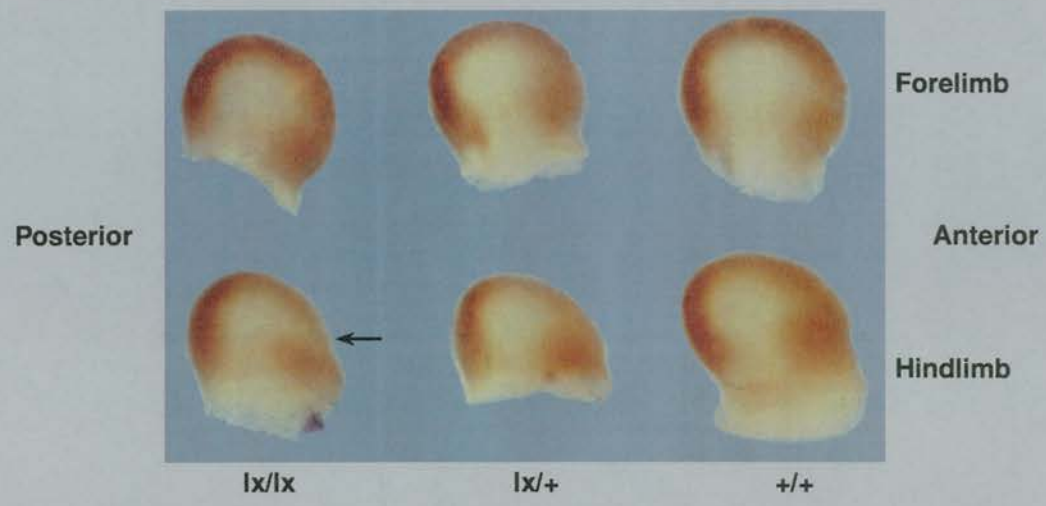


Figure 3.18: *Dach1* expression in the developing limbs of luxate (*lx*) E11.5 embryos.
The forelimbs of both *lx/lx* and *lx/+* animals show wildtype *Dach1* expression while the hindlimbs of *lx/lx* homozygotes have a small gap in the anterior region of expression (arrow). This is also seen in a proportion of *lx/+* hindlimbs but is never present in forelimbs.

3.3.10 *Dach1* expression in *luxate* limbs

Due to the intriguing pattern of expression observed in the limbs it was decided to investigate the expression pattern of *Dach1* in limbs of the luxate (*lx*) mutant. This mutant exhibits pre-axial polydactyly in the hindlimbs of the homozygote, the forelimbs never being affected. The phenotype is variably penetrant in the heterozygote with polydactyly being observed in a proportion of cases, (although not as severe as in the homozygotes) while other animals are morphologically normal. In the hindlimbs of *lx/lx* animals there is a small gap in the anterior region of *Dach1* expression, which is not present in the forelimbs or in wildtype mice (see figure 3.18). This pattern was also seen in a proportion of heterozygous hindlimbs. It is not known what causes the luxate mutant, however *Sonic hedgehog (Shh)* is ectopically expressed in the anterior of homozygous hindlimbs and also in a proportion of heterozygotes.

3.4 Results : Chromosomal localisation of *Dach1*

To map murine *Dach1* a genomic clone was localised to a metaphase mouse chromosome spread by fluorescence *in-situ* hybridisation (FISH). The genomic clone was isolated by screening approximately $1x10^6$ clones of a mouse λ-get library (from Dr T.Boehm) with a probe made from the Dach-box N region (nucleotides 138 - 382) of the mouse cDNA sequence. This region was isolated by PCR using primers P396 and P432 as in appendix 1 and library screening carried out as in section 6.5. The resulting clone pλDach1 was partially sequenced to confirm its identity which revealed an exact match with the 5' end of the *Dach1* cDNA contig.

pλDach1 was labelled with bio-16-dUTP and hybridised to mouse metaphase chromosomes (Fantes et al .,1992). 28 cells were scored which all gave a signal on chromosome 14 band E3 (figure 3.7). This was confirmed by repeating the hybridisation in the presence of FITC labelled chromosome 14 specific paint and pλDach1 always localised to the distal fifth of the painted chromosome (labelling of probe, FISH and scoring was all carried out by Muriel Lee). At the same time human *DACH1* was also mapped by Dr Isabel Hanson to chromosome13q21-22 which is in a well characterised region of synteny with the distal portion of mouse chromosome

14 where *Dach1* maps (figure 3.7). There are no mouse genes mapping to this region which have a specific eye or limb defect but there are neural crest type mutants.

3.5 Discussion

3.5.1 *Dach1* is homologous to *dac* and contains two functional domains

When murine *Dach1* is compared to *Drosophila dac* two regions of high homology are seen. One located toward the N terminus which is referred to as Dach-box N and one toward the C-terminus, referred to as Dach-box C. These regions are likely to represent important functional domains. Southern blotting has suggested that there are unlikely to be a large family of *Dach* genes, however a second *dac* related gene, *Dach2* has been isolated (Tabin et al., 1999) and it cannot be ruled out that there may be further members of this family.

3.5.2 *Dach1* is part of a superfamily with the *Ski* genes

The proto-oncogene *Ski* and a *Ski* family member, *Sno*, have N-terminal domains with significant sequence identity to *Dach1* in an 83 amino acid region which corresponds exactly to Dach-box N (figure 3.4). A C-terminal domain of the *Ski* genes also shows similarity to Dach-box C based on a shared α-helical motif rather than extensive sequence similarity. *Dach1, dac, Ski* and *Sno* are all predicted to form an extended α-helical motif with a strong tendency to interact with other helices to form a coiled coil structure. On the basis of these similarities it seems likely that *Dach, dac, Ski* and *Sno* are members of the same gene superfamily and that structural and functional parallels can be drawn between them.

3.5.3 Dach-box C

Hydrophobic amino acids are seen in Dach-box C with a heptad periodicity characteristic of coiled-coil proteins. This is shown in figure 3.5 in a helical wheel projection (Landschulz et al., 1988), face (a) being highly hydrophobic, with leucine predominant. Coiled-coil motifs are also found in, for example, structural proteins such as myosin which may have upwards of 100 repeats and in leucine zipper DNA-binding proteins like fos and jun which have only four heptad repeats (Landschulz et al., 1988; Lupas et al., 1991; Lupas, 1996). Ski, Dach and dac have 14, 10 and 8

heptads respectively. The *Ski* and *Dach* genes are unusual however, in that they have a heptad which results in alternating basic and hydrophobic amino acids along face (a) of the extended helix (figure 3.5; Sleeman and Laskey, 1993). In the Dach proteins there is also a complementary heptad of alternating acidic and hydrophobic amino acids forming face (d) of the helix. This probably represents a novel zipper motif and the positive and negative side chains may interact to stabilise the helix or protein dimers.

Ski homodimerisation is essential for interactions with the transcription factor NF-1 to take place and is known to mediated by the α-helical coiled coil domain (Tarapore et al., 1997; Zheng et al., 1997a). By analogy to this it is probable that Dach may form functionally important dimers through this region. *Drosophila* dac is known from *in-vitro* studies to interact with eya through a region which includes Dach-box C and the α-helical motif (Chen et al., 1997). As the eya protein does not have coiled coil potential however (P=0 GCG Coilscan algorithm, Dr I.Hanson-personal communication) it seems likely that this interaction is mediated by the conserved amino acids in Dach-box C immediately 5' to the coiled coil motif rather than through a zipper mechanism involving the α-helix itself.

3.5.4 Dach-box N

Dach-box N does not appear to contain any known structural motifs, however, there is evidence that it may be involved in transcriptional activation. The *Drosophila* dac protein has been shown to have transcriptional activation activity within a region containing Dach-box N (Chen et al., 1997) and a region of *v-Ski* containing this domain has been shown to have transforming and myogenic activity (Zheng et al., 1997b). This is also the region of Ski which interacts directly with the NF-1 family of transcription factors (Tarapore et al., 1997). The N-terminal domain of Dach1 may therefore function by interacting directly with proteins involved with the transcriptional apparatus.

3.5.5 Speculative functions for *Dach1* at the molecular level.

Drosophila dac is thought to function as part of a protein complex, binding to eya, so and other factors but not binding directly to DNA (Chen et al., 1997).

Consistent with this no DNA binding motifs are seen in Dach1 and sequence analysis has suggested two domains of Dach1 which could be involved in protein-protein interactions. Ski is known to interact with N-CoR through Dach-box N and with mSin3A through Dach-box C, forming the histone deacetylase complex (HDAC) with these and other proteins (Nomura et al., 1999). By analogy, Dach1 could act to link proteins in a multiprotein complex, interacting with Eya through the Dach-box C region and with another factor through Dach-box N.

Dach1 may also, like Ski, dimerise through the α-helical coiled-coil region and might perhaps interact in alternative complexes to perform different functions as Ski appears to do. Ski interacts with NF-1 through the N-terminal region to perform a transcriptional activation function (Tarapore et al., 1997). When it is part of the HDAC complex, however, Ski has repressor activity (Nomura et al., 1999).

3.5.6 *Dach1* expression is similar in mouse and fly

Drosophila dac is found in the leg primordia, eye primordia and CNS including the optic lobe of the larval brain. Similarly mouse *Dach1* expression is found in the embryonic eye, limb, and brain. Expression in the leg and eye is especially relevant as these two structures are abnormal in *Drosophila* loss of function mutations.

Limb expression

The *Drosophila dac*$^{-/-}$ mutant lacks intermediate leg segments, the distal and proximal elements being fused. This corresponds to larval *dac* expression which is found in a medial ring of cells in the imaginal disc, from which intermediate leg segments develop.

In the E10.5 mouse, *Dach1* expression is found within an anterior region of the central mesenchyme of the limb bud. This may be the region from which the long bones of the zeugopod or the tarsals and carpals of the footplate will form. Mesenchymal condensations which indicate chondrogenesis are initially detected at around E11.5 (Wright et al., 1995), approximately a day after *Dach1* is found in the central mesenchyme. By this stage *Dach1* expression has resolved into the distal mesenchyme of the footplate. If *Dach1* is involved in the development of

intermediate limb structures it is therefore likely to be specifying these elements rather than regulating chondrogenesis. The later expression of *Dach1* in the distal mesenchyme may be indicative of a role in outgrowth, particularly at the tips of the digits.

In luxate mice, *Dach1* expression was altered very subtly, in hindlimb buds only. In homozygotes and also in some heterozygotes a small gap in the anterior expression domain was seen which is not normally observed. The luxate phenotype correlates with this pattern as only the hindlimbs of homozygotes and some heterozygotes exhibit the mutant phenotype of polydactly which is combined with hemimelia (missing tibia). The change in *Dach1* expression is very subtle however, and it is possible that this is simply a secondary effect of tissue mispecification. If *Dach1* is indeed involved in the specification of the long bones this hemimelia could be a result of, or at least marked by, the missing region of anterior *Dach1* expression.

Dach1 expression in heterozygous Hemimelic extra toes *(Hx/+)* mice has been reported by Caubit et al. (1999). A large region of ectopic *Dach1* expression was observed in both fore and hindlimbs, corresponding to the ectopic tissue characteristic of this mutant. The mutation responsible for *Hx* is not known and no significant conclusions were drawn as the increased *Dach1* expression may well be a result of the ectopic tissue rather than a causative factor. No significant alteration of *Dach1* expression was seen in extra toes mice *(Xt)* which are mutated in *Gli3,* a homologue of *Drosophila cubitus interruptus* which is involved in the *hedgehog* signalling pathway (Caubit et al., 1999).

Eye expression

The eye is either missing or severely reduced in size in *Drosophila dac* mutants and head cuticle replaces all retinal structures. A roughened appearance in these reduced eyes is caused by abnormal photoreceptor cells. Mutations in *Drosophila ey*, the homologue of the mouse *Pax6* gene, results in a similar phenotype and evidence suggests early eye development is mutually dependent on both *ey* and *dac*. By analogy with *Drosophila* it might be predicted that *Dach1* in mouse would function either at an early stage, in neuroretina formation or later, in photoreceptor cell differentiation. *Dach1* is expressed in the undifferentiated neuroretina in a

similar pattern to, and at similar stages to, *Pax6* which has a role at the earliest stages of neuroretinal formation (Quinn et al., 1996). If the relationship between *Dach1* and *Pax6* is similar in mouse and *Drosophila* it would therefore be expected that *Dach1* would have a role in early retinal formation.

Dach1 is also expressed in the peri-optic mesenchyme which is partially neural crest derived and is the source of many specialised eye structures including ocular muscles, the sclera and choroid. The possibility exists that this mesenchyme is involved in inductive interactions specifying the layers of the early optic cup, particularly the RPE (section 1.4.3).

3.5.7 Speculative functions for *Dach1*.

Dach1 is found in a wide variety of regions in the developing mouse and it is unclear if there is one unifying factor linking all these regions. Some links do however become obvious when the data presented here are taken together with the early *Dach1* expression data obtained by Caubit et al. (1999) who reported expression from E8.0 onwards.

A neural crest function?

Notably Caubit et al. (1999) report *Dach1* expression in the pre-migratory, migratory and, in some cases, post-migratory neural crest, and note that *Dach1* expression is seen before *Slug*, one of the earliest hitherto known neural crest markers. At E8.25 expression is seen in the dorsal neural fold and in segmented patches of neural crest cells which migrate laterally over the somites. By E9.5 *Dach1* is found in large regions of head mesenchyme much of which has a neural crest origin. In both Caubit et al. and the work presented in this thesis *Dach1* is expressed in many regions which contain at least some cells of neural crest origin; the perioptic mesenchyme, the pharyngeal pouches, the dorsal root ganglia, the sympathetic ganglia (Caubit et al., 1999 only), some components of the inner ear, trigeminal ganglion, glossopharangeal-Vagal ganglion complex and the nasal process (Gilbert et al., 1997). It would be interesting to investigate further if other neural crest derivatives express *Dach1*, and what happens to *Dach1* expression in mutants which

disrupt neural crest migration such as *Hoxa-3* and *Hoxb-1* mutants (Gilbert et al., 1997).

A proliferative function?

Dach1 expression is found in many regions of high proliferative activity. The ventricular zone of the telencephalon, the neuroretina, the progress zone of the limb bud and the genital eminence are all such regions. In *Drosophila* too, *dac* is found in highly proliferative regions of the developing eye and leg, and in $dac^{-/-}$ mutants an excess of cell death was seen in the region of the leg imaginal disc where *dac* is normally expressed (Marden et al., 1994). This does not necessarily indicate a proliferative function however, as programmed cell death may occur simply because specification and differentiation have failed. In the fly however, *eya* and *so*, which are known to interact with *dac,* do have a role in eye disc cell proliferation (Chen et al., 1997; Pignoni et al., 1997) suggesting that here at least *dac* may also. Interestingly mice which lack *Ski* show excessive programmed cell death in the neural epithelium (Zheng et al., 1997) and by analogy *Dach1* may also have a role in proliferation.

3.5.8 *Dach1* interacting genes?

Dach1 is found in large regions of the forebrain and hindbrain but strikingly not in the midline, and indeed *Dach1* is not seen in the floorplate of the neural tube either. The expression of midline markers such as BMP4 would appear to correspond to the region of *Dach1* exclusion (Caubit et al., 1999). Caubit et al. also speculate that the ventral restriction of *Dach1* could be caused by *Shh* as *Dach1* is absent from the area around the *Shh* secreting notochord. In the limb bud however it appears that *Dach1* may be independent of *Shh* as no obvious differences in *Dach1* expression were seen in extra toes mice which contain an ectopic anterior *Shh* source. Caubit et al. also suggest that it may not be coincidence that *Dach1* is expressed adjacent to sources of FGF, not only in the neural crest where *Slug,* a known neural crest marker, is known to be induced by FGF, but also in the mesenchyme beneath the AER of the limb bud and in the brain.

3.5.9 *Dach1* expression is not directly controlled by *Pax6*: is *Dach1* part of a conserved network between *Drosophila* and mammals?

Halder et al.(1995) suggested that since *ey* can induce ectopic eye structures in *Drosophila, Pax6* must be at the top of a genetic hierarchy controlling eye development. Other genes, *dac* and *eya*, have since been shown to produce ectopic retinal development (Shen and Mardon, 1997, Bonini et al.,1997) indicating that there is a complex network of interactions rather than a straightforward linear pathway. When any of the three genes, *dac, ey* or *eya*, are ectopically expressed the remaining two are induced in the ectopic eye region, therefore positive feedback loops must exist. Both genetic and protein-protein interactions have also been shown between *dac* and *eya* (Chen et al.,1997), suggesting that complex formation and genetic interactions within a network are important for *Drosophila* eye formation.

It was hence of interest to investigate the possibility that *Dach1* may be involved in a similar network in mammals. Many *Drosophila* eye developmental genes are conserved between mammals and *Drosophila* and now *Dach1* is included in this group. *Dach, Pax6, Six3/Optx2* and three members of the *Eya* family are expressed in the embryonic mouse eye so several members of the proposed network are present. The data presented here suggests that even where both *Pax6* and *Dach1* are expressed together, *Pax6* is not necessarily required for *Dach1* expression. The expression of *Dach1* within the E12.5 and E11.5 small eye telencephalon is the same as in the wildtype brain, so in this tissue *Dach1* expression is either independent of, or upstream of *Pax6* which is also expressed in the telencephalon in a similar pattern to *Dach1*. Interactions may occur between these two gene products and it is likely at this level that they operate in the genetic network. Also in the majority of the tissues in which *Pax6* and *Dach1* are found their expression domains do not overlap; for example a striking pattern is observed in the neural tube, where *Dach1* and *Pax6* are expressed in complementary domains. Similarly, *Drosophila dac* has a function in the limb independent of *ey*. In the developing optic cup however, *Pax6* may be required for *Dach1* expression as *Dach1* could not be detected in the degenerating optic cup of an E12.5 small eye mouse.

It is likely, from this and the preceding discussion, that *Dach1* will prove to have a variety of functions within the developing mammal and that the relationship with *Pax6* may be important in specifying this function within a few of these tissues whereas in others *Dach1* will act independently of *Pax6*.

3.5.10 *Dach1* maps to a region containing no specific eye and limb defects

Dach1 maps to chromosome 14E3 in the mouse near to the endothelin-B receptor (*Ednrb)* gene. No specific limb and eye defects map to this region, however several *piebald* mutants are known which delete regions around the *Ednrb* gene but have phenotypes more extensive than the *Ednrb* knockout. The spectrum of defects not attributable to a lack of *Ednrb* in $S^{l5DttMb}$ and S^{31Pub} includes CNS, neural crest, dorsal spinal chord and digit defects (O'Brien et al., 1996).

Human *DACH1* has been mapped to region 13q22 (Dr I.Hanson in Hammond et al., 1999; Caubit et al., 1999; Kozmik et al., 1999) which is in the region syntenic with mouse chromosome 14E3. Again deletions in this region cause neural crest deficiencies, growth retardation, mental retardation, limb defects and also rarely micro-ophthalmia (Tranebjaerg et al., 1988; Rivas et al., 1984). Some but not all of these defects are attributable to the absence of the *EDNRB* gene. In addition postaxial polydactyly type A2 maps to 13q and is a good candidate disease for a *Dach1* mutation given the expression of *Dach1* in the limb bud (OMIM 602085). Kozmik et al., also suggest that *DACH1* is a good candidate for Bardet-Biedel syndrome which is characterised by retinal degeneration and polydactyly. Four genes known to be responsible for this disease do not map to 13q, however there is evidence that at least one more gene is involved (Kozmik et al., 1999)

Chapter 4 : Cloning and characterisation of zebrafish *(Danio rerio) Dachshund* genes

4.1 Introduction

4.1.1 Cloning and analysis of zebrafish *Dachshund* genes

The cloning and analysis of three zebrafish *Dachshund* genes by homology to the mouse gene *Dach1* (see chapter 3) is presented in this chapter. This study includes analysis of the expression pattern of all three genes and preliminary over-expression assays carried out with one of these genes, *zfDachA*.

Previously Oliver et al. (1996) and Loosli et al. (1998) over-expressed *Six3*, a gene important for vertebrate eye development, in medaka resulting in the induction of ectopic lens and retina from the otic vesicle and brain respectively. When similar experiments were carried out in the zebrafish an enlarged forebrain was seen but no ectopic eye structures were reported (Kobayashi et al.,1998). Ectopic eye structures have also been observed when either *Pax6* or *Rx1* is over-expressed in *Xenopus* (Chow et al., 1999; Mathers et al., 1997).

dac, the *Drosophila* counterpart of the vertebrate *Dachshund* genes, is one of a conserved group of genes which can produce ectopic eyes when over-expressed in *Drosophila* imaginal discs. *Pax6* and *Six3* are vertebrate homologues of two others, *ey* and *so* (discussed in sections 1 and 3.1). It was therefore of interest to investigate possible eye functions for vertebrate *Dachshund* and the over-expression assays presented here are a first step in this direction.

4.1.2 The zebrafish system

The zebrafish provides a good system for both genetics and the study of development. As a developmental system advantages include the fact that many eggs are produced per mating, fertilisation is external which aids embryo collection and the development of the fish is rapid. Eye development in particular is very fast, all retinal cell types being present and differentiated by 60 hours post fertilisation (hpf). The optical clarity of the embryo also aids observation of both living embryos and those which have been fixed and stained. Over-expression assays are also relatively

straightforward to perform in fish and a quick result can be obtained due to the rapid development. There are however disadvantages of the system. As yet specific genes cannot be targeted and knocked out in fish for loss of function analysis, although gene function can sometimes be removed by other approaches such as the use of antisense or dominant negative constructs. There also appears to have been a great deal of gene duplication in the zebrafish genome compared to that of mammals. For instance, two *Pax6* genes have been isolated from zebrafish but only one from mammals (Krauss et al., 1991; Nornes et al., 1998; Ton et al., 1991; Walther and Gruss 1991) and fish *eng2* and *eng3* seem equally similar to murine *En2* (Postlethwait et al., 1998). Most convincingly, more *Hox* genes have been isolated in the fish than in mammals and three of these are not present within the four mammalian *Hox* clusters (Prince et al., 1998b). This gene duplication can however, be an advantage in dissecting the functions of a gene which has multiple functions in mammals, because these functions may be carried out by separate genes in the zebrafish (Detrich et al., 1999)

From a genetic point of view many mutants have been created by large scale mutagenesis screens (Development 123, 1996). Specifically there is a large pool of eye development mutations affecting neurogenesis, neuronal survival, electrophysiology, retinotectal projections and morphogenesis. There are also more generalised mutants affecting eye size (Baier et al., 1996; Malicki et al., 1996; Karlstrom et al., 1996; Trowe et al., 1996).

4.1.3 Zebrafish eye development

Teleost eye development is broadly similar to mammalian eye development, but the presence of a solid neural keel instead of the hollow neural tube of mammals means that the fish retina begins life as a solid optic lobe rather than a hollow optic vesicle. At about 11.5hpf a thickening appears in the anterior brain which is initially flat and protrudes on either side of the brain. At 13.5hpf the posterior part of the lobe separates from the brain; the anterior region, which remains attached, will later form the optic stalk. The lobe also begins to turn on its axis so that the lower surface now points to the brain and the upper surface becomes the external surface. At 15hpf the external surface begins to invaginate to form the optic cup and a thickening develops

in the surface ectoderm adjacent to the invagination, this is the lens placode. By 24hpf the lens has become internalised and detached from the surface ectoderm, which differs in mechanism from mammalian development where the lens placode invaginates. The two layers of the optic cup, the inner neuroretina and the outer retinal pigmented epithelium (RPE), can also be easily identified by this stage in the fish. The first neurons, the ganglion cells, are recognisable between 29 and 34hpf. Virtually all neurogenesis is complete by 60hpf, all neuron types being generated at roughly the same time, which contrasts with the sequential development seen in the mammalian eye. In the zebrafish a small region of mitotic cells, the marginal zone persists throughout life (Easter and Nicola, 1996; Westerfield et al., 1993; Malicki., 1999; Schmitt and Dowling 1994).

4.2 Cloning and sequence analysis of zebrafish *Dachshund* genes

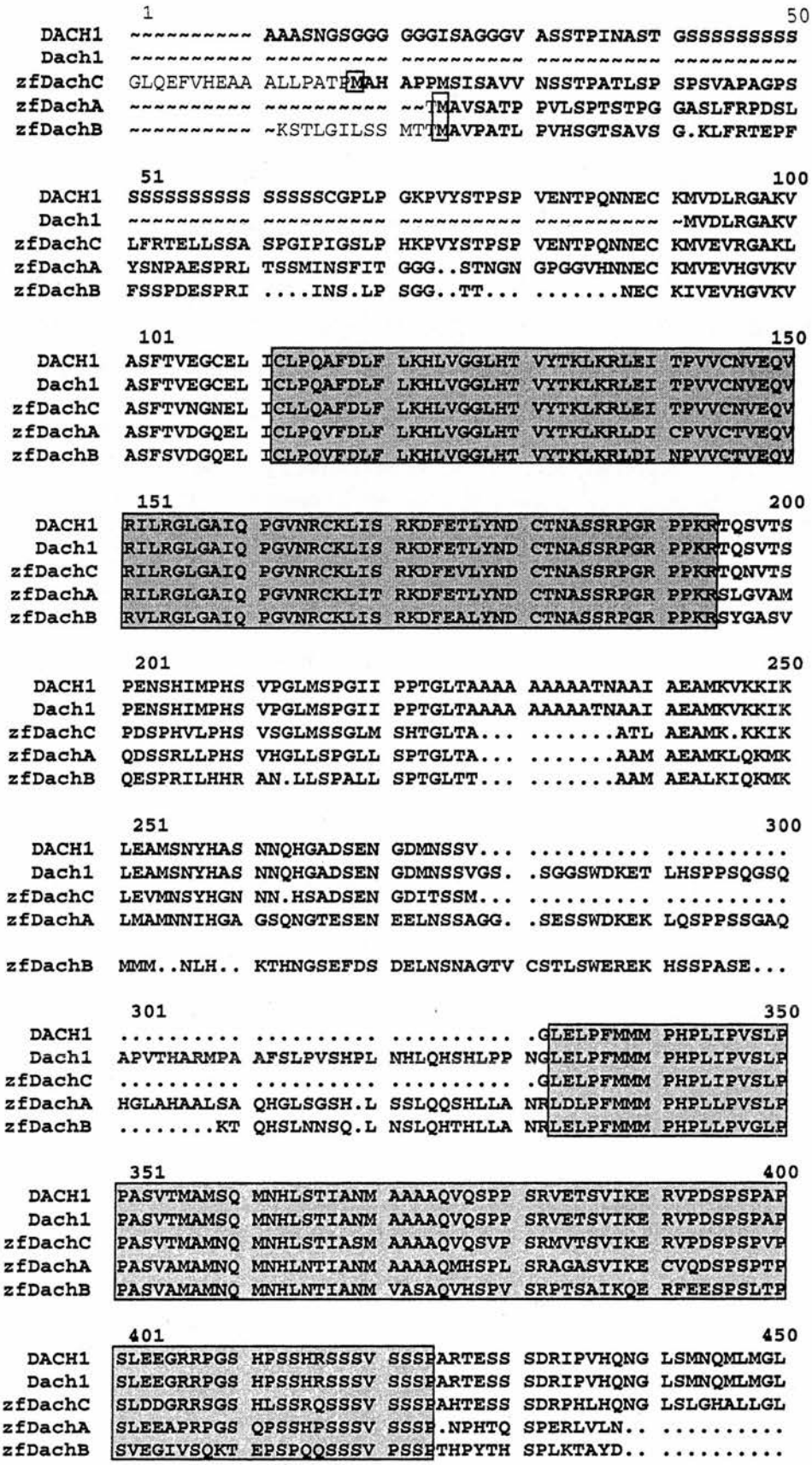
4.2.1 Cloning zebrafish *dachshund* homologues

A zebrafish 15-19 hour (28.5°C) cDNA library (a gift from Bruce Appel, University of Oregon) was screened with nucleotides 138 to 382 of murine *Dach1* using a hybridisation temperature of 60°C. This region contains Dach-box N, one of two highly conserved domains between *Drosophila dac* and murine *Dach1* (see chapter 3). One full length zebrafish gene was isolated in this way and designated *zfDachA*. Nucleotides 372 to 636 of this fish clone were then PCR amplified and used to re-screen the library. Two further full length genes were isolated in this way and named *zfDachB* and *zfDachC*.

4.2.2 All three fish clones contain complete open reading frames (ORFs).

The *zfDachA*, *zfDachB* and *zfDachC* clones are 2883bp, 2377bp and 2072bp respectively and all appear to contain a full length open reading frame. These are 602, 564 and 576 amino acids long respectively (see figure 4.1 and appendix 2).

There is little doubt that the ORFs of *zfDachA* and *zfDachB* are complete as the presumptive initiating codons are both preceeded by two in frame stop codons. In addition the first three amino acids of the protein sequence, methionine, alanine, and valine, are conserved between these two fish genes and the full length mouse sequence as reported by Caubit et al. (1999) and Kozmik et al. (1999). It is however,


1 50
DACH1 ~~~~~~~~~~ AAASNGSGGG GGGISAGGGV ASSTPINAST GSSSSSSSSS
Dach1 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
zfDachC GLQEFVHEAA ALLPATPMAH APPMSISAVV NSSTPATLSP SPSVAPAGPS
zfDachA ~~~~~~~~~~ ~~~~~~~~~~ ~~TMAVSATP PVLSPTSTPG GASLFRPDSL
zfDachB ~~~~~~~~~~ ~KSTLGILSS MTTMAVPATL PVHSGTSAVS G.KLFRTEPF
51 100
DACH1 SSSSSSSSSS SSSSSCGPLP GKPVYSTPSP VENTPQNNEC KMVDLRGAKV
Dach1 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~MVDLRGAKV
zfDachC LFRTELLSSA SPGIPIGSLP HKPVYSTPSP VENTPQNNEC KMVEVRGAKL
zfDachA YSNPAESPRL TSSMINSFIT GGG..STNGN GPGGVHNNEC KMVEVHGVKV
zfDachB FSSPDESPRI ....INS.LP SGG..TT... ....NEC KIVEVHGVKV
101 150
DACH1 ASFTVEGCEL ICLPQAFDLF LKHLVGGLHT VYTKLKRLEI TPVVCNVEQV
Dach1 ASFTVEGCEL ICLPQAFDLF LKHLVGGLHT VYTKLKRLEI TPVVCNVEQV
zfDachC ASFTVNGNEL ICLLQAFDLF LKHLVGGLHT VYTKLKRLEI TPVVCNVEQV
zfDachA ASFTVDGQEL ICLPQVFDLF LKHLVGGLHT VYTKLKRLDI CPVVCTVEQV
zfDachB ASFSVDGQEL ICLPQVFDLF LKHLVGGLHT VYTKLKRLDI NPVVCTVEQV
151 200
DACH1 RILRGLGAIQ PGVNRCKLIS RKDFETLYND CTNASSRPGR PPKRTQSVTS
Dach1 RILRGLGAIQ PGVNRCKLIS RKDFETLYND CTNASSRPGR PPKRTQSVTS
zfDachC RILRGLGAIQ PGVNRCKLIS RKDFEVLYND CTNASSRPGR PPKRTQNVTS
zfDachA RILRGLGAIQ PGVNRCKLIT RKDFETLYND CTNASSRPGR PPKRSLGVAM
zfDachB RVLRGLGAIQ PGVNRCKLIS RKDFEALYND CTNASSRPGR PPKRSYGASV
201 250
DACH1 PENSHIMPHS VPGLMSPGII PPTGLTAAAA AAAAATNAAI AEAMKVKKIK
Dach1 PENSHIMPHS VPGLMSPGII PPTGLTAAAA AAAAATNAAI AEAMKVKKIK
zfDachC PDSPHVLPHS VSGLMSSGLM SHTGLTA... ....ATL AEAMK.KKIK
zfDachA QDSSRLLPHS VHGLLSPGLL SPTGLTA... ....AAM AEAMKLQKMK
zfDachB QESPRILHHR AN.LLSPALL SPTGLTT... ....AAM AEALKIQKMK
251 300
DACH1 LEAMSNYHAS NNQHGADSEN GDMNSSV... ....
Dach1 LEAMSNYHAS NNQHGADSEN GDMNSSVGS. .SGGSWDKET LHSPPSQGSQ
zfDachC LEVMNSYHGN NN.HSADSEN GDITSSM... ....
zfDachA LMAMNNIHGA GSQNGTESEN EELNSSAGG. .SESSWDKEK LQSPPSSGAQ
zfDachB MMM..NLH.. KTHNGSEFDS DELNSNAGTV CSTLSWEREK HSSPASE...
301 350
DACH1 .... .GLELPFMMM PHPLIPVSLP
Dach1 APVTHARMPA AFSLPVSHPL NHLQHSHLPP NGLELPFMMM PHPLIPVSLP
zfDachC .... .GLELPFMMM PHPLIPVSLP
zfDachA HGLAHAALSA QHGLSGSH.L SSLQQSHLLA NRLDLPFMMM PHPLLPVSLP
zfDachB .....KT QHSLNNSQ.L NSLQHTHLLA NRLELPFMMM PHPLLPVGLP
351 400
DACH1 PASVTMAMSQ MNHLSTIANM AAAAQVQSPP SRVETSVIKE RVPDSPSPAP
Dach1 PASVTMAMSQ MNHLSTIANM AAAAQVQSPP SRVETSVIKE RVPDSPSPAP
zfDachC PASVTMAMNQ MNHLSTIASM AAAAQVQSVP SRMVTSVIKE RVPDSPSPVP
zfDachA PASVAMAMNQ MNHLNTIANM AAAAQMHSPL SRAGASVIKE CVQDSPSPTP
zfDachB PASVAMAMNQ MNHLNTIANM VASAQVHSPV SRPTSAIKQE RFEESPSLTP
401 450
DACH1 SLEEGRRPGS HPSSHRSSSV SSSPARTESS SDRIPVHQNG LSMNQMLMGL
Dach1 SLEEGRRPGS HPSSHRSSSV SSSPARTESS SDRIPVHQNG LSMNQMLMGL
zfDachC SLDDGRRSGS HLSSRQSSSV SSSPAHTESS SDRPHLHQNG LSLGHALLGL
zfDachA SLEEAPRPGS QPSSHPSSSV SSSP.NPHTQ SPERLVLN.. .....
zfDachB SVEGIVSQKT EPSPQQSSSV PSSPTHPYTH SPLKTAYD.. .....

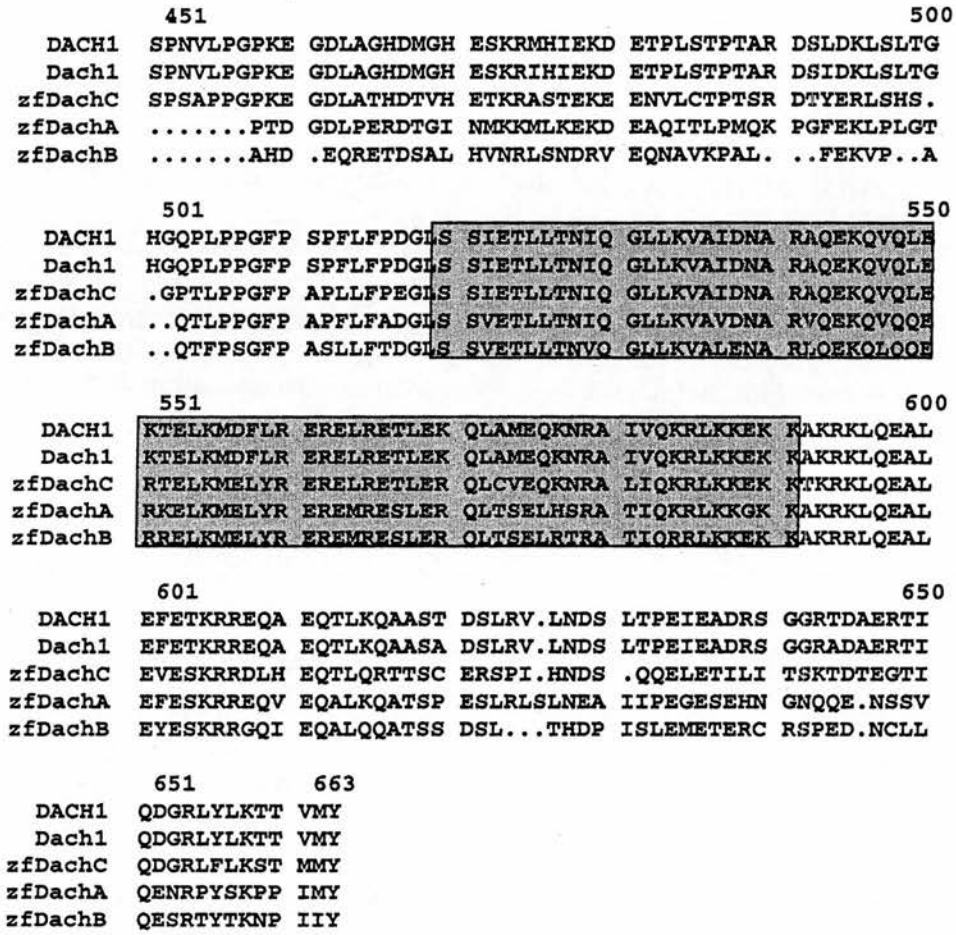


Figure 4.1 Sequence alignment of the protein sequence of zfDachA, zfDachB, zfDachC, Dach1 and DACH1.

Dach-box N (numbered 111–194) and Dach-box C (no. 520–591), two regions of homology between all known *dac* homologues, are boxed in dark grey. A region of homology between vertebrate *dac* homologues which is not present in *Drosophila* (Dach-box M) is boxed in light grey (no. 333-424). The probable start sites of the three zebrafish genes (boxed) are shown.

The mouse protein has an insertion of 52 amino acids compared to the human protein (no. 277-331) which appears to correspond to an alternatively spliced exon, as discussed in chapter 3. This insertion is shared by zfDachA and partially by zfDachB but is absent from zfDachC. A further region 3' to Dach-box M (no. 439-457) is present in Dach1, DACH1 and zfDachC but absent from zfDachA and zfDachB. An Alanine repeat (no. 228-237) present in the mammalian genes is absent from all three zebrafish genes.

less clear that the *zfDachC* clone contains a complete ORF. Although the putative initiating methionine is followed by an alanine residue, the *zfDachC* clone isolated extends only 17 amino acids 5' of this and contains no upstream stop codons within this region.

All three fish genes, like the mammalian genes, contain a 3' stop codon preceded by a tyrosine residue and followed by multiple stop codons.

4.2.3 A third region is conserved between the vertebrate *dac* homologues.

Two regions which are highly conserved between mammalian and *Drosophila dachshund* are also found in all three zebrafish homologues (see figure 4.1 and 3.4). Identity with mammalian Dach1 at the amino acid level is 94%, 93% and 98% for zfDachA, zfDachB and zfDachC respectively in Dach-box N and 72%, 68% and 87% in Dach-box C.

A third region of high homology, Dach-box M, is also apparent between the vertebrate genes and is positioned between Dach-box N and Dach-box C (see figure 4.1 and 4.2). This region is 92 amino acids long and is not shared by *Drosophila* dac. Identity to Dach1 is 79.3%, 63.0% and 85.9% for zfDachA, zfDachB and zfDachC respectively within this region.

4.2.4 Phylogenetic analysis of the *Dachshund* family.

Phylogenetic analysis was carried out using programs available through GCG ('paupsearch' and 'paupdisplay'). This allowed trees based on parsimony and on genetic distance to be constructed. Trees were constructed based on amino acid sequence to give an idea of the relationships between genes.

Phylogenetic analysis was performed based on the entire *Dachshund* gene and separately using the three individual conserved regions. This was to ensure that gaps in the sequence alignment, which may represent alternative splice sites rather than true genetic differences, did not affect the result unduly. This also allowed the partial *C.elegans* sequence and the *Drosophila* sequence, which is much longer than the mammalian genes, to be included in the analysis. Figure 4.3 shows the trees produced by analysis of genetic distance, all four are very similar. *zfDachC* is the most similar fish gene to *Dach1* and *DACH1*, *zfDachB* being the least similar. *zfDachA* and

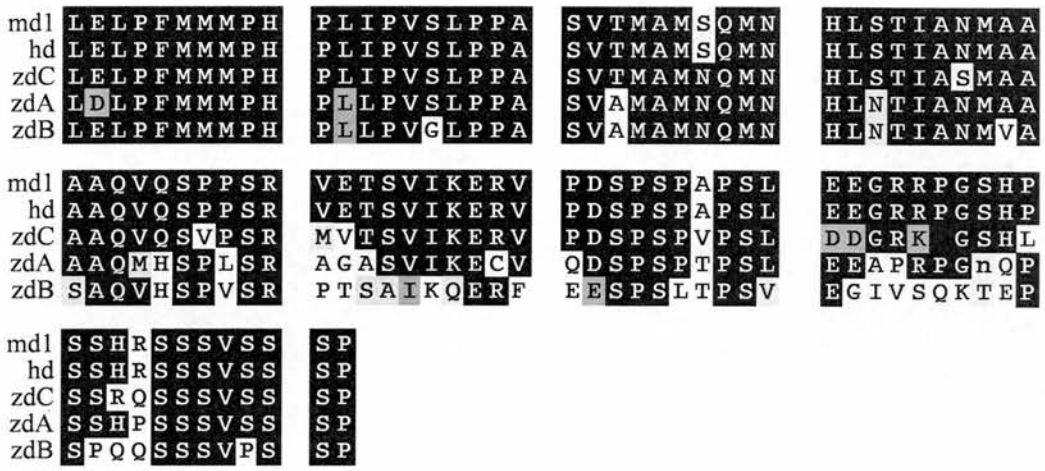


Figure 4.2 : Conserved amino acids between vertebrate homologues of dachshund.

Dach-box M which spans 92 amino acids between Dach-box N and Dach-box C and is conserved between mammalian Dachshund and all three zebrafish genes. It is not however, present in *Drosophila* dac. **md1:** mouse Dach1, **hd :** human DACH1, **zdA:** zebrafish DachA, **zdB:** zebrafish DachB, **zdC:** zebrafish DachC.

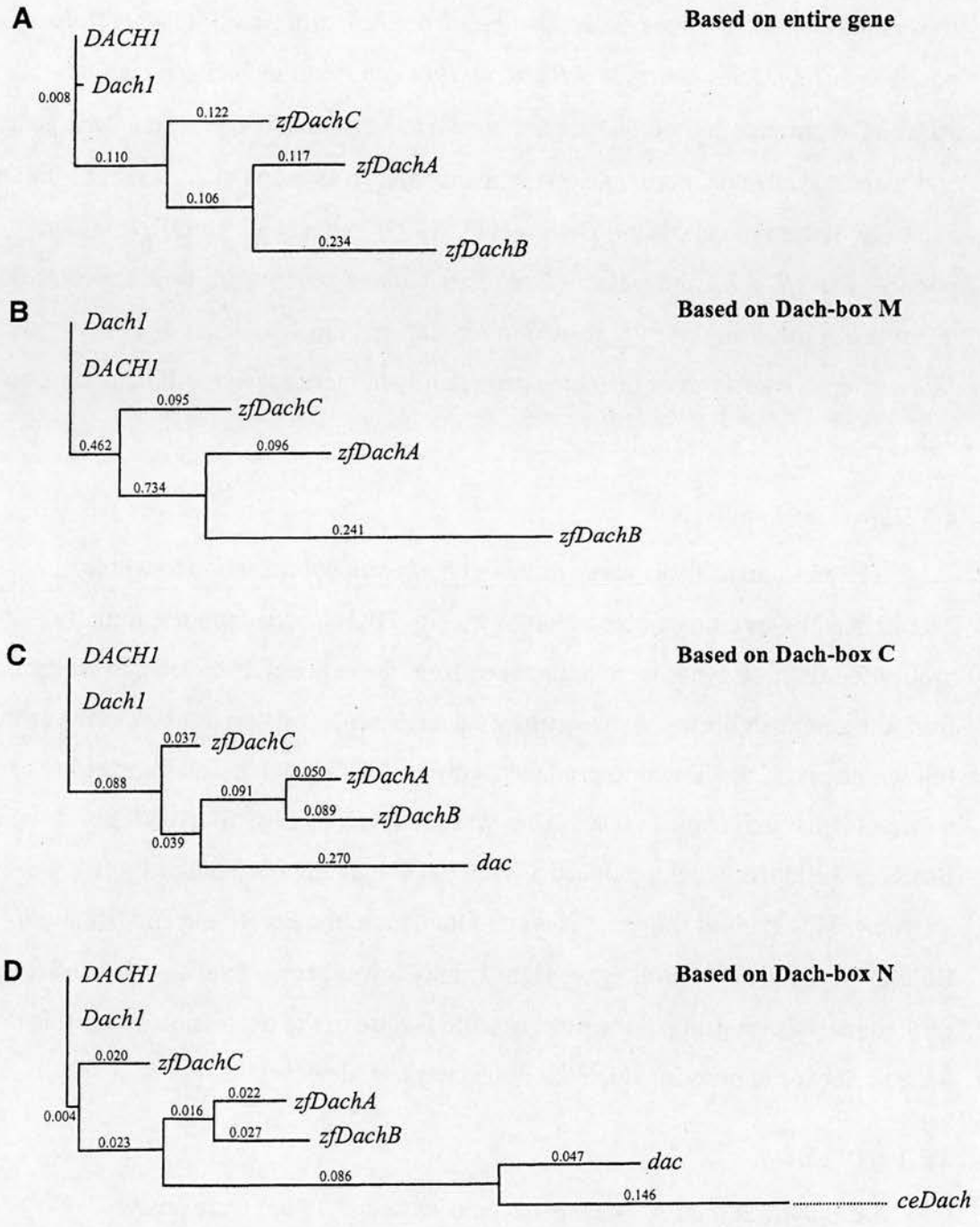


Figure 4.3 : Phylogenetic analysis of the *Dachshund* gene family based on amino acid sequence. Trees were produced using programs available through GCG ('paupsearch' and 'paupdisplay') based on minimum evolutionary distance. Branch length is roughly proportional to the evolutionary distance represented, but is not exactly to scale. Relative evolutionary distance is indicated on each branch. The deduced evolutionary relationship between mammalian and zebrafish *Dachshund* genes is similar whichever region of the gene is compared.
Dach1: mouse *Dachshund*; *DACH1*: human *Dachshund*; *zfDachA*: zebrafish *Dachshund A*; *zfDachB*: zebrafish *Dachshund B*; *zfDachC*: zebrafish *Dachshund C*; *dac*: *Drosophila dachshund*; *ceDach*: *C.elegans dachshund*.

zfDachB may also be more similar to each other than to the other family members (this is indicated in all trees except that based on the entire gene). It is therefore possible that *zfDachA* and *zfDachB* are related to one mammalian gene whilst *zfDachC* represents another (There are known to be at least two chicken *Dach* genes and there may also be more than one in mammals) [Hammond et al., 1998; Caubit et al., 1999; Kozmik et al., 1999; Davies et al., 1999; Tabin et al., 1999]). It is also possible that *zfDachA* and *zfDachC* are related to one mammalian gene and *zfDachB* to another, or that all three are related to separate mammalian genes or to only one. More extensive analysis of the *Dach* gene family in these species will help to resolve this.

4.3 Expression analysis.

Expression analysis was carried out by *in-situ* hybridisation to whole zebrafish embryos using antisense digoxygenin (DIG) labelled probes. Initially probes for all three genes were transcribed from the entire cDNA clone, as isolated from the zebrafish library. Subsequently a shorter probe was used for *zfDachA* as the full length probe was found to produce significant background. The truncated *zfDachA* construct, named zfDachA/hr, was produced by digestion of *zfDachA* with EcoRI and HindIII which produced a fragment containing nucleotides 0 – 1464 of *zfDachA*. This excised fragment was sub-cloned into the EcoRI and HindIII sites of Bluescript SK+/-. This probe gave significantly lower background so was used for all further analysis. In general, the most notable feature of the expression patterns is the wide variety of regions in which the three genes are detected.

4.3.1 *zfDachA*

Embryos at intervals between shield stage and 72 hpf were analysed. No expression was detected at shield or tailbud stage. A small amount of expression was seen by five somites but was extremely weak and difficult to localise although it appeared to be within the head region and the neural keel. By eight somites expression was detectable within the neural keel and the anterior portion of the embryo, in the developing eye region and brain. Expression appeared to be restricted to the forebrain and the hindbrain (data not shown).

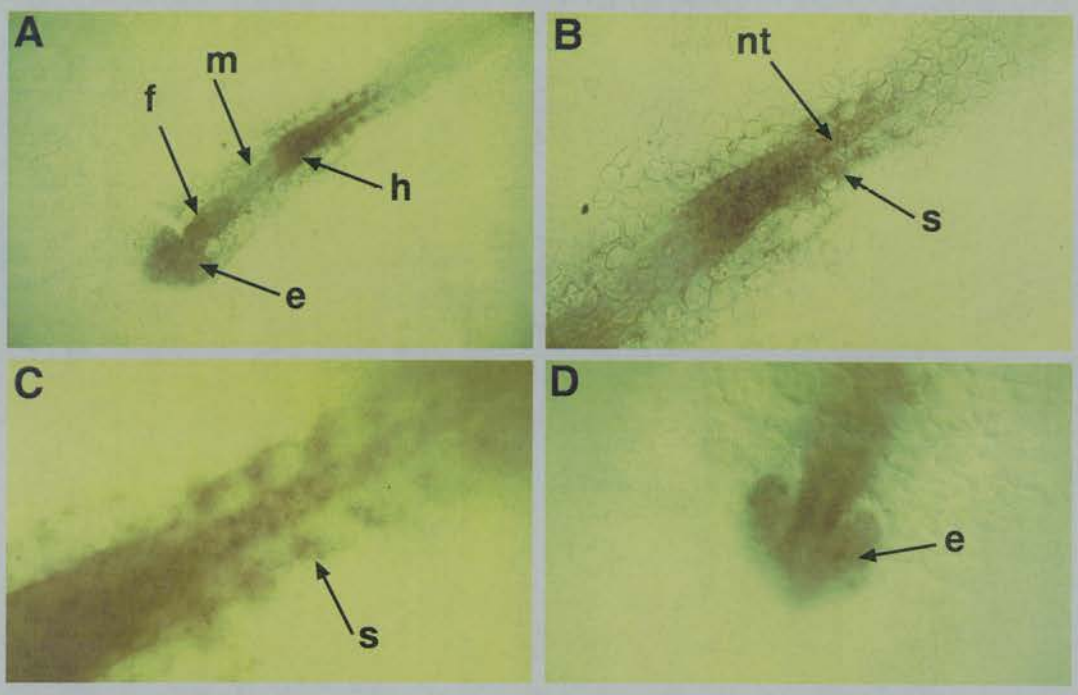


Figure 4.4 *zfDachA* expression in 14 somite zebrafish embryos.
Expression is seen in the forebrain (**f**) and eye (**e**), hindbrain (**h**), neural tube and the anterior most somites (**s**)
Dorsal views (anterior is to left)

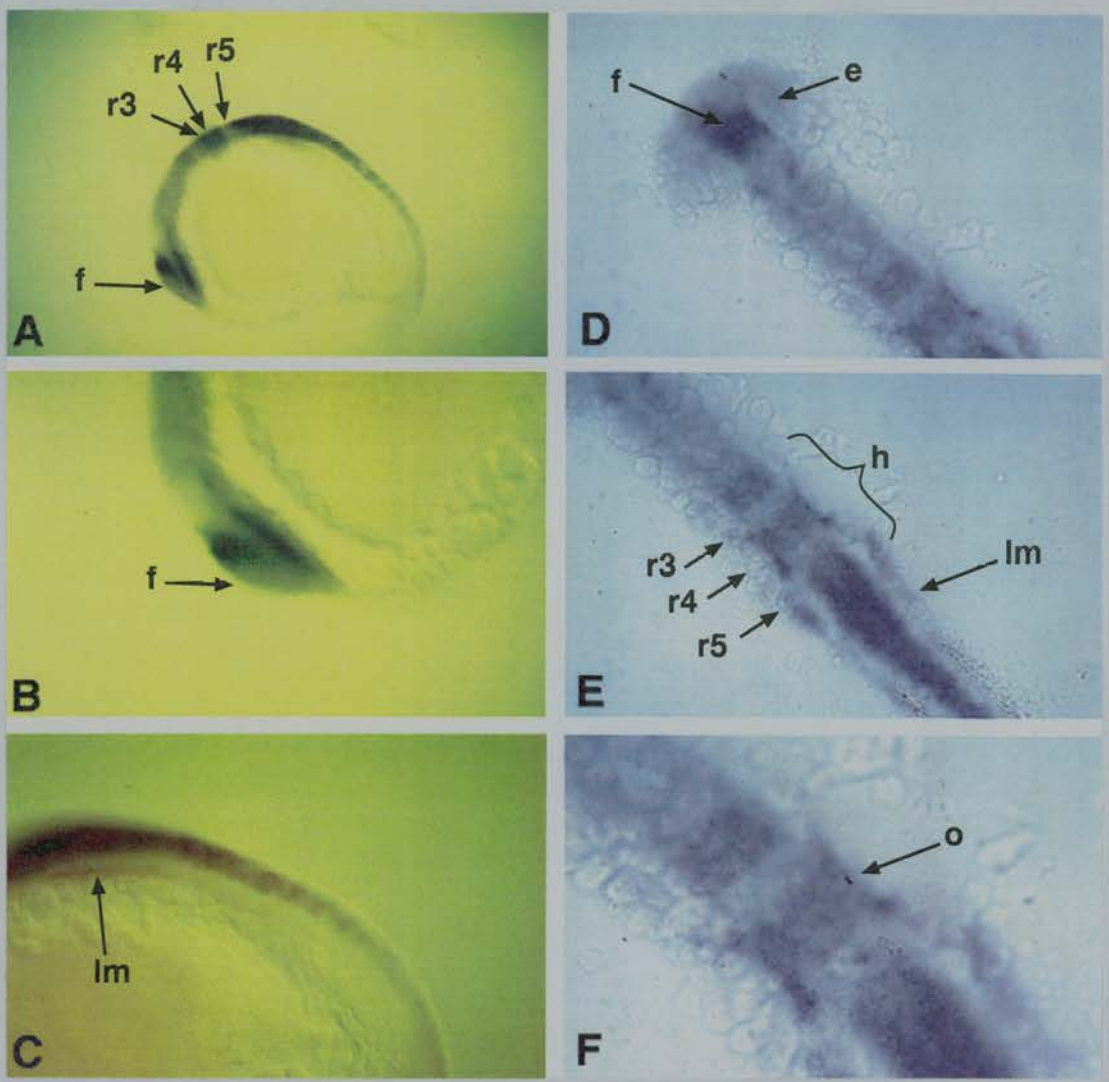


Figure 4.5 *zfDachA* expression in 20 somite zebrafish embryos.
Expression is seen in the forebrain (**f**), eye (**e**), otic vesicle (**o**), lateral mesoderm (**lm**), hindbrain (**h**) and in the rhombomeres of the hindbrain excluding rhombomeres 3 and 5 (**r3 and 5**).
A-C) side view (dorsal to top, anterior to left)
D-F) dorsal view (anterior to top left)

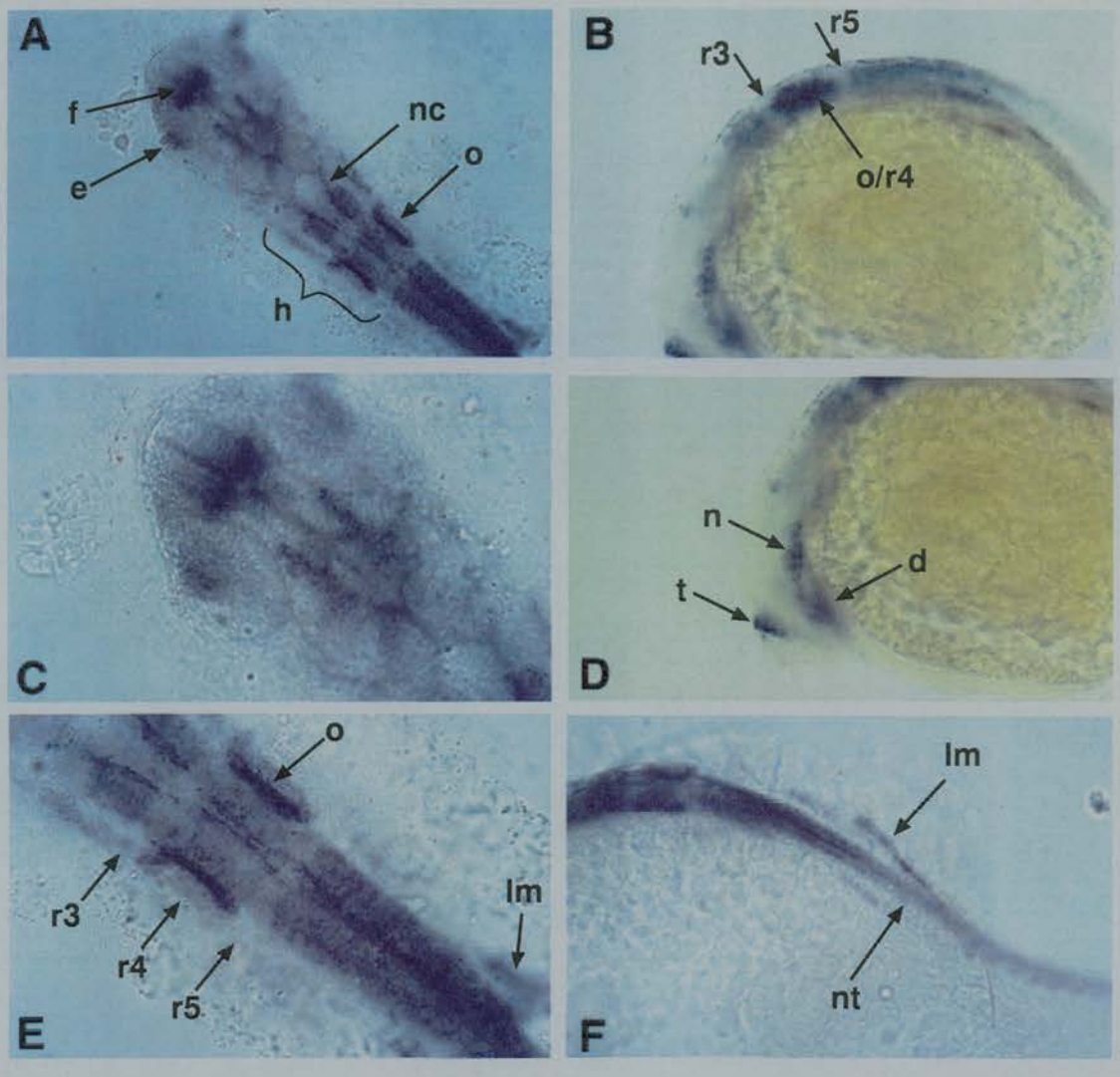


Figure 4.6 *zfDachA* expression in 24hpf zebrafish embryos.
Expression is seen in the eye (**e**), neural crest (**nc**), otic vesicle (**o**), lateral mesoderm (**lm**), neural tube (**nt**) and specific neurones (**n**). Expression in the brain is detected in the telencephalon (**t**) and diencephalon (**d**) of the forebrain (**f**) and in the hindbrain (**h**) specifically excluded from rhombomeres 3 and 5 (**r3,5**)
A,C,E) Dorsal view (anterior to top left)
B,D,F) Side view (dorsal to top, anterior to left)

Figure 4.6b *zfDachA* expression in the zebrafish eye.
Transverse 10μm wax sections.
A) 24hpf embryo, expression is detected in the outer lens (**L**)
B) 48hpf embryo, expression is detected in the ganglion cell layer (**gc**)
C) 48hpf embryo with H&E counterstaining, showing expression in the ganglion cell layer (*gc*) but not within the lens (**L**).

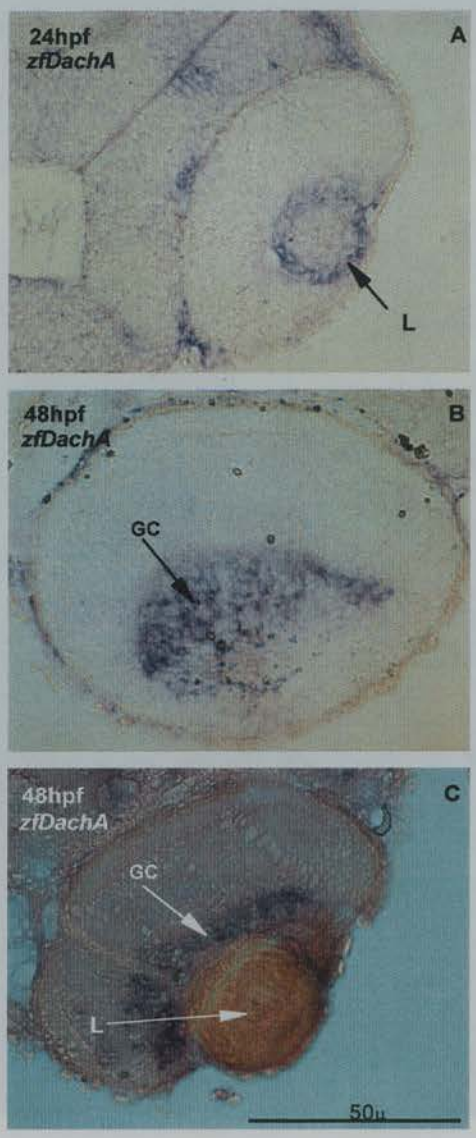

24hpf
zfDachA
A
L
48hpf
zfDachA
B
GC
48hpf
zfDachA
C
GC
L
50u

Figure 4.7 *zfDachA* expression in 48hpf zebrafish embryos.
Expression is seen in the hindbrain (**h**), otic vesicle (**o**), ganglion cell layer (**gc**) of the eye (**e**) but not in the lens (**l**); in localised, scattered surface regions of the anterior head , probably representing melanocytes (**m**) and in two anterior regions believed to represent olfactory placodes (**ol**).
A-F, H) Dorsal view (anterior to top)
G,I) Side view (Dorsal left, anterior to top)

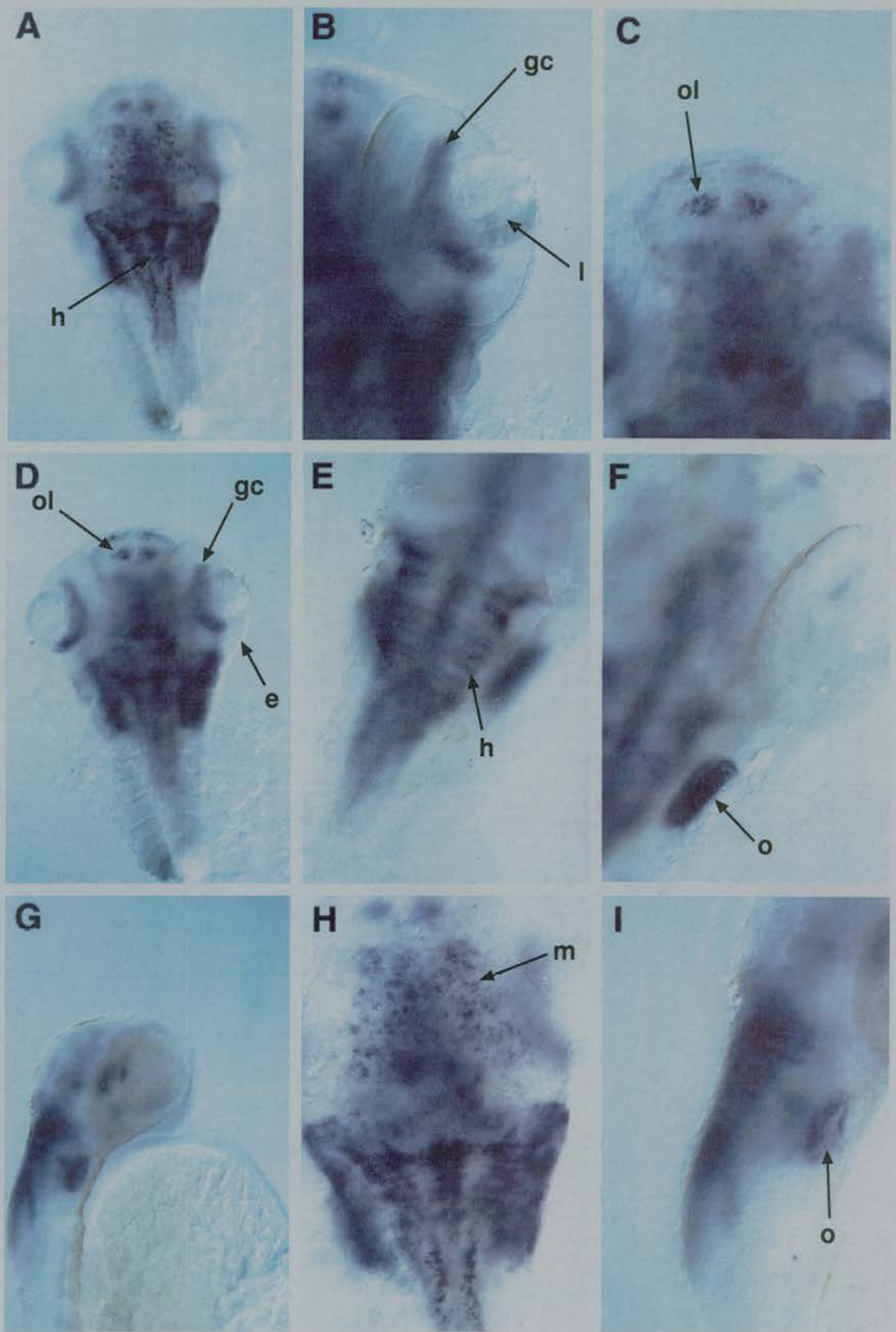


Figure 4.7 *zfDachA* expression. 48 hours post fertilisation.
ol : olfactory region, h : hindbrain, o : otic vesicle, gc : ganglion cell layer,
e : eye, l : lens, m : melanocytes

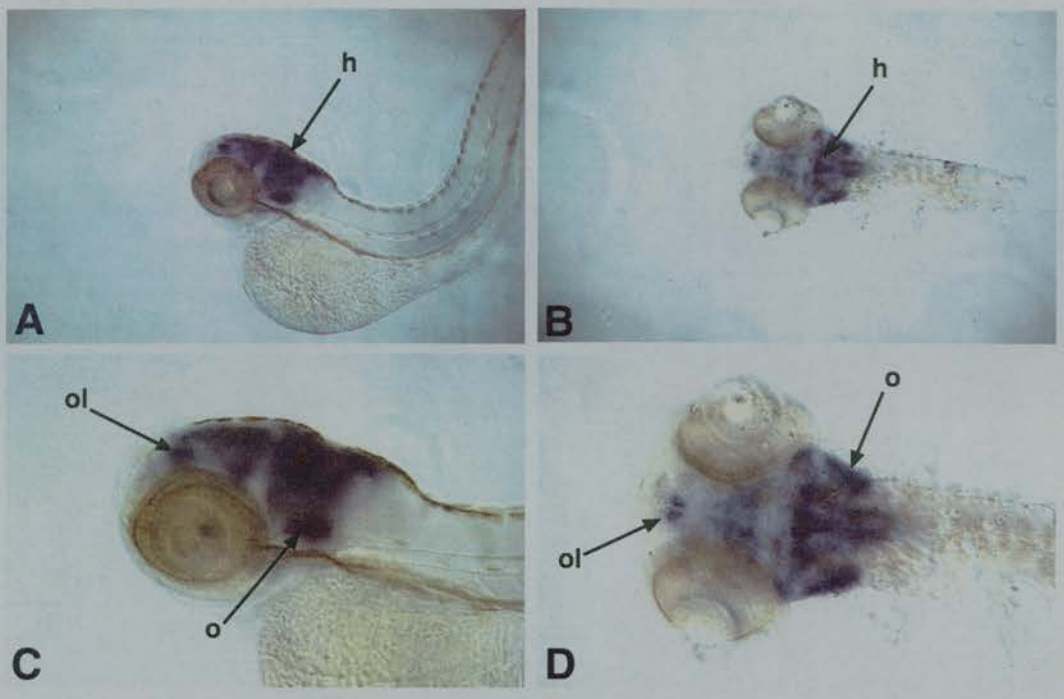


Figure 4.8 *zfDachA* expression in 72hpf zebrafish embryos
Expression is seen in the hindbrain (**h**) , otic vesicle (o) and the olfactory region (**ol**)
A,C) Side view (Dorsal to top, anterior to left)
B,D) Dorsal view (anterior to left)

At 14 somites (figure 4.4) expression is seen in the forebrain and developing eye, in the hindbrain, the neural keel, the lateral mesoderm and in the anterior portion of the five anterior most somites. At 16 somites expression is generally similar to that found at 14 somites although the somite expression has been lost. A rhombomere specific pattern is also beginning to appear in the hindbrain; expression is specifically excluded from rhombomeres three and five. Expression also becomes obvious in the otic vesicle at this point. This pattern of hindbrain and otic vesicle expression is maintained at 18 and 20 somites (figure 4.5) and at 24 hours (figure 4.6).

At 20 somites, in addition to the rhombomere specific hindbrain expression, *zfDachA* remains detectable in the otic vesicle, the eye region, the neural keel and in the lateral mesoderm. In the forebrain distinct areas of expression can be identified, believed to be the caudal telencephalon and the diencephalon. The expression pattern is very similar at 24hpf (figure 4.6) but expression in more specific regions of the brain can by now be identified. Specifically *zfDachA* is present in the caudal telencephalon, in the diencephalon and in a row of neurones just posterior to this. In the eye expression is confined to the outer layer of the developing lens (figure 4.6b). Expression is also found in the neural crest cells.

By 48hours (figure 4.7) expression is no longer seen throughout the length of the neural tube and the rhombomere specific expression seen at earlier stages is no longer obvious, instead *zfDachA* appears to be expressed in all rhombomeres of the hindbrain. Strong expression remains detectable in the otic vesicle (figures 4.7) and in the forebrain. Two strong regions of expression at the anterior of the embryo may correspond to the nasal placodes and other localised regions of expression within the forebrain are likely to correspond to ganglia. Surface expression within the head which may correspond to developing melanocytes is also detected. Striking expression is also seen in a region of the retina adjacent to the lens which corresponds to the ganglion cell layer (figures 4.7 and 4.6b). At this stage no expression was observed in the lens.

At 72hours (figure 4.8) expression remains in the forebrain and hindbrain but in a more restricted pattern. Strong expression in the otic vesicle remains detectable.

4.3.2 *zfDachB*

zfDachB expression was analysed at stages from 24hpf to 72hpf. At 24hpf expression is seen in the migrating lateral line primordium (figure 4.9) and in localised, mainly ventral regions of the CNS, many of which represent specific neurones. Two of these are believed to represent epiphyseal neurones and the post-optic commisure. At 48 hours expression is detected in the hindbrain and otic vesicle and a few scattered regions near the surface of the anterior head. Expression within the pharangeal arches is also observed (figure 4.10). By 72 hours no expression was detected. Expression was not detected in the eye at any stage examined.

4.3.3 *zfDachC*

zfDachC expression was analysed from 24hpf to 72hpf. At 24hpf (figure 4.11) expression was seen in the forebrain, in rhombomere three of the hindbrain, in the midbrain/hindbrain boundary region, in the otic vesicle, in the neural crest and in specific neurones including the reticulo-spinals. Strong expression was also detected in two bilateral stripes on either side of the fishes body which represent the pronephric ducts. By 48 hours (figure 4.12) expression remains detectable in the forebrain and hindbrain and in the otic vesicle. Expression is also found in the midbrain and a small region on the ventral anterior surface of the embryo which may represent the mouth. By 72 hours (figure 4.13) a small region of hindbrain expression remained detectable. Expression was not detected in the eye at any stage examined.

4.4 Overexpression analysis of *zfDachA* function.

4.4.1 Introduction / experimental design.

In order to analyse *zfDach* function two experimental approaches were taken. *zfDachA* was chosen for investigation in relation to this study as it is the only *zfDach* gene to be expressed in the eye.

Firstly, gain of function assays were carried out by the overexpression of full length *zfDachA* RNA transcripts in fish embryos. Over-expression studies of this nature do not necessarily produce a phenotype because of restricted competence of cells to respond to the gene product. *In-vivo* degradation of the transcript can often

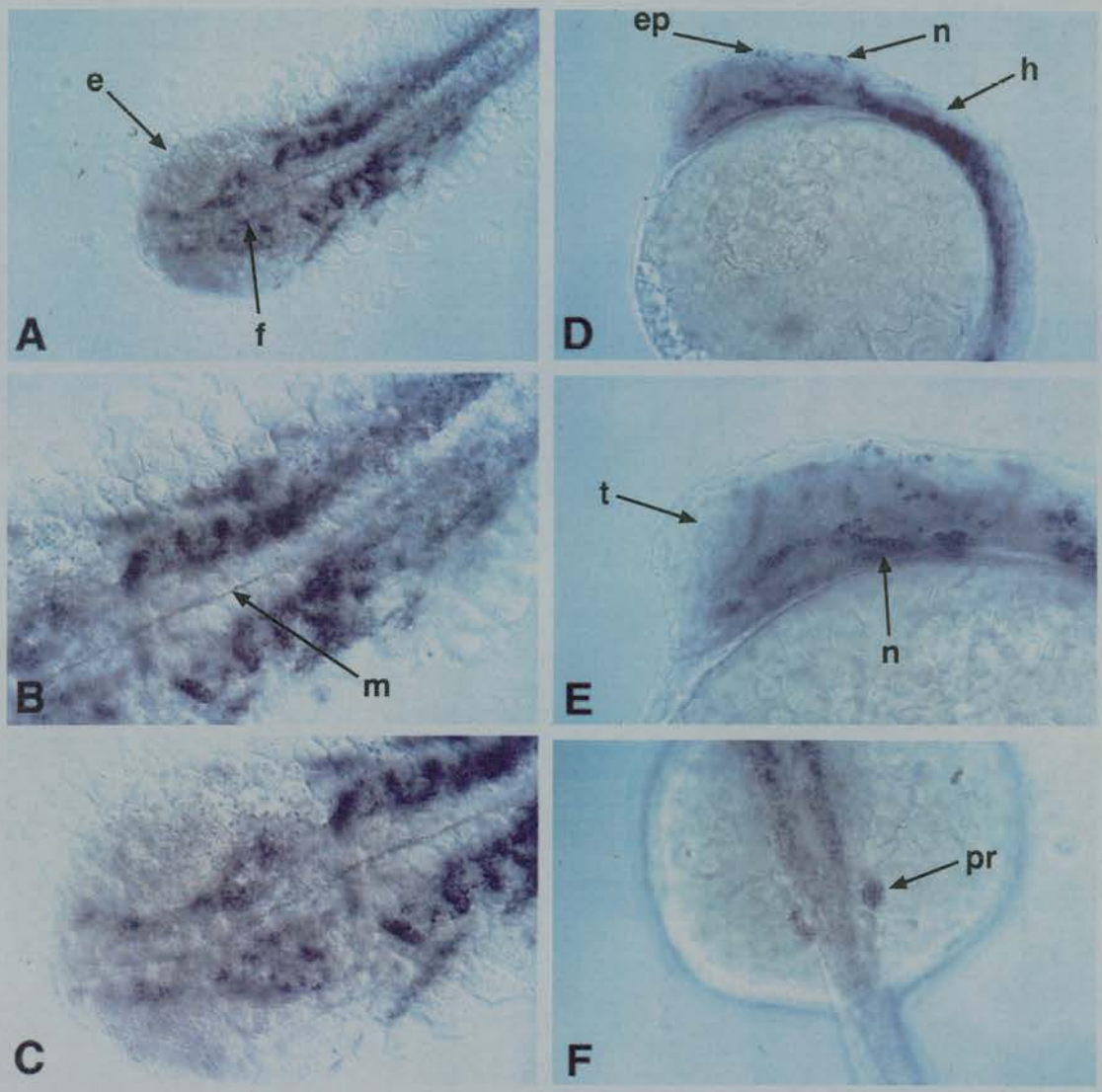


Figure 4.9 *zfDachB* expression in 24hpf zebrafish embryos
Expression is detected in the migrating lateral line primordium (**lp**), in the hindbrain (**h**) and in many localised regions representing individual neurones (**n**). Adjacent to the midline (**m**) expression is not seen.
(**e**) eye, (**f**) forebrain, (**ep**) epiphysis, (**t**) telencephalon.
A-C) Dorsal view (anterior to bottom left)
D-E) Side view (dorsal to top, anterior to left)
F) Dorsal view (anterior to top)

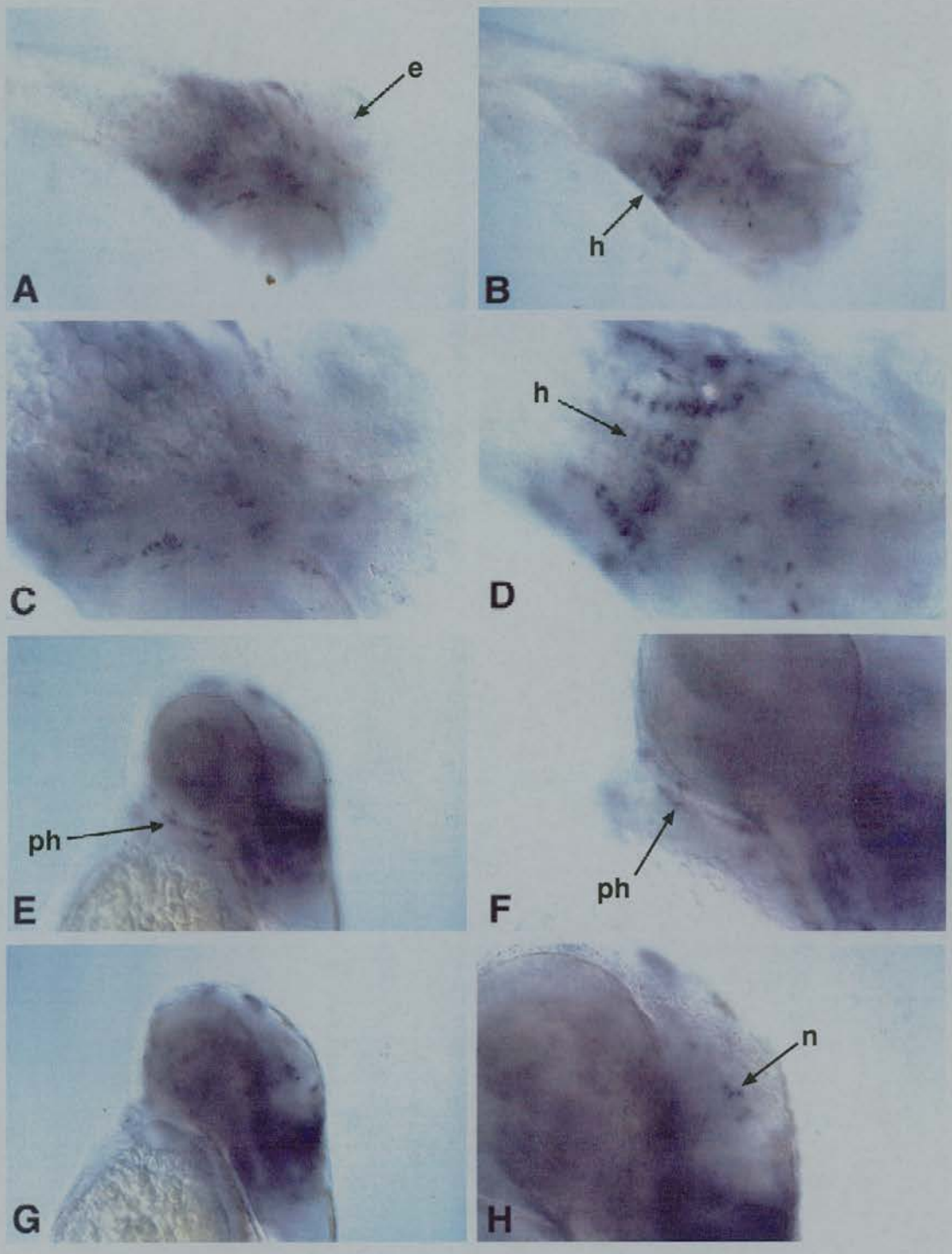


Figure 4.10 *zfDachB* expression in 48hpf zebrafish embryos.
Expression is seen in the hindbrain (**h**), pharangeal arches (**p**), specific neurones (**n**) and in surface regions of the dorsal head.
(**e**) eye.
A-D) Dorsal view (anterior to right)
E-H) Side view (dorsal to top, anterior to left)

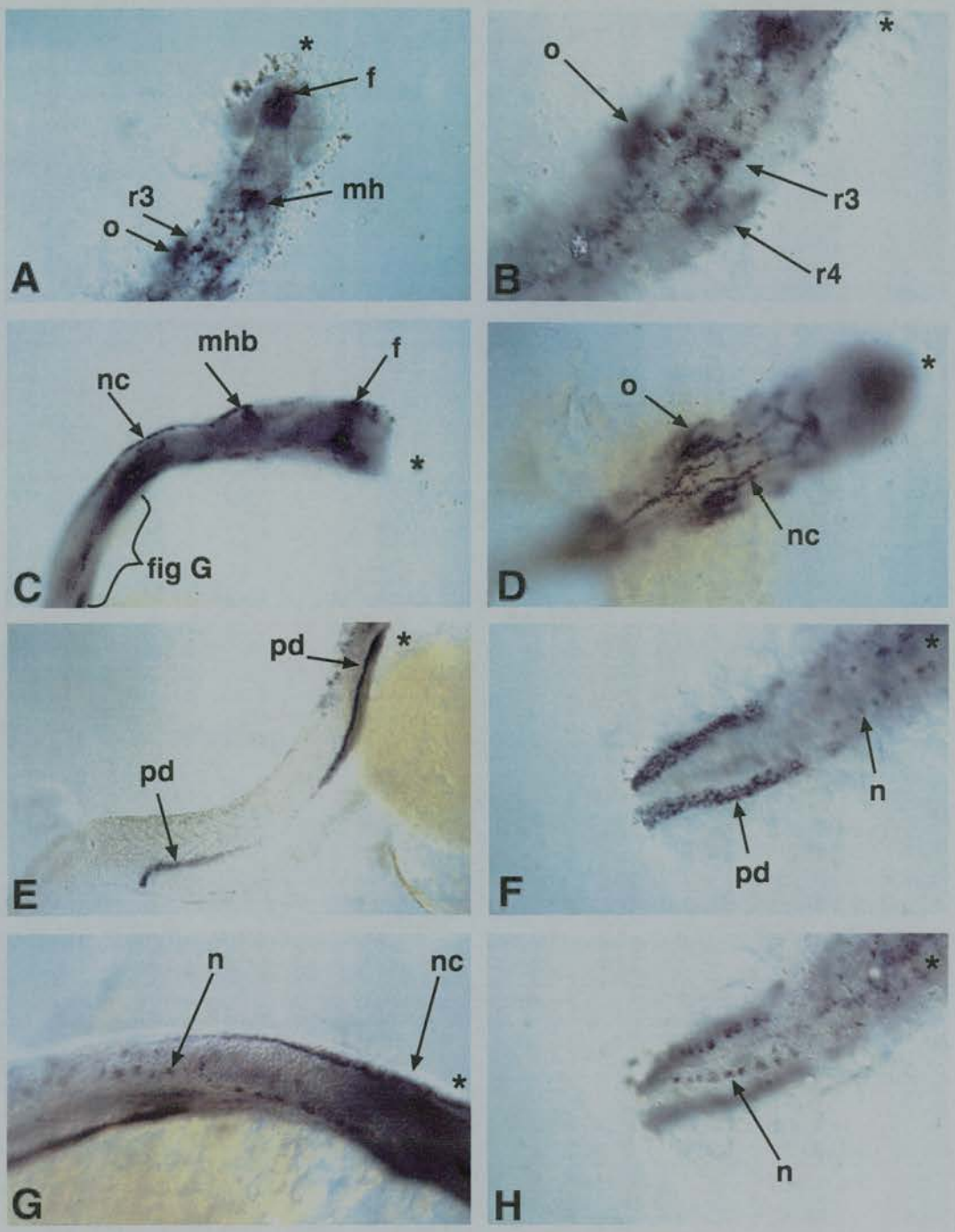


Figure 4.11 *zfDachC* expression in 24hpf wildtype zebrafish embryos.
Anterior is to the right and is marked*
A,B,D,E,H) are dorsal views
C,F,I) are side views (dorsal to top)
zfDachA expression is detected in the forebrain (**f**), midbrain/hindbrain boundary region (**mhb**)(shown in **A** and **C**), rhombomere three (**r3**) of the hindbrain (**A** and at higher magnification in **B**), otic vesicle (**o**) (**A,B** and **D**), neural crest (**D**, a surface view of a similar region to **B**), various neurones (**n**) (**F, G** and **H**) and in the pronephric ducts (**pd**) (**E** and **F**).

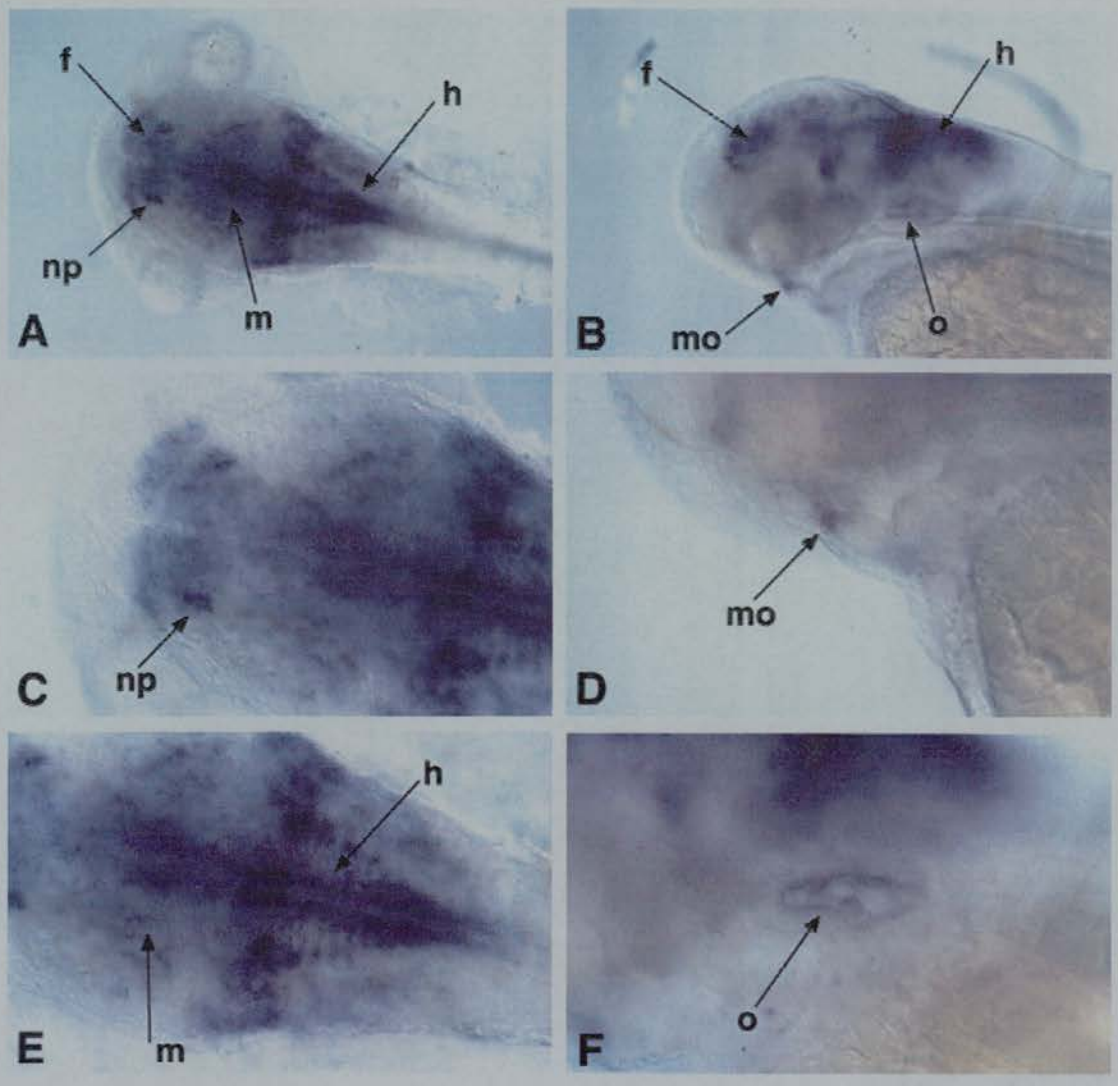


Figure 4.12 ***zfDachC*** **expression in 48hpf wildtype zebrafish embryos.**
Anterior is to left
A,C,E) Dorsal view ; (**B,D,F**) side view (dorsal to top)
C and **E** are higher magnifications of **A**
D and **F** are higher magnifications of **B**
zfDachA expression is seen in the forebrain (**f**), midbrain (**m**), hindbrain (**h**), otic vesicle (**o**), in two bilateral spots probably representing nasal placodes (**np**) and in an anterior ventral region which may be the mouth (**mo**).

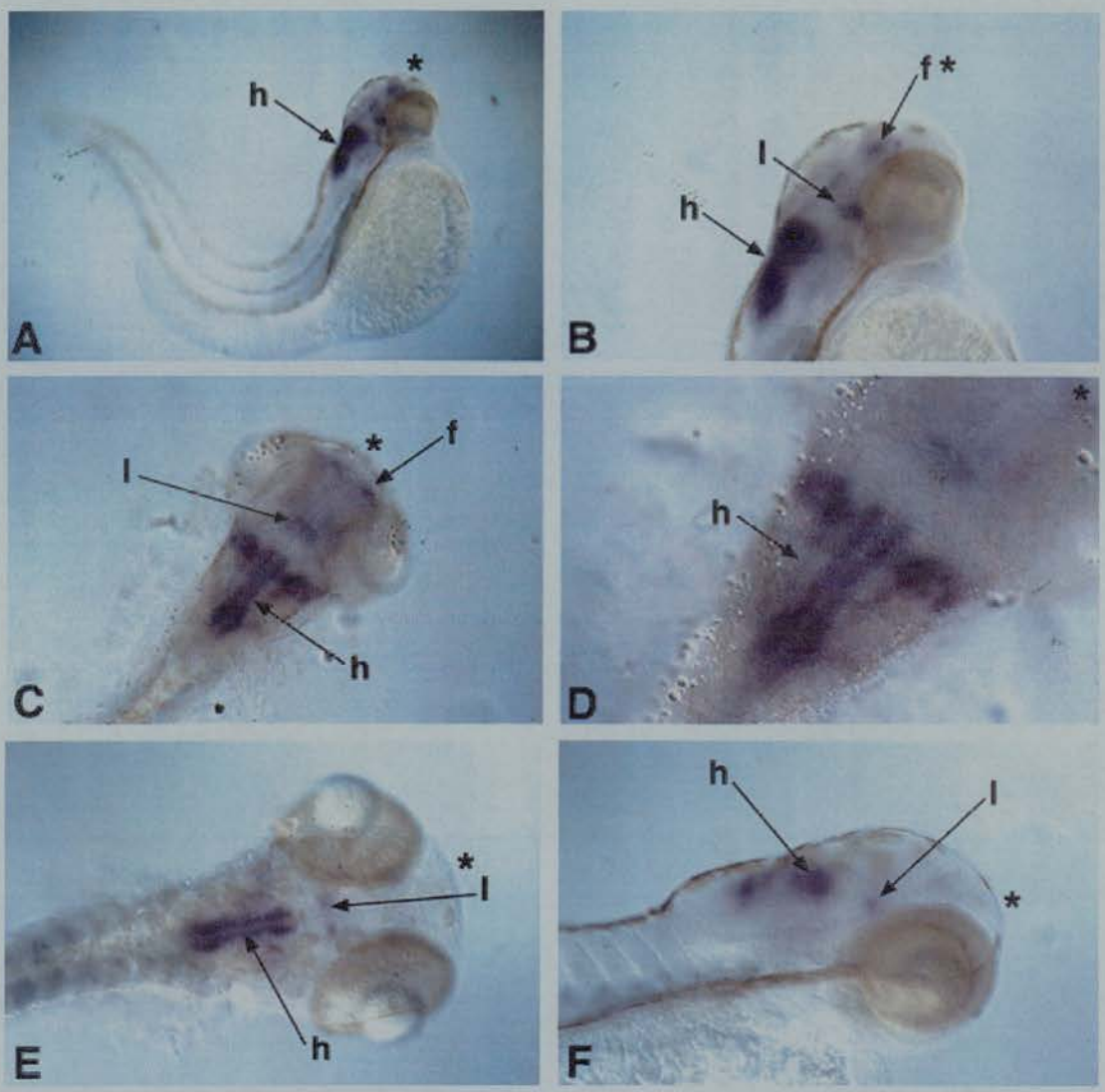


Figure 4.13 *zfDachC* expression in 60hpf [A-D] and 70hpf [E-F] wildtype zebrafish embryos.
Anterior is to right (marked *).
A,B,F) side view (dorsal to top); (**C,D,E**) dorsal view
A - D) at 60hpf *zfDachC* expression is seen in the hindbrain (**h**) in two medial stripes and two lateral regions, in the forebrain (**f**) and in a localised region behind the eye (**l**)
E - F) at 72hpf expression remains in the medial hindbrain (**h**) and a localised region behind the eye (**l**) but is absent from the forebrain.

also be a problem. Alternatively effects may be widespread and non-specific because translation of exogenous RNA is initiated soon after fertilisation, at the midblastula transition. The latter scenario was certainly a possibility in this case since *zfDachA* is expressed in many tissues and might therefore be expected to have widespread effects (section 4.3). If however, *zfDachA*, like *Drosophila dac*, is fundamental to early eye development it might be expected to have a specific effect on development of this organ. *dac* induces ectopic eye formation in *Drosophila* which indicates that in certain situations it is sufficient to determine retinal fate (Mardon et al., 1994). Other *Drosophila* genes also do this, and mammalian homologues of two of these, *Pax6* and *Six3*, can induce ectopic retina formation from the vertebrate brain (Chow et al., 1999; Loosli et al., 1998; Bonini et al., 1997; Halder et al., 1995). Similarly, perhaps *zfDachA* overexpression in the zebrafish might produce ectopic retina. Alternatively the endogenous eye could be affected in some way, perhaps increasing or decreasing in size or exhibiting alterations to specific tissues.

The second experimental approach utilised the injection of a predicted dominant negative construct to interfere with and remove endogenous gene function. *Dachshund* is thought to act as part of a protein complex, interactions occurring through the Dach-box C and Dach-box N regions (Chen et al., 1997; see chapter 3). A construct lacking either Dach-box could therefore interact with only part of the complex. This would presumably result in a non-functional complex which still contains, and so removes from circulation, some or all of the endogenous proteins which are needed to mediate *Dach* function.

A construct was therefore designed which lacks Dach-box C but contains Dach-box N. *v-ski*, a C-terminally truncated form of *Ski* (part of the same superfamily as *Dach*) which also lacks the Dach-box C region, is known to act in a dominant negative manner. *Ski* has repressor activity as part of the histone deacetylase complex. *v-ski* disrupts this complex and so removes repressor activity resulting in cellular transformation (Nomura et al., 1999; Zheng et al., 1997b). By analogy, C-terminally truncated *Dach* would be expected to exhibit dominant negative activity. It is also possible that full length *zfDachA* might sufficiently disrupt complex stoichometry that it would itself act in a dominant negative manner.

Although the truncated *zfDachA* is predicted to have dominant negative activity it could potentially have alternative effects. It might carry out a subset of the normal functions of *zfDachA*. Alternatively, it may have a more potent effect than injection of the full length gene if it can act on downstream targets without the need for additional genes with which it must normally complex.

Suitable controls are necessary to ensure that the effects of injected RNA are specific. Injections were therefore performed using β-gal marker RNA alone, at an equivalent concentration to the RNA used for *zfDachA* injections. This was to ensure that any phenotype produced was not due to mechanical damage or to non-specific effects of the presence of large amounts of RNA. *zfDachB* and/or *zfDachC* could also have been injected to ensure that any effects observed were specific to *zfDachA*. It may of course be interesting to inject these two genes in any case.

4.4.2 Production of injection constructs

Various constructs were prepared for injections. The first consisted of *zfDachA* in Bluescript SK+/- exactly as excised from the cDNA library. This *zfDachA* clone has long 5' and 3'UTRs and includes a polyA tail which help to resist *in-vivo* degradation of the injected transcript. It was therefore not thought necessary to sub-clone this gene into a vector such as pCS2+ which allows the addition of stabilisation sequences, described below, to the transcript.

The second construct consisted of a C-terminal truncation of *zfDachA,* named zfDachA/hr, produced by digestion of *zfDachA* with HindIII (see figure 4.14). This construct terminates at nucleotide 1464 within the open reading frame of the gene so contains no 3'UTR or polyA tail. This transcript would therefore be extremely unstable *in-vivo* so was sub-cloned into pCS2+. This vector contains an SV40 poly-adenylation site 3' to the polylinker, and a 27bp *Xenopus* β-globin sequence 5' to the polylinker, both of which are transcribed with the insert. The addition of these sequences to the transcript has been shown to effectively stabilise the transcript by providing a short 5'UTR and allowing the addition of a polyA tail (Hammerschmidt et al., 1999). The truncated *zfDachA* gene had previously been inserted into the EcoRI and HindIII sites of pBluescript SK (section 4.3), so was now excised with

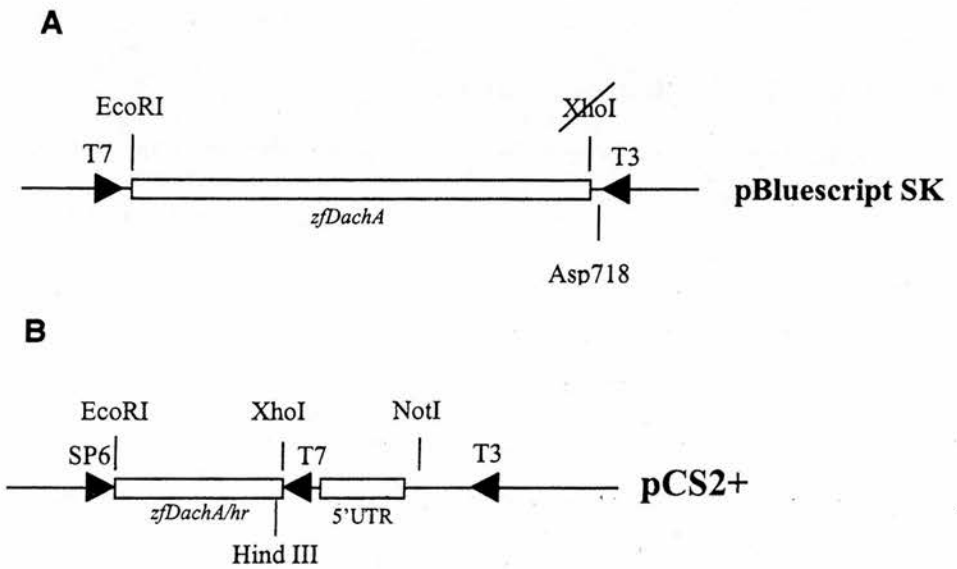


Figure 4.14 Constructs used to produce transcripts for injection
A) The *zfDachA* construct is exactly as excised from a zebrafish cDNA λ-ZAP library (see section 4.2) with the insert cloned into the EcoRI and XhoI sites of pBluescript SK+/-. The XhoI site has however been corrupted at some stage so linearisation was achieved using Asp718. RNA was transcribed with T3 polymerase.
B) *zfDachA*/hr is C-terminally truncated *zfDachA* containing nucleotides 0 to 1464 inserted into the EcoRI and XhoI sites of pCS2+. 3' of the insert is an SV40 polyadenylation signal which is transcribed with the insert, stabilising the transcript. Linearisation was carried out with NotI, sited 3' to both insert and SV40 sequence. RNA was transcribed with SP6 polymerase.
SP6; SP6 promoter, **T3**; T3 promoter, **T7**; T7 promoter, **5'UTR**; SV40 polyadenylation site.

XhoI and EcoRI and sub-cloned into the XhoI and EcoRI sites of pCS2+ (figure 4.14).

When any RNA transcript was prepared, a 7-methyl guanosine cap was incorporated at the 5' end of the transcript to ensure efficient translation (section 6.12).

4.4.3 Injection of full length *zfDachA* RNA

zfDachA transcript was injected into 4 – 16 cell zebrafish embryos. Initially injections were carried out at the 2 cell stage but this caused a high incidence of early death. By injecting at the 4 – 16 cell stage ectopic *zfDachA* expression was more localised, allowing more embryos to develop to 24hpf when analysis was carried out. A β-gal transcript was co-injected with the *zfDachA* RNA to allow the identification of regions containing injected RNA by staining for lacZ.

zfDachA RNA was injected at three concentrations, 30ng/μl, 60ng/μl and 120ng/μl. This corresponds to approximately 60pg, 120pg and 240pg per embryo as 1μl was found to be sufficient for about 500 injections. β-gal RNA was used at 120ng/μl in all cases. Two phenotypes were observed, one affecting the somites and the other the brain. These are described below.

A control experiment was also performed where β-gal RNA alone was injected at 240ng/μl. This was twice the usual β-gal concentration to mimic the total concentration of *zfDachA* RNA plus β-gal RNA when the highest concentration of *zfDachA* RNA was injected. No embryos with either the somite or brain phenotype described below were observed. This indicates that both are caused specifically by *zfDachA* RNA rather than mechanical damage or a high concentration of non-specific RNA.

4.3.3.1 Brain phenotype

Of 172 embryos injected with varying *zfDachA* RNA concentrations (see figure 4.18 and table 4.2) overgrowth or ectopic tissue was observed in the midbrain region of 67. In some cases this was quite dramatic. The severity of the overgrowth varied from dramatic outgrowths into the ventricles (figure 4.15 b and c) to small blips (figure 4.15d). Initially it was thought possible that this ectopic tissue could be

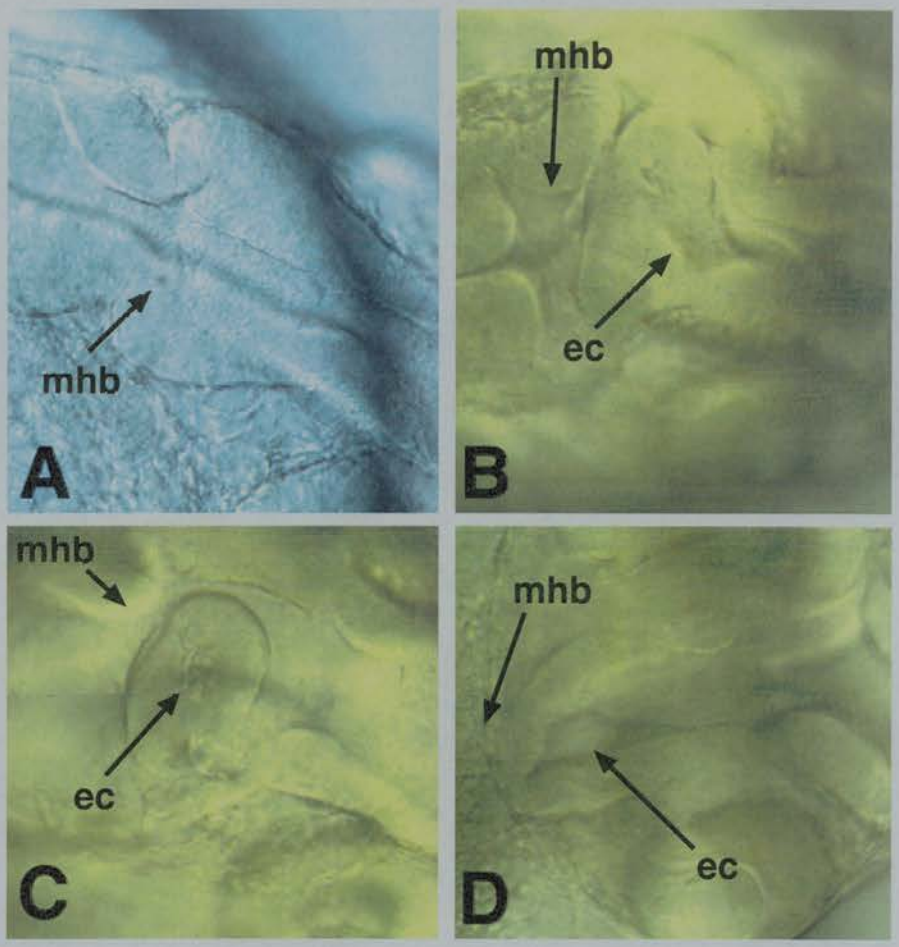


Figure 4.15 Ectopic midbrain tissue caused by overexpression of *zfDachA* in zebrafish embryos by RNA injection.
Anterior is to the right; all views are dorsal
A) Wildtype 24hpf midbrain/hindbrain boundary region.
B/C) Two separate *zfDachA* injected 24hpf embryos showing severe outgrowth of the ventricle walls in the midbrain region.
D) *zfDachA* injected 24hpf embryo showing a small outgrowth of the ventricle wall.
mhb; midbrain/hindbrain boundary, **ec**; ectopic tissue

of ocular origin as superficially some outgrowths resembled retinal tissue. For this reason a series of eye and brain markers, *Rx1*, *Pax6* and *eng3* were used to further characterise the phenotype.

Rx1 is a specific marker of retinal tissue at 24hpf (Mathers et al., 1997). In *Six3* overexpression assays *Rx2* was used to show the presence of ectopic retinal tissue in the brain (Loosli et al., 1998) however there should be little difference between *Rx1* and *Rx2* as retinal markers as they are expressed in the same way at 24hpf.

Pax6 was used as a second marker of eye fate and also as a brain marker. At 24hpf *Pax6* is expressed in the diencephalon, telencephalon and hindbrain (Krauss et al., 1991; Nornes et al., 1998).

eng3 is a specific marker of the midbrain-hindbrain boundary region at 24hpf (Ekker et al., 1992). All three *engrailed (eng)* genes are expressed in the midbrain-hindbrain region at 24hpf but *eng3* is expressed in the widest area so was selected for this study.

Marker expression in midbrain outgrowths

Rx1 expression was not detected in ectopic midbrain tissue in any case (10 embryos were stained for *Rx1*) although expression was detected in the endogenous retina. This suggests that these brain outgrowths are extremely unlikely to represent ectopic retina.

Pax6 was used as a marker of forebrain and hindbrain regions and also to confirm that the ectopic midbrain tissue was not of retinal origin. It was however unlikely that *Pax6* would be detected in ectopic retina in the absence of *Rx1* expression as *Rx1* is found at 11hpf, one hour earlier than reported *Pax6* expression (Mathers et al., 1999; Krauss et al., 1991; Nornes et al., 1998) In addition, in *Six3* overexpression studies (Loosli et al., 1998) *Pax6* was found in a smaller proportion of ectopic eyes than *Rx2*.

Pax6 expression appeared normal in all nine embryos examined. The midbrain overgrowth, which is outside the normal *Pax6* expression domain, did not express *Pax6*. This suggests that it has not adopted either forebrain of hindbrain fate.

Eng3 expression was seen in the midbrain/hindbrain boundary region as normal in all four cases examined. Regions of ectopic tissue near to the midbrain/hindbrain boundary expressed *eng3* although overgrowth anterior to this did not (Figure 4.17). This suggests that the ectopic tissue is overgrowth of the midbrain region which has not changed its fate in any way.

4.4.3.2 Somite phenotype

At a gross morphological level some embryos were observed to be bent in one or more places. The more severely affected embryos were also shortened in an anterior-posterior direction. On closer inspection the somites were seen to have lost their characteristic chevron shape. Out of 172 embryos injected with *zfDachA,* 84 had somites which were abnormal to some degree. In the most severe cases the somites appeared block like and irregular while in less severely affected embryos they were U-shaped (figure 4.16). In the less affected embryos many somites had normal morphology while a few, generally in the region of the bend, were abnormal. In the more severely affected, shortened embryos all somites tended to be affected to some degree. The notochord was present in these animals and appeared normal as far as could be ascertained using Normarski optics.

4.4.3.3 Correlations and further phenotypes

The embryos with the most severe somite defects generally also had brain defects. Many of these embryos were so badly affected however, that necrosis was beginning to occur and it was possible that the brain defects were secondary to this. In the less severely affected embryos both the brain and somite phenotypes were observed independently indicating that one phenotype is not secondary to the other.

It is also possible that other phenotypes induced by injections of *zfDachA* RNA were present but not detected. Hindbrain defects for instance may have been difficult to spot without the aid of markers of rhombomere identity such as *Krox20.* At the gross morphological level no eye phenotype was observed but again subtle changes affecting perhaps a single layer of the retina would be unlikely to be detected without markers and histological examination.

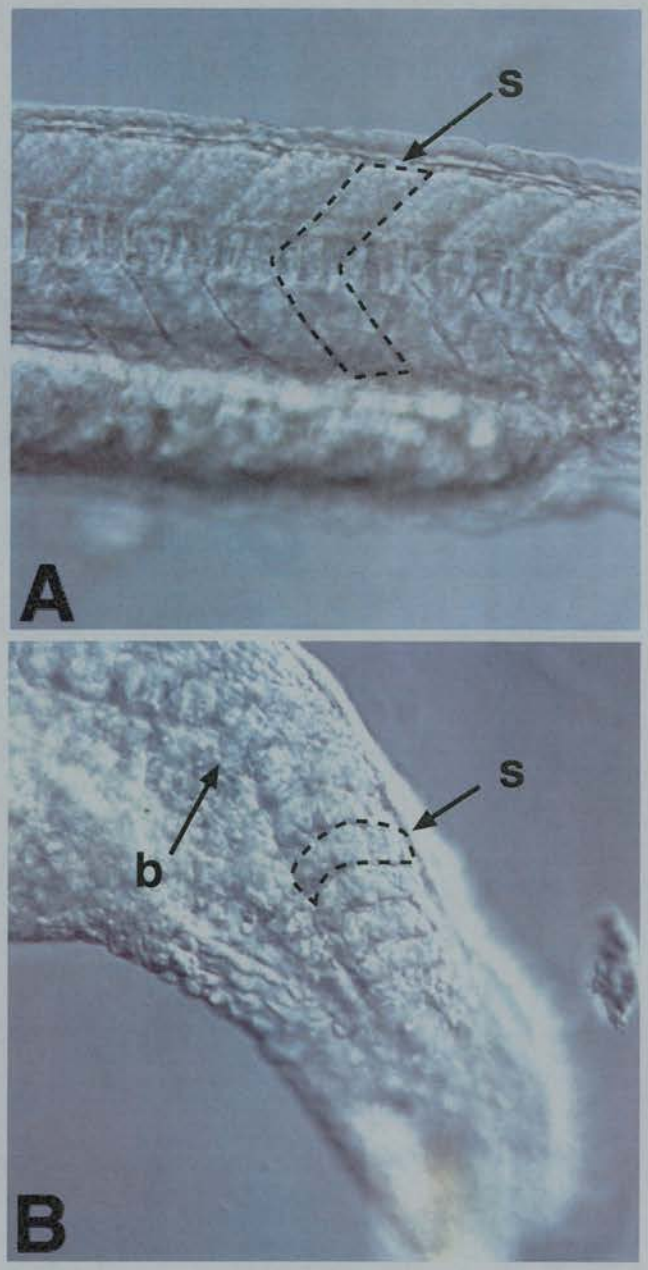


Figure 4.16 Somites lose their chevron shape on overexpression of *zfDachA* in zebrafish embryos by RNA injection.
Anterior is to the left; both are side views
region enclosed by dotted line represents a single somite
A) somites (**s**) in a wildtype 24hpf embryo showing a clear chevron shape.
B) somites (**s**) in a *zfDachA* injected embryo. The tail is bent (**b**) and shortened in an anterior-posterior direction. Somites have lost their characteristic chevron shape.

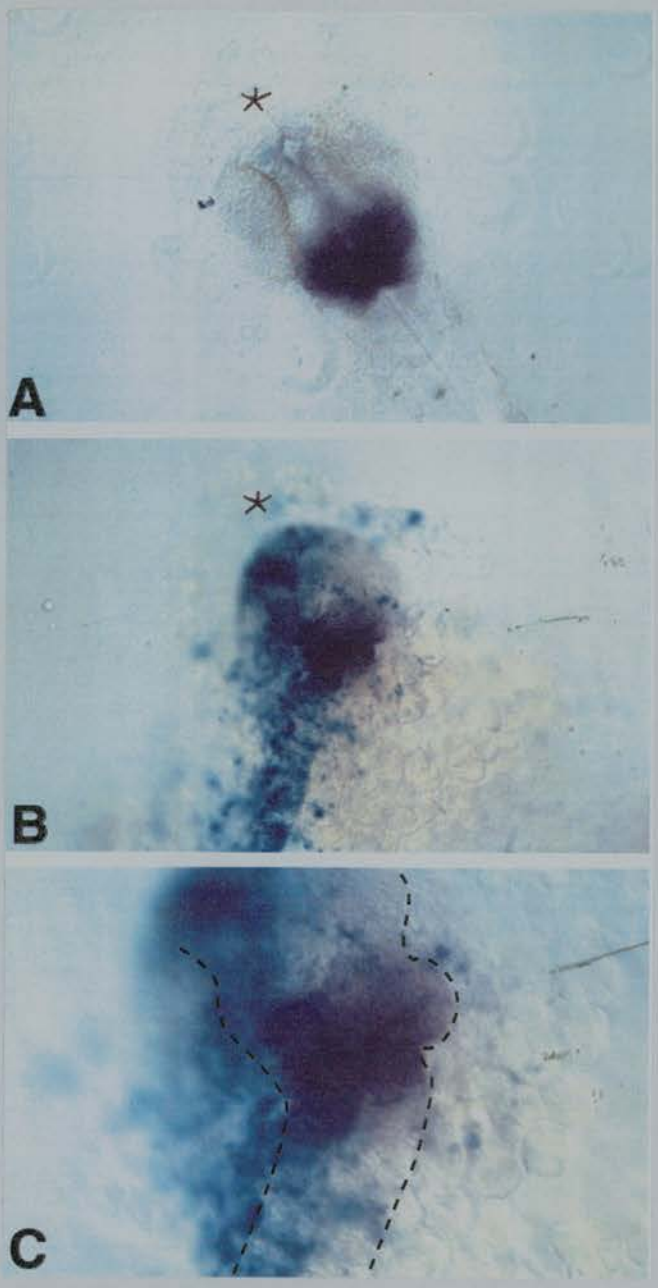


Figure 4.17 ***Engrailed 3 (Eng3)*** **expression in the midbrain/hindbrain region of 24hpf zebrafish embryos.**
Anterior is to the top of the figures marked * in **A** and **B**
Dotted lines show the extent of the brain tissue.
A) Wildtype 24hpf embryo showing expression at the midbrain/hindbrain boundary
B) 24hpf embryo in which *zfDachA* has been overexpressed by RNA injection. *Eng3* expression is seen within the midbrain/hindbrain region as normal and overgrown tissue which falls within this region expresses *Eng3*.
C) Higher magnification of figure (**B**) to show midbrain/hindbrain boundary region

4.4.3.4 Concentration effects : on the somite phenotype

The data shown in table 4.1 were collected from two separate experiments and the somite phenotype classified according to the scale shown in the table 4.1 legend. In the first experiment, three concentrations were injected (blue in figure 4.18a) and in the second, two (orange in figure 4.18a). In the second experiment a fungal infection occurred, which may have affected the data obtained as many embryos were extremely necrotic when analysed. These were all counted as a severe phenotype although the phenotype may not actually have been a specific somite one. This would account for the large excess in the severe category. If however, each experiment is analysed separately, it appears that RNA concentration within the range used makes little difference to the proportion of each phenotype produced.

4.4.3.5 Concentration effects : on the brain phenotype

Similar analysis was carried out for the brain phenotype (data in table 4.2 and figure 4.18b). Here the phenotype was split into 3 categories as detailed in the table 4.2 legend. The data shown are from the two experiments described above. No definite conclusions can be drawn from this data, in part because it was not always obvious whether the brain phenotype in severely abnormal embryos was specific or a secondary effect of gross morphological defects.

4.4.4 Injection of zfDachA/hr, a C-terminally truncated *zfDachA* RNA

C-terminally truncated *zfDachA* was injected into 4-16 cell zebrafish embryos at two concentrations, 60ng/µl and 120 ng/µl. β-gal RNA was co-injected at 120ng/µl. A similar somite phenotype to that seen with full length *zfDachA* RNA was observed, although this was much less severe. No specific brain phenotype was detected and no phenotypes were seen in zfDachA/hr injected embryos which were not observed in *zfDachA* injections. The embryos obtained were categorised for somite phenotype in the same way as for *zfDachA* injections (see table 4.2 legend). The data obtained are shown in table 4.3 and figure 4.19

Table 4.1 : *zfDachA* injections: Somite phenotype.
The data were spit into five categories according to the following scale:
Wildtype: completely normal with respect to somites (Brain defects in the absence of a somite phenotype were placed in this category.)
Mild: slight kinks in tail, not shortened, many normal somites.
Medium: major bends in tail, may have shortened appearance, some somites may be block like.
Severe: very severely bent, shortened, block like irregular somites, often necrotic, head structures may be reduced, may be small in size, generally morphologically highly abnormal.
Dead
If there was any doubt as to which category an embryo should be placed in it was counted in the less severe category.
When percentages were calculated dead embryos were not included as this figure can vary widely for many reasons other than effects of injected RNA

	ExperimentA [120ng]	Experiment A [60ng]	Experiment A [30ng]	Experiment B [60ng]	Experiment B [30ng]
Wildtype	11 (50%)	11 (55%)	7 (38.9%)	1 (3.6%)	2 (7.1%)
Mild	4 (18.2%)	2 (10%)	4 (22.2%)	2 (7.1%)	1 (3.6%)
Medium	0 (0%)	1 (5%)	1 (5.6%)	4 (14.3%)	2 (7.1%)
Severe	7 (31.8%)	6 (30%)	6 (33.3%)	21 (75%)	23 (82.2%)
Dead	4	10	7	22	13
Total	**26**	**30**	**25**	**50**	**41**

Table 4.2 : *zfDachA* injections : Brain phenotype
The data were split into three categories according to the following scale.
Wildtype: wildtype with respect to brain (somite phenotype with no brain phenotype was included)
Brain: A specific brain phenotype (overgrowth or irregular appearance in the midbrain region).
Brain + severe: A definite brain phenotype but accompanied by other severe abnormalities.
Dead embryos were not included in percentage calculations

	Experiment A [120ng]	ExperimentA [60ng]	Experiment A [30ng]	Experiment B [60ng]	Experiment B [30ng]
Wildtype	12 (54.5%)	11 (55%)	10 (55.5%)	6 (21.4%)	10 (35.7%)
Brain	4 (18.2%)	6 (30%)	1 (5.6%)	8 (28.6%)	3 (10.7%)
Brain + severe	6 (27.3%)	3 (15%)	7 (38.9%)	14 (50%)	15 (53.6%)
Dead	4	10	7	22	13
Total	**26**	**30**	**25**	**50**	**41**

Figure 4.18 (a) : *zfDachA* injections (somite phenotype)

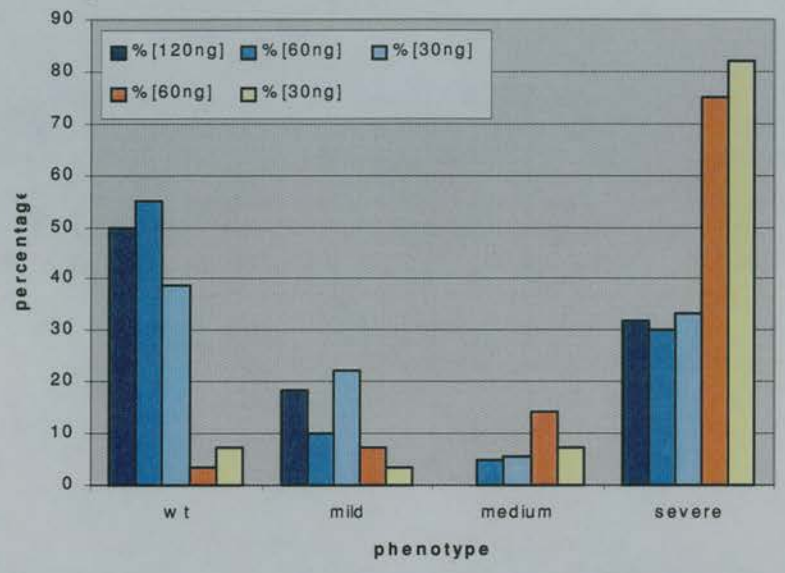


Figure 4.18 (b) : *zfDachA* injections (brain phenotype)

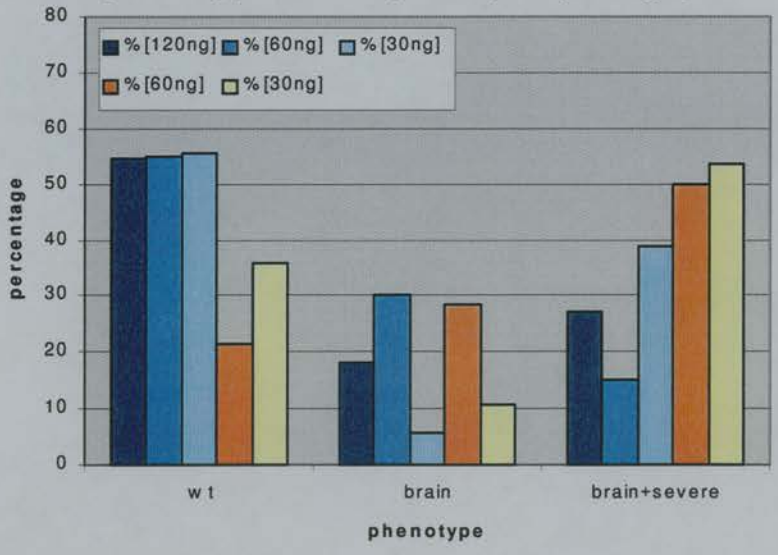


Figure 19 : zfDachA/hr (C-terminally truncated zfDachA) injections

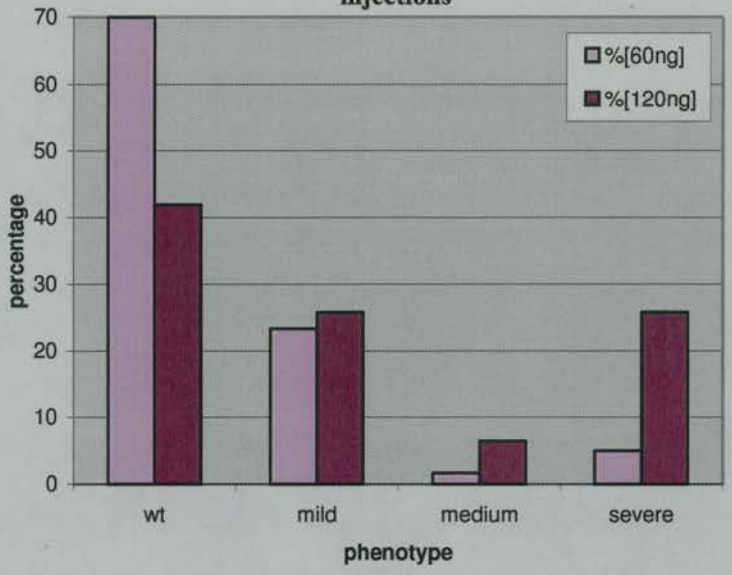


Table 4.3 : zfDachA/hr (C-terminally truncated *zfDachA*) injections; somite phenotype.

The data were classified into five categories using the scale shown in the table 4.1 legend. Dead embryos were not included in percentage calculations

RNA Concentration	**[60ng]**	**[120ng]**
Wildtype	42 (70%)	13 (41.9%)
Mild	14 (23.3%)	8 (25.8%)
Medium	1 (1.7%)	2 (6.5%)
Severe	3 (5%)	8 (25.8%)
Dead	28	25
Total	**88**	**56**

In this case it seems that the higher concentration of zfDachA/hr does cause a more extreme somite phenotype, producing a higher percentage of embryos in every phenotypic category except wildtype (figure 4.19).

4.5 Discussion

4.5.1 *zfDachA, zfDachB* and *zfDachC* are homologues of *Drosophila dac* and mammalian *Dachshund* genes

zfDachA, zfDachB and *zfDachC* share two highly conserved domains, Dach-box N and Dach-box C, with *Drosphila dac* and mammalian *Dach* (figure 3.3). The fish genes also have high similarity with *Dach1* at the amino acid level throughout their length; 66.5%, 56.8% and 76.3% identity for *zfDachA*, *zfDachB* and *zfDachC* respectively. All three zebrafish open reading frames, like *Dach1* and *DACH1*, begin with the amino acids methionine and alanine, finish with a tyrosine residue, and share a further highly conserved domain, Dach-box M. Taken together this evidence indicates that *zfDachA*, *zfDachB* and *zfDachC* are members of the *Dachshund* family.

4.5.2 *zfDachA* and *zfDachC* may be homologous to *Dach1* while *zfDachB* is the homologue of a further murine *Dach* gene

Evidence from phylogenetic analysis

Only one mammalian *Dach* gene, *Dach1*, has been reported (Hammond et al., 1998; Caubit et al., 1999; Davis et al., 1999; Kozmik et al., 1999) although two are known in the chick (Tabin et al., 1999). A greater number of fish genes have been isolated but this is not an unusual situation as increased duplication appears to have occurred in the zebrafish lineage compared to that of the mammals, resulting in an increased level of genetic redundancy (Postlethwait et al., 1999; Detrich et al., 1999). It is of course possible that as yet unreported mammalian and zebrafish *Dachshund* genes exist.

Phylogenetic analysis reveals that *zfDachC* is the fish gene most similar to *Dach1* while *zfDachB* is the most distant. *zfDachA* is a roughly equal evolutionary distance between *zfDachB* and *zfDachC*. Based on this and expression data discussed below, *zfDachA* and *zfDachC* are likely to be related to *Dach1* while *zfDachB* is

different and may be related to a second mammalian *Dach* gene. Without further investigation of the mammalian and fish *Dach* gene families firm conclusions cannot be drawn. It is of course possible that the three fish genes are related to separate mammalian genes or to only one.

Evidence from expression patterns

zfDachA and *zfDachC* are both expressed in the neural crest, the fore and hind brain, the otic vesicle and in discrete regions of the CNS which represent specific neurons and ganglia. *zfDachC* is also found in the pronephric ducts, while *zfDachA* is found in the eye, the nasal region, the neural tube, branchial arches and the lateral mesoderm. Equivalent regions express *Dach1* in the mouse (Hammond et al., 1998; Caubit et al., 1999; Davis et al., 1999; Kozmik et al., 1999) *zfDachB* however, is found mainly in discrete ventral regions of the CNS while mouse *Dach1* seems to be more dorsal. The most obvious region of *zfDachB* expression is within the lateral line primordia which has no homologous structure in the mouse. *zfDachB* expression is however, found in the pharangeal arches where *Dach1* expression is also detected.

Taking both phylogenetic analysis and the known expression patterns into consideration *zfDachA* and *zfDachC* are likely to be related to *Dach1*. *zfDachC* is the most similar at the sequence level and has significant similarity to *Dach1* at the expression level. *zfDachA* is sufficiently similar to *Dach1* at the expression level to be considered an equivalent although from the sequence alone some doubt would arise. *zfDachB* is sufficiently different from *Dach1* at both the expression and sequence levels to make it likely that this gene is related to a further mammalian *Dachshund* gene, although it could be a third divergent relative of *Dach1*.

4.5.3 An eye function for zebrafish *Dachshund* genes?

zfDachA was detected in the developing eye region of zebrafish embryos from eight somites onwards. By 24hpf expression was found specifically in the outer layer of the lens and by 48hpf specifically in the inner layer of the retina, which corresponds to the ganglion cell layer. In the mouse, *Dach1* is detectable throughout the retina at the earliest stages of development, later being expressed more strongly in the layer of the neuroretina closest to the lens (Hammond et al., 1998 and this thesis;

Caubit et al., 1999) and in the anterior lens epithelium (Davis et al., 1999). The fish expression pattern is therefore similar but not identical.

Early in development from 12hpf onwards *Pax6* is seen in the optic primordia. At 24hpf *Pax6* is expressed in all layers of the developing fish eye and in the anterior three quarters of the optic stalk; later, in one week old fish it is localised to the ganglion cell layer and to the bipolar cell layer (Krauss et al., 1991). Zebrafish *Six3* and *Six6* are both expressed in the optic primordia early in development, *Six3* turning off by 14 somites. *Six6* continues to be expressed in lens and retina until about 20 somites and by 24hpf is present mainly in the ventral retina. Staining was also detected in the optic stalks. This pattern remains at 48hpf (Seo et al., 1998).

zfDachA expression therefore overlaps with, but is not identical to, either *Pax6* or *Six3/6* expression. At 48 hours the ganglion cell layer expression is similar to that of *Pax6* and overlaps with *Six6*. *zfDachA* could therefore interact with either of these genes in this area. It is however the very early functions of these genes which are of particular interest. All three are present from an early stage in optic primordia formation and so could potentially function in specifying eye fate.

Six3 and *Pax6* can produce ectopic eye structures when overexpressed in medaka and *Xenopus* respectively (Loosli et al., 1998; Chow et al., 1999) When *zfDachA* was ectopically expressed in the zebrafish, ectopic tissue formed in the midbrain which superficially resembled retinal tissue. This tissue did not express *Rx1* or *Pax6* however, so is unlikely to represent ocular tissue. This does not necessarily mean that *zfDachA* is not needed for eye specification although it perhaps indicates that it is not sufficient.

No gross morphological phenotype was observed in the endogenous eye when *zfDachA* was injected but subtle phenotypes such as alterations in a single retinal layer would not necessarily have been identified. This therefore requires further characterisation including the use of markers and histological techniques. Even if no eye phenotype is detected this may be because the cells are not competent to respond to increased *zfDachA* levels. Loss of function analysis will therefore also be important (see section 4.5.7). In addition, because interactions between the retinal fate inducing genes are important in the fly (Chen et al., 1997; Pignoni et al., 1997),

it may be that overexpressing more than one of these potential retinal determination genes together (ie *Six3*, *Pax6* and *Eya* which has not yet been isolated in the fish) will cause an interesting phenotype. Even if *zfDachA* is not sufficient to specify retinal fate it might act synergistically with one or more of the other genes.

4.5.4 Brain functions for zebrafish *Dachshund* genes?

All three zebrafish *Dach* genes are expressed in the brain and CNS and could potentially have important patterning roles. Overexpression of *zfDachA* caused ectopic tissue to form in the midbrain. This tissue is not retinal, as discussed above, and the absence of *Pax6* expression within the overgrowth indicates that it is unlikely to have adopted hindbrain or forebrain fate. At 24hpf *Pax6* is expressed in the diencephalon, part of the telencephalon and throughout the hindbrain of wildtype uninjected embryos.

Eng3, which is expressed at the midbrain/hindbrain boundary, is detected in any regions of ectopic tissue which are within its normal expression domain. This suggests that the overgrowth has retained its midbrain fate. The overgrowth might occur due to *Dach* having a proliferative function as is suggested by *dac* loss of function studies in *Drosophila* and circumstantial evidence in the mouse (Mardon et al., 1994; chapter 3). BrdU (bromodeoxyuridine) labelling, which marks dividing cells, would be useful to indicate whether extra proliferation is in fact occurring.

In addition to the overexpression phenotype a striking pattern of *zfDachA* expression is observed in the hindbrain, specifically being excluded from rhombomeres three and five. This pattern is reminiscent of the *Hox* genes although no reported *Hox* gene has exactly this pattern (Prince et al., 1998). Similarly *zfDachC* is restricted to rhombomere three. Perhaps *zfDachA* and *zfDachC* might themselves be involved in specifying rhombomere identity. In the analysis presented here the hindbrain was not investigated in detail. No obvious hindbrain aberrations, such as overgrowth, were detected in the *zfDachA* and zfDachA/hr injected embryos but subtle changes resulting in misspecification of one or more rhombomeres would have been overlooked. This could manifest as a transformation of one rhombomere to another, or perhaps more likely, as disrupted rhombomere (R) formation. In the zebrafish mutant *valentino*, R5 and R6 fail to separate from one another as a result of

a lack of *valentino* gene product which is usually present in R5 and R6 (Chitnis and Dawid, 1999; Moens et al., 1996) This mutant was identified by the absence in R5 of *Krox20,* a marker of R3 and R5 fate (Oxtoby and Jowett, 1993). The use of markers such as *Krox20* and the *Hox* genes could reveal phenotypes of this nature in the *zfDachA* injected embryos. In view of the striking hindbrain expression pattern of *zfDachA* in particular, this would be an extremely interesting investigation to carry out.

All three fish *Dach* genes are also expressed in localised regions of the CNS which represent individual neurones and ganglia so may be involved in the specification or differentiation of neural pathways. The use of a marker which highlights individual neurone tracts, such as acetylated alpha tubulin (Chitnis and Kuwanda, 1990) would reveal any misspecifications induced by *zfDachA* overexpression.

4.5.5 Neural crest functions for zebrafish *Dachshund* genes?

zfDachA and *zfDachC* are expressed in the neural crest and in structures which have a neural crest derived component, the ear, the branchial arches and various ganglia. A neural crest function is therefore probable and as in the mouse *zfDachA* and *zfDachC* are potentially good neural crest markers. *zfDachA* overexpression analysis produced no obvious neural crest phenotype but a detailed analysis of this was not performed. *zfDachB* was not detected in the neural crest.

4.5.6 A somite function for zebrafish *Dachshund*?

zfDachA was detected transiently in the anterior region of the five anterior most somites at the 14 somite stage. In the zebrafish these five somites are distinct in that they develop synchronously, further somites being added sequentially posterior to the initial five. Similarly mammalian *Dach1* is expressed in somites and chick *Dach2* is thought to have a major role here (Davis et al., 1999; Tabin et al., 1999).

When *zfDachA* RNA was overexpressed a striking somite phenotype was produced. The somites lost their characteristic chevron shape, the less severely affected taking on a U shape while the more severely affected appeared block like and irregular. The notochord appeared normal which is an important observation as

notochord lacking mutants such as *floating head* and *no tail* have defective somites as a result (reviewed in Eisen et al., 1996). To ascertain the underlying cause of this phenotype markers need to be used to locate various portions of the somites. *Engl* for instance marks muscle pioneer cells, and the absence or presence of too many of this cell type is known to cause U-shaped somites (reviewed in Eisen et al., 1996).

When zfDachA/hr (truncated *zfDachA* which lacks Dach-box C) RNA was injected the phenotype resembled the somite phenotype produced by overexpression of full length *zfDachA*, although not as severe. It cannot necessarily be assumed that both phenotypes are identical without further characterisation, but preliminarily this suggests that *zfDachA* can perform at least part of its function without the need for Dach-box C. Alternatively both zfDachA/hr and full length *zfDachA* may be acting in a dominant negative manner. If *zfDachA* normally functions as part of a complex, overexpression of either the full length or truncated transcript could upset the stoichometry of the complex components, thus removing endogenous *zfDachA* function.

4.5.7 Further investigations

Initially it is important to complete analysis of the expression of all three zebrafish *Dach* genes. Double *in-situ* hybridisation or antibody staining may be useful to pinpoint exactly which regions of the embryo express these genes and may help to identify expression relationships with other genes. *Emx*, *Pax6, Pax2,* acetylated alpha tubulin and *Krox20* may all be useful markers.

Emx1 and *emx2* are expressed in the dorsal telencephalon and *emx2* is also found in regions of the diencephalon. These genes may therefore be useful to identify the rostral extent of *zfDach* expression (Morita et al., 1995). Acetylated alpha tubulin stains neurone tracts and so would be especially useful for the identification of specific neurones which express *zfDach*. As the position of the acetylated alpha tubulin stained neurones has been well documented this would also be useful in the identification of the larger brain expression domains (Chitnis and Kuwanda, 1990).

Pax2 is expressed in the optic stalks, the telencephalon and midbrain and *Pax6* is found in the eye, the diencephalon, the hindbrain and in regions of the telencephalon (Krauss et al., 1999a and b; Nornes et al., 1998). Both of these genes

would therefore be useful in defining brain and eye expression domains. *Krox20* is expressed in rhombomeres three and five of the hindbrain and so could be used to confirm the rhombomere specific expression of *zfDachA* and *zfDachC* (Oxtoby and Jowett, 1993). As has already been discussed, some if not all of these genes might also be useful in further investigating the overexpression phenotypes of *zfDachA* and zfDachA/hr.

In-situ hybridisation of *zfDach* genes to mutant fish may also be informative. Various somite and brain mutants exist which could be useful. *zfDach* genes might prove useful as markers in some of these mutants in any case, for instance *zfDachC* as a neural crest marker.

Mapping all three fish *Dach* genes is also an important step to take. Although this may not be immediately useful, in the long term this will facilitate the identification of mutants corresponding to these genes. It may also help to resolve the question of which genes are related to which mammalian genes. It might prove possible to identify mutant(s) corresponding to one or more of the *zfDach* genes by the candidate gene approach. This is unlikely to be a trivial exercise but may provide the best basis for functional analysis of these genes.

Apart from the further characterisation of the phenotypes caused by overexpression of *zfDachA,* as discussed above, it will be important to know whether these phenotypes are specific to *zfDachA*. A first step will be to ascertain whether or not *zfDachB* and *zfDachC* are ectopically activated by injection of *zfDachA.* A simple way to do this is to perform *in-situ* hybridisation analysis with *zfDachB* and *zfDachC* on embryos which have been injected with *zfDachA*. Overexpression of *zfDachB* and *zfDachC* could also answer this question and it might also be interesting to perform this analysis in an attempt to elucidate functions for these two genes.

Mosaic analysis, where a gene is expressed under the control of an inducible promoter, may be useful in the analysis of the overexpression phenotypes (Hammershmidt et al., 1999). This technique allows expression to be induced at specific times in localised areas so that non-specific effects and early death, which may be caused by widespread early overexpression, can be avoided. Autonomy of gene action can also be investigated in this way.

To fully understand the function of these genes it will also be necessary to perform loss of function analysis. As yet targeted knockouts cannot be produced in zebrafish, but methods such as the application of antisense RNA have often been successful in removing functional gene products in organisms such as *C.elegans*. There is one report of successful application of this method to zebrafish. In this case *Alx*, the zebrafish *Chx10* homologue, was affected by the application of antisense oligonucleotides, resulting in a similar phenotype to ocular retardation, the mouse *Chx10* mutant (Hammerschmidt et al., 1999; Barabino et al., 1997). Dominant negative constructs can also prove useful in certain cases and this approach could potentially work for *zfDach* genes as discussed in section 4.4.1.

Chapter 5: Conclusions

Pax6, *Eya1-3* and *Six3* are the previously known vertebrate homologues of *ey, eya* and *so,* three of a group of four interacting *Drosophila* genes fundamental to eye development. These are sometimes referred to as the retinal determination (RD) genes. The vertebrate genes have also been implicated in eye development (Hill et al., 1991; Xu et al., 1998; Oliver et al., 1995; Loosli et al., 1998). In this study small eye chimeras were used to further characterise the eye functions of *Pax6.* Secondly, the possibility that vertebrate homologues of *dac*, the fourth *Drosophila* RD gene, might also be involved in eye development was investigated.

One mouse *dac* homologue, *Dach1*, and three zebrafish *dac* homologues, *zfDachA*, *zfDachB* and *zfDachC* were identified in this study. All share two domains of high sequence conservation with *Drosophila dac,* one near the N-terminus, Dach-box N, and one near the C-terminus, Dach-box C, which contains a novel zipper motif. The *Dach* genes also have significant similarity to the *Ski* genes within these regions and on this basis the *Dachshund* genes and the *Ski* family genes are proposed to form a superfamily.

In the developing eye *Dach1* is expressed in the optic vesicle and perioptic mesenchyme, later being expressed in the anterior lens epithelium and becoming restricted to the neuroretinal layer nearest to the lens. In the zebrafish the situation is similar, *zfDachA* being expressed in the developing optic lobe. Later, *zfDachA* is found in the same tissues as *Dach1* but in a different sequence which may reflect differences in the relative timing of fish and mouse eye development. *zfDachA* expression disappears from the optic cup by 24hpf then reappears in the ganglion cell layer by 48hpf. Lens epithelium expression is seen at 24hpf, comparatively earlier than in the mouse. Based on these expression patterns *Dach* may have an early role in retinal determination but is also likely to have a later role in the development and differentiation of specific tissues.

With the identification of *Dach,* vertebrate homologues of all four *Drosophila* RD genes have been detected in the developing eye. Expression of *Dach1* overlaps with that of *Pax6, Six3* and *Eya2/3* in the mouse optic vesicle/neuroretina (Hammond et al., 1998; Grindley et al., 1995; Oliver et al., 1995; Xu et al., 1998), and *zfDachA*

expression overlaps with *Pax6* and *Six3/6* (Krauss et al., 1991; Seo et al 1998a) in the zebrafish optic lobe/neuroretina (*Eya 2/3* homologues have yet to be isolated in this organism). These genes could therefore, potentially, interact in a similar way to the *Drosophila* RD genes which are thought to form a protein complex consisting of dac, eya, so and some other factor(s). *Ey* is needed along with this complex to determine retinal fate (Chen et al., 1997; Pignoni et al., 1997).

dac/Dach has been shown, *in-vitro,* to interact with *eya/Eya* through the Dach-box C region and is thought to interact with a further factor through Dach-box N, thus linking members of the complex (Chen et al., 1997; Pignoni et al., 1997). *Ski*, which is likely to function in a similar way to *Dach*, is known to form part of a protein complex, the histone deacetylase (HDAC) complex, by interacting with N-CoR through Dach-box N and with m-Sin3A through Dach-box C (Nomura et al., 1999). By analogy Dach1 could well function as part of a similar complex.

Apart from its role in the HDAC complex Ski is known to interact with NF-1, a transcription factor, through a region containing Dach-box N and forms homodimers through Dach-box C. When complexed with NF-1 Ski has trancriptional activation activity while as part of the HDAC complex it has a repressor function (Tarapore et al., 1997; Nomura et al., 1999). *Ski* then, has different functions when complexed with different factors, and by analogy *Dachshund* is also likely to have multiple functions facilitated by interactions with a variety of gene products.

It has been speculated, as discussed in chapters three and four, that *Dach* may have roles in retinal fate specification, outgrowth and specification in the limb, CNS patterning and in neural crest and somite development. In the retina *Dach* probably interacts with *Eya2/3*, *Pax6* and *Six3* or *Optx2* but these factors are not expressed in all other regions where *Dach* is found. Other members of the *Pax, Eya* and *Six* families are, however, present in many regions. *Pax3, Eya1/2/4* and *Six1,* for instance, are all expressed in the somites (Abdelehak et al., 1997; Borsani et al., 1999) where *Dach1*, *zfDachA* and chick *Dach2* (Tabin et al. 1999) are detected. Overexpression of *zfDachA* in the zebrafish has indicated a function for *zfDachA* in the somites and *Dach2* has been shown to interact with *Pax3* (Tabin et al., 1999). This suggest that interactions with various different members of the *Pax, Eya* and *Six*

gene families will facilitate a variety of *Dach* functions. Exactly what these roles will be has however, yet to be determined. Increased cell death in the *dac*$^{-/-}$ *Drosophila* mutant lead to the suggestion that *dac* may be involved in proliferation and indeed *Dach1* expression is detected in regions with high proliferative potential in the mouse (this study; Mardon et al., 1994; Hammond et al., 1998 and this thesis; Caubit et al., 1999; Davies et al., 1999; Kozmik et al., 1999). Overexpression of *zfDachA* in zebrafish embryos produced overgrowth in the midbrain region which might tend to confirm this proliferative role. A proliferative function cannot explain the presence of *Dach* in all areas however.

The RD genes, are expressed in very similar regions in flies and vertebrates, *Dach* now joining this group. *Dach1* expression in the eye and limb of the mouse has obvious similarities to the eye and leg expression of *Drosophila dac*. In particular *Dach1* and *dac* are both found in the presumptive medial portions of the developing limb, *Dach1* within the mouse limb bud and *dac* in the fly imaginal disc (Mardon et al., 1994: Hammond et al., 1998 and this thesis). The legs and eyes of flies and mammals are not however homologous structures; they develop differently and have very different morphologies in the two species. Eyes in particular are so morphologically diverse across the phyla that they have long been held to be an example of convergent evolution having arisen independently many times (Hill and Davidson. 1994; Nilsson and Pelger, 1994). Bearing this in mind, the apparent similarities in RD gene expression are intriguing. RD gene conservation might suggest that hitherto unknown evolutionary links actually exist between flies and vertebrates, and as more conserved RD genes are isolated this becomes increasingly likely.

It has been suggested that *Pax6* was present in a primordial light sensitive structure which was subsequently modified into the diverse eye types found today (Land., 1992; Hill and Davidson, 1994; Nilsson., 1996; Ikeo and Gehring, 1999). Perhaps the ancestral 'eye' also contained the remaining RD genes since it is hard to envisage that they would all have been independently acquired so many times.

Future prospects

As yet, little is known about the functions of the vertebrate RD homologues with the exception of *Pax6,* and even in this case exact functions and targets are not known. This study has indicated a function for *Pax6* in RPE differentiation but the potential exists to exploit the small eye chimera system to a far greater extent. This was discussed in detail in chapter 2.

As far as the remaining RD genes are concerned it is important to investigate their individual roles and particularly how they interact with one another. Interactions between the vertebrate RD genes and their relatives could initially be investigated using in-vitro assays such as the yeast-two-hybrid system. Loss of function analysis will also be important, although production and analysis of targeted *Dach1* knockouts might be complicated by the existence of a second *Dach* gene. Double knockouts may therefore be required to avoid any effects of redundancy. In the zebrafish, antisense approaches could prove useful. Alternatively mutants in one or more *zfDach* gene might have been produced in the recent large scale mutagenesis screens, this would not however, be trivial to investigate. An important first step would be to map the three fish genes. At an immediate level, as discussed in chapter 4, it will also be important to complete the analysis of the *zfDachA* overexpression phenotypes, especially the phenotype found in the somites but also the possibility that brain patterning defects may be occurring.

The study of *Pax6* in a variety of species has lead not only to a greater understanding of *Pax6* function in its own right, but also to an increased knowledge of the mechanisms of eye development. Further analysis of RD gene functions, through the study of the *Six, Eya* and *Dach* genes in addition to *Pax6,* should further greatly increase this body of knowledge. Analysis of these genes in a wide variety of species will also be extremely important in addressing the questions of eye evolution.

Chapter 6 Materials and Methods

All chemicals were obtained from Sigma unless otherwise stated

6.1 Mouse strains

BALB/c : BALB/c / Eumm

AAF_1 : BALB/c x A/J; F1 hybrid

Pax6$^{Sey\text{-}neu}$: CBA/Ca-*Pax6*$^{Sey\text{-}Neu}$

Pax6^{Sey}; *Tg*$^{+/+}$: CBA/Ca-*Pax6*^{Sey} x TGB

CF_1 : F_1 hybrid derived from crosses between two congenic strains C57BL and BALB/c

TGB (*Tg*$^{+/+}$): derived from crosses between strain 83 and (C57BL x CBA/Ca)F_1 (Keighren and West, 1995b; Lo et al., 1986, 1987)

CBA : CBA/Ca

6.2 Chimera Production and Typing

Chimeras were produced as outlined in section 2.2. The general scheme is shown in table 6.2 and the mouse strains used in table 6.1

Pregnant mares serum gonadotrophin (PMSG): 50units/ml

Human chorionic gonadotrophin (hCG): 50units/ml

Table 6.1 : Mouse Strains

Strain	*Gpi1*	Pigment	β-globin Transgene	Use in this study
Pax6^{Sey}	*b / b*	*C / C*	*Tg* $^{+/+}$	Donor Male
Pax6$^{Sey\text{-}Neu}$	*b / b*	*C / C*	*Tg* $^{-/-}$	Donor Female
BALB/c	*a / a*	*c / c*	*Tg* $^{-/-}$	Donor Male & Female
AAF_1	*a / a*	*c / c*	*Tg* $^{-/-}$	Donor Male & Female
CF_1	*c / c*	*c / c*	*Tg* $^{-/-}$	Recipient Female

Table 6.2 : Experimental scheme for chimera production

Day	Procedure	Technique
1	Donor females superovulation.(1) [*Pax6*$^{Sey\text{-}Neu}$ and BALB/c or AAF_1 used]	Inject 0.1ml PMSG intraperitoneally at 12 noon.
2	Recipient females superovulation (1) [CF_1]	Inject PMSG as above
3	Donor females superovulation (2) Mate donor females to appropriate male:	Inject 0.1ml hCG intraperitoneally at 12 noon **Female** **Male** BALB/c x BALB/c AAF_1 x AAF_1 *Pax6*$^{Sey\text{-}Neu/+}$ x *Pax6*$^{Sey/+}$
4	Recipient females superovulation (2) Mate recipient female to vasectomised male Confirm mating of donor females	Inject hCG as above Check for vaginal plug
5	Confirm mating of recipient females	Check for vaginal plug
6	Collect and aggregate embryos from donor females Culture overnight	See section 6.2.1
7	Transfer aggregated embryos to uterus of recipient female. This is taken to be day 2.5 of pregnancy.	See section 6.2.2
17	Collect and analyse E12.5 chimeric embryos	See section 6.2.3

6.2.1 collection aggregation and culture of embryos

Stock A (10x): 1M NaCl, 0.05M KCl, 1.2M KH_2PO_4, 0.01M $MgSO_4.7H_2O$, 0.23M Na lactase (60% solution), 5.5mM glucose, 10^5 units penicillin, 750 units/mg streptomycin.

Stock B (10x): 0.25M $NaHCO_3$, 0.01g phenol red

Stock C (100x): 0.33M sodium pyruvate

Stock D (100x): 0.17M $CaCl_2.2H_20$

Stock E (10x): 0.25M HEPES (Ultrapure Calbiochem), 0.01g phenol red. Adjusted to pH 7.4 with 5M NaOH then made to 100ml.

Stocks were made up with sterile culture grade H_2O and filter sterilised using a 0.22μm millipore filter. Stocks A, D and E were stored at 4°C for up to 3 months and B and C stored at 4°C for up to 2 weeks.

M2 handling medium: 1ml stock A, 0.16ml stock B, 0.1ml stock C, 0.1ml stock D, 0.84ml stock E. 7.8ml double distilled H_2O (BDH), 4mg/ml BSA. Filter sterilised before use.

Acid Tyrode's solution: 0.14M NaCl, 2.6mM KCl, 1.4mM $CaCl_2$, 0.5mM $MgCl_2.6H_2O$, 0.2mM$NaH_2PO_4.H_2O$, 5.5mM glucose, 0.12M $NaHCO_3$, 0.4% polyvinyl pyrrolidone. Adjust to pH 2.5 with 5MHCl, filter sterilise and store at 4°C.

M16 culture medium: 1ml stock A, 1ml stock B, 0.1ml stock C, 0.1ml stock D, 7.8ml double distilled H_2O, 4mg/ml BSA. Filter sterilise before use.

Phytohaemagglutinin (PHA): M form, GIBCO

Embryos were collected from the donor females by flushing the oviducts and uteri with M2 using a needle and syringe. The embryos obtained were placed in fresh M2.

8 cell embryos were selected and the zona pelucida removed from each by placing into pre-warmed Acid Tyrode's solution at 37°C for a few seconds. They were removed as soon as the zona started to disappear as death occurs if the embryos are left beyond this point. The embryos were then washed in M2 medium to remove all traces of Acid Tyrode's. Pairs of embryos were transferred to M2/PHA (19:1 v:v) to aid aggregation, and gently pushed together. Pairs were left to aggregate for 2-3 minutes then washed in M2. They were then transferred to pre-equilibrated drops of M16 under oil and allowed to develop overnight at 37°C in 5% CO_2.

6.2.2 Transfer of embryos

Hypnorm : 0.315 mg/ml fentanyl citrate and 10mg/ml fluanisone; Janssen pharmaceuticals

Hypnovel : 2mg/ml midazlam hydrochloride; Roche

Pseudopregnant females were anaesthetised with 0.25ml per 30g body weight of a 1:1 mixture of Hypnorm:Hypnovel. Both Hynorm and Hypnovel were diluted 1:1 with sterile distilled water before mixing together.

Embryos were collected in a fine glass pipette in a small amount of M2 medium. Embryos were surgically inserted into the uterus via a small incision made with a fine needle as close as possible to the oviduct (McLaren and Mitchie 1956).

6.2.3 Collection and Analysis of Chimeras

Dissection

PBS: 0.85 NaCl, 0.02%KCl, 0.02% MPO_4 (pH 7.3)

Embryos were dissected at E12.5 into ice cold PBS. Various statistics were collected for each embryo: weight of entire conceptus, weight of placenta, weight of fetus, crown-rump length of the fetus and the stage of the fetus by hind limb development. The percentage of pigmentation in the eye was also recorded, any obvious abnormalities noted and photographs of the embryo taken. Once this data was collected, the forelimbs and tail were removed for GPI analysis; the yolk sac,

amnion and placenta also being kept for this purpose. The head was removed for histology and the remaining trunk tissue taken for PCR genotyping.

The head was fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4°C before processing for histology as in section 6.10.1. The trunk tissue was frozen at -20°C for later digestion with proteinase K. The forelimbs, tail, placenta, yolk sac and amnion were mixed with 20µl 50% glycerol (200µl for the placenta) and frozen to -20°C for GPI analysis.

GPI Electrophoresis

Electrophoresis buffer : 0.02M Tris, 0.2M Glycine (Sigma)

Tris-Citrate buffer: 0.3M Tris, 0.08M citric acid

Stain : 1.5ml Glycerol/$MgCl_2$ (50%Glycerol/0.1% $MgCl_2$), 170µl Tris Citrate buffer, 170µl 20 mg/ml Fructose-6-phosphate (Sigma), 170µl 2.7 mg/ml Nitro-blue-tetrazolium (Sigma), 170µl 2.7 mg/ml NADP (Sigma), 20µl 2.5mg/ml Phenozine methosulphate (Sigma), 6-10 units glucose-6-phosphate-dehydrogenase (Sigma). This is enough for 1 plate.

Titan III cellulose acetate plates (Helena laboratories) were soaked in electrophoresis buffer for 30 minutes before use. Tissue samples were frozen and thawed three times to ensure cell lysis, then diluted 1:5 in water and applied to the plates using a super Z-8 applicator (Helena laboratories). Electrophoresis was carried out from anode to cathode at 200V for 1 hour before staining for glucose phosphate isomerase (GPI) activity in staining solution for up to 20 minutes in the dark at 37°C. The plates were then rinsed in water, fixed in 5% acetic acid for 5 minutes then washed in dH_2O for 15 minutes and allowed to air dry in the dark.
Densitometry was then carried out using a Helena Process-24 gel scanner (Helana laboratories) to determine the relative proportions of GPI-1A, GPI-1B and GPI-1C in the samples (West et al., 1994)

PCR Genotyping

Proteinase K digestion of trunk samples

5x Proteinase K buffer : 50mM Tris, 250mM KCl, 25mM $MgCl_2$, 2.25% NP40 (Nonidet-P40)(Sigma), 2.25% Tween (Polyoxyethelene-sorbitan-monolaurate)(Sigma), 0.5mg/ml gelatin (Sigma)

The trunk sample was placed in an eppendorf tube containing 0.5mls proteinase K buffer and 30µl of 10mg/ml proteinase K. The samples were incubated overnight at 55°C then vortexed, heated to 97°C for 3 minutes and cooled on ice. Tubes were centrifuged at 13000rpm for 10 minutes to pellet debris before PCR analysis.

PCR amplification of the *Pax6*^{Sey} and *Pax6*$^{Sey\text{-}Neu}$ regions

1µl trunk lysate was PCR amplified as in section 6.5 using primers Q800 and Q801 to amplify a 220 bp fragment for *Pax6*$^{Sey\text{-}Neu}$ and primers Q802 and Q803 to amplify a 140bp fragment for *Pax6*^{Sey}. An annealing temperature of 59°C was used. 10µl of the resulting PCR product was run on a 1.2% agarose gel as in section 6.3.6 to ensure the specific amplification of the correct sized band had occurred.

Restriction digestion of PCR fragments

30µl of PCR product was digested as in section 6.3.5 in a total volume of 40µl using HindII for *Pax6*$^{Sey\text{-}Neu}$ and DdeI for *Pax6*^{Sey} .

Table 6.3 : Restriction fragment sizes for chimera genotyping

	***Pax6*$^{Sey\text{-}Neu}$ amplification**	***Pax6*^{Sey} amplification**
Wildtype	220bp (no digestion)	83bp,74bp
Mutant	140bp, 80bp	83bp, 55bp, 19bp

Polyacrylamide gel electrophoresis of the digested PCR fragments

The digested PCR fragments were run out on 1.0mm thick 15% polyacrylamide gels using Hoefer vertical slab gels (Pharmacia Biotech). The gel was made from 15ml 40% 19:1 (v:v) acrylamide:bisacrylamide (Northumbria Biologicals Ltd), 300µl 10% ammonium persulphate, 30µl TEMED (N,N,N',N'-Tetramethylethylenediamine, Sigma), 2µl 20x TBE (section 6.3.6) in a final volume of 40mls. Samples were mixed with $1/10^{th}$ volume of orange G loading buffer (section 6.3.6) before running. The entire digest was loaded alongside the remaining 10µl undigested PCR product and Msp1 size ladder (New England Biolabs). Gels were run in 1x TBE for 3 hours at 280V.

Silver staining polyacrylamide gels

Solution 1: 10% ethanol, 0.5% acetic acid in dH_2O

Solution 2: 1g $AgNO_3$ in 1 litre dH_2O

Stain : 15g NaOH, 0.1g $NaBH_4$ and 4ml 37% formaldehyde in 1 litre dH_2O

Fix : 7.5g Na_2CO_3 in 1 litre dH_2O

To visualise the DNA, the gels were silver stained. The gel was placed in a tray on a horizontal shaker throughout and all solutions were made up immediately before use. Initially gels were washed twice for three minutes in solution 1, then incubated for 10 minutes in solution 2. They were then washed twice in water before staining for 20 minutes. A small amount of stain was poured onto the gel which formed a precipitate, this was poured off and the rest of the staining solution added. After staining the gel was fixed for 10 minutes and photographed.

6.3 DNA and RNA preparation and purification.

6.3.1 Bacterial Culture Media and additives

Culture Media

Culture media was prepared and autoclaved by the medium preparation service.

L-Broth : 10g bactotryptone (Difco), 5g bacto-yeast extract (Difco), 10g NaCl (pH7.2) in 1 litre of dH_2O.

L-Broth^{Mg2+} : As above with the addition of 2.46g $MgSO_4$.

L-Agar/L-Agar^{Mg2+} : As L-Broth/L-Broth^{Mg2+} with the addition of 15g Agar

CY-Top Agarose : 10g casamino acids, 5g yeast extract, 3g NaCl, 2g KCl (pH7.0) in 1 litre dH_2O. 0.6% agarose.

Additives/solutions

Ampicillin : 50mg/ml stock solution in dH_2O. Added to media/agar to a final concentration of 50μg/ml.

Tetracycline : 50mg/ml stock solution in ethanol. Added to media to a final concentration of 50μg/ml.

X-GAL : (5-Bromo-chloro-indoyl-β-galactopyranoside, Melford Laboratories) 20mg/ml stock solution in Dimethylformamide (BDH). Added to agar to a final concentration of 40μl/ml

IPTG : (isopropyl-β-galactopyranoside, Melford laboratories) 20mg/ml stock solution in dH_2O. Added to agar to a final concentration of 40μl/ml.

Maltose: 20% stock solution in dH_2O sterilised through a 0.2μm filter (Gelman Sciences). Added to media at 0.2% when preparing library plating cells.

$MgSO_4$: 10mM stock in dH_2O used to prepare plating cells for λ library screening. Autoclaved before use.

λ-buffer : 10mM Tris.HCl (pH7.5), 10mM $MgSO_4$ (Used for short term storage of λ bacteriophage stocks). Sterilised by autoclaving.

TE : 10mM Tris (pH8.0) 1mM EDTA

6.3.2 Growing and storing bacteria

Liquid culture and plates were grown at 37°C in appropriate media. Liquid cultures were grown in an orbital shaker at approximately 225rpm. Where necessary a colorimeter was used to ascertain the optical density (OD) of the culture before harvesting the cells.

For long term storage bacteria were kept at -70°C in 15% glycerol / 85% L-Broth prepared as in Sambrook et al., 1987. To grow bacteria from these frozen stocks a scraping was streaked onto appropriate selective media and incubated at 37°C.

6.3.3 Plasmid DNA Preparations

Plasmid preparations were carried out using a QIA-prep spin miniprep kit (Qiagen) for small scale preparations and a Qiagen plasmid Maxi kit for larger scale preparations. Both kits were used according to manufacturers instructions, incorporating optional steps. The resulting DNA was re-suspended in 50µl dH_2O for minipreps and in 1ml dH_2O for maxipreps.

In general both methods provided DNA which was suitable for enzymatic digestion, PCR, RNA transcription and manual sequencing. Miniprep DNA was poor for ABI automated sequencing although Maxiprep DNA worked well.

6.3.4 Phenol/Chloroform purification.

Phenol/Chloroform purification was performed according to Sambrook et al. (1987) and was used to purify both DNA and RNA samples. DNA/RNA was extracted twice with an equal volume of a 1:1 (v:v) phenol:chloroform mixture then once with an equal volume of a 24:1 (v:v) chloroform: Isoamyl alcohol mixture. In each case the two liquids were mixed, microcentrifuged at 13000rpm for one minute and the top, aqueous, DNA/RNA containing phase removed.

DNA/RNA was precipitated at -20°C with 1/10 volume 3M NaOAc (pH 5.2) and 2 volumes 100% ethanol. It was then pelleted at 13000rpm for 10 minutes, washed in 70% ethanol, dried and re-suspended in dH_2O

6.3.5 Restriction Digestion of DNA

Carlo's Awe Inspiring Buffer (x10): 200mM Tris (pH7.5), 1mM KCl, 70mM MgCl, 10mM DTT, 1mg/ml Gelatin

This was carried out using enzymes and buffers supplied by Boehringer Mannheim.

For small scale digests up to 1µg of DNA was digested with 1µl (10 units) enzyme in a total volume of 50µl containing 5 µl of the appropriate buffer. This was incubated for 1.5 hours at the optimal temperature for that enzyme. Larger scale digests were carried out in larger volumes, generally 5 or 6µg was cut overnight in a total volume of 200µl with 2µl (20 units) enzyme.

When 2 different enzymes were required which cut in the same buffer digestions were carried out simultaneously. Otherwise Carlo's Buffer was used in place of the commercial buffer. This appeared adequate in all cases encountered.

6.3.6 Agarose gel electrophoresis

20x TBE : 1M Tris-HCl (pH 8.0); 20mM EDTA; 1M boric acid, pH8.3.
20x TAE : 0.8M Tris-HCl (pH 8.0); 20mM EDTA; 0.4M acetic acid.
10x Loading buffer : 15% Ficoll in TE; orange G

Separation of DNA and RNA molecules was achieved by horizontal agarose gel electrophoresis. The size of the gel and percentage of agarose used were dependant on the resolution required. 1% agarose (Biogene Ltd. Hi-Pure Low EEO Agarose) was used for fragments between 300bp and 4 kb, 1.2% for smaller bands down to 100bp and 0.8% for larger fragments up to approximately 7kb. Either 1kb ladder (GibcoBRL 500bp -12kb) or ϕX174-HaeIII (Boehringer Mannheim, 70bp-1.3kb) were run alongside samples.

All gels were made and run in 1x TBE except those from which DNA was to be extracted using a Geneclean II kit (see section 6.3.7) which were made and run in 1x TAE. All DNA extraction gels were made from low melting point agarose (UltraPURE LMP agarose, GibcoBRL). 1μg/ml Ethidium bromide was added to all gels to visualise DNA and 1/10 volume of loading buffer was added to each sample before loading. DNA/RNA bands were visualised using a UV transilluminator (305nm).

6.3.7 Extraction and purification of DNA from agarose

DNA was run out in low melting point agarose (TBE for Qiagen; TAE for Geneclean) and the appropriate band excised with a minimum of excess agarose. DNA was then extracted from the agarose using either a QIA-quick gel extraction kit (Qiagen) or a Geneclean II kit (AnaChem) according to manufacturers instructions and including all optional steps. QIA-quick was used except when DNA was to be used for production of capped RNA (section 6.12.1).

6. 4 Cloning DNA fragments

pBluescript II SK +/- : Contains T7 and T3 RNA polymerase sites on either side of the polylinker. Ampicillin resistance and blue/white selection. Used for creation of in-situ probes (sense and anti-sense)

pCS2+ : Contains an SP6 polymerase site and *Xenopus* β-globin sequence 5' to the polylinker, SV40 polyA sequence and linearisation sites 3' to this. Ampicillin resistance. Used for transcription of zebrafish injection RNA.

PCR 2.1 : (InVitrogen) Contains a T7 polymerase site 3' to polylinker. Ampicillin resistance and blue/white selection. Used for cloning PCR fragments using a TA cloning Kit (InVitrogen).

Vector and insert were digested as in section 6.3.5 with enzymes which allow the ligation of one to the other. When this was not possible linkers were added containing suitable restriction sites.

6.4.1 CIP treatment to prevent re-ligation.

Linearised vectors were treated with calf alkaline phosphatase (CIP) (Boehringer Mannheim) to prevent self religation. For ligation to occur at least one of the two ends must be phosphorylated. DNA was mixed with 10μl dephosphorylation buffer, 1μl CIP and water to 100μl. The mixture was incubated at 37°C for 30 minutes for 5' overhanging ends or for 15 minutes at 37°C then 15 minutes at 56°C for blunt ends and 5'recessed ends. A further 1μl CIP was then added and the incubation repeated. The reaction was stopped by the addition of 2μl EDTA and incubation at 65°C for 10 minutes, then phenol chloroform extraction was carried out to remove the enzymes (section 6.3.4).

6.4.2 Linkers

To ligate insert into pCS2+ vector, linkers were often needed. Linkers used were made from two oligonucleotides (Genosys) which were annealed together before use. 5μl of each oligo (1μg/μl) were mixed together with 2μl of ligation buffer (supplied with T4 DNA ligase - Boehringer Mannheim) and 8μl water. This was boiled for 5 minutes and allowed to cool slowly to room temperature.

Each linker produced contained a unique internal restriction site so that its presence in a construct could be confirmed.

Table 6.4 : linkers

Oligo 1	Oligo 2	End restriction sites	Internal restriction sites
V471 (GAGCTCTAGAA GTG)	V470 (AATTCACTTCTAGAGCTC TGCA)	PstI, EcoRI	SacI
V468 (CTCCAGATATC TGC)	V469 (TCGAGCAGATATCTGGA GGTAC)	Asp718, XhoI	EcoRV

6.4.3 Ligation

Vector and insert were mixed in a ratio of 3:1 insert to vector with 1µl T4 DNA ligase (Boehringer Mannheim), 1µl ligation buffer and water to 10µl. Ligation was carried out overnight at 16°C for 5' overhangs and recessed ends, 22°C for blunt ends. When linkers were used 0.25µg was added to the reaction mixture (ie 0.5µl of the annealing reaction above)

The reaction was heat inactivated at 65°C for 20 minutes then desalted using 0.025µm nitrocellulose discs (Millipore) over water for a further 20 minutes.

6.4.4 Competent cells for transformation (Electrocompetent)

1 litre of L-broth was inoculated with a single colony of INV Fα cells (InVitrogen) and grown at 37°C until an OD of 0.5 to 1 was reached. The culture was chilled on ice for 20 minutes then pelleted at 3000 x g for 15 minutes. This pellet was re-suspended in 1 volume cold sterile dH_2O and re-pelleted then the process repeated once (second resuspention in ½ vol). The cells were then re-suspended in 3ml 10% glycerol and rapidly frozen in 40µl aliquots on dry ice. Aliquots were stored at -70°C.

6.4.5 Transformation

2µl of desalted ligation was mixed with 40µl competent cells in an electroporation cuvette (Flowgen) on ice. The Gene Pulser apparatus was set to 25µF, 2.5 kV and the pulse controller set to 200Ω. The cuvette was pulsed once (a time constant of 4.5 to 5 msec was ideal), then immediately 1 ml of L-Broth^{Mg2+} was added and the cells incubated for 1 hour at 37°C. Cells were then plated on suitable selective medium and incubated at 37°C overnight.

6.4.6 Colony PCR

To identify colonies containing inserts in pBluescript and PCR2.1 prior to DNA preparation, PCR amplification of the insert was carried out using primers to the M13 reverse and T7 polymerase sites which flank the polylinker. The colony was streaked onto a reference plate, incubated overnight, then part of the streaked colony was picked into 200µl dH_2O and boiled for 10 minutes, cooled on ice and 4µl PCR amplified as in section 6.5.

6.5 Polymerase Chain Reaction (PCR)

PCR was used to amplify specific regions of DNA. Primers complementary to the two 5' ends of the DNA strand to be replicated, were annealed to the template DNA. This enables Taq DNA polymerase, which is thermostable, to extend the DNA from the primers.

Multiple cycles of template denaturation, primer annealing and DNA extension allows the exponential amplification of a specific region of DNA.

PCR conditions : Amplification was carried out in a Hybaid Omnigene thermal cycler. Each reaction was set up in 50µl total volume containing 1 unit AmpliTaq (Perkin Elmer), 5µl 10x reaction buffer (supplied by manufacturer), 5µl 25mM $MgCl_2$ solution (supplied by manufacturer), 100ng of each primer, 200µM dNTPs (1:1:1:1 A:C:G:T) and template DNA. Plasmid DNA was used at a concentration of approximately 10ng and genomic DNA was used at about 100ng. Amplification from bacteriphage-λ eluate used 1µl of eluate, prepared as in section 6.7.4. Each reaction was covered with a drop of mineral oil (Sigma) to prevent evaporation.

Primers : Primers for PCR were designed so the T_m's of the primer pair were as nearly as possible the same. In general primers were 18 - 22 nucleotides in length with T_m's of 55-65°C. Primers used are shown in appendix 1

PCR programmes : An initial step of 3 minutes at 94 °C was used to denature the DNA template. Then 35 cycles of a three step process was used to exponentially amplify the DNA:

a) Denaturation : 30 seconds at 94°C to denature the DNA produced in each cycle so it was available for re-amplification.
b) Annealing : 30 seconds at a temperature approximately 4°C lower than the melting temperature (T_m) of the primer with the lower T_m. This step allows the primers to anneal to the template. T_m was calculated according to the equation:

T_m at 1M Na^+ concentration = 4 (G+C) + 2 (A+T)

Annealing temperature is crucial to the success of PCR and in some cases had to be modified to optimise amplification. If non-specific bands were seen the annealing temperature was raised until only specific product was seen, and if no product was initially amplified annealing temperature was reduced until a band was produced.
c)Extension : At 72°C to allow the polymerase to extend the DNA. Length of time was normally 1 minute for products of 1kb and under, this time extended by 1 minute for each further kb of product required.

After 35 cycles a further extension step of 3 minutes at 72°C was used. When a TA cloning kit was to be used this step was extended to 8 minutes according to manufacturers instructions.

6.5.2 Reverse Transcription (RT) PCR

Preparation of total RNA from mouse tissues using RNAzol

All solutions and equipment were kept RNAse free throughout. Solutions and glassware were treated with Diethyl pyrocarbonate (DEPC, Sigma) which is a potent inhibitor of RNAse. Solutions were treated with 1µl DEPC to 1ml solution then

autoclaved to remove the DEPC. Glassware was rinsed in Decon (Decon Labs Ltd) then ethanol, then DEPC dH_2O before baking at 200°C for 2 hours. All plastic ware was purchased RNAse free.

Harvested tissue was immediately frozen in liquid nitrogen and stored at -70°C until required. 200µl RNAzol (Biogenesis) was added to the still frozen tissue and the sample homogenised. Up to 300µl extra RNAzol was added depending on tissue size. 1/10 volume chloroform was added, the tube vortexed and incubated on ice for 5 minutes. The sample was then spun at 13000rpm in a 4°C microfuge and the top, aqueous layer removed to a new tube. An equal volume of propan-2-ol was mixed with the aqueous layer and the tube chilled on ice for 15 minutes. RNA was then pelleted at room temperature at 13000rpm. The pellet was washed with 70% ethanol, dried on ice then re-suspended in 200µl DNAse1 buffer (20mM Tris-HCl pH8.0, 10mM $MgCl_2$) and 1µl DNAse1. This was incubated at room temperature for 1 hour then the RNA phenol chloroform extracted as in section 6.3.4. Final re-suspension was in 40µl of DEPC treated dH_2O.

1st Strand cDNA synthesis

First strand cDNA synthesis was carried out using a First strand cDNA synthesis kit (Pharmacia Biotech) according to manufacturers instructions. 1-5µg RNA in RNAse free water to 20µl was heated to 65°C for 10 minutes to denature the RNA and then chilled on ice. 11µl Bulk First-Strand cDNA Reaction Mix (supplied by manufacturer), 1µl of $pd(N)_6$ primer (random hexamer mix - supplied by manufacture) and 1µl dithiothreitol (DTT) (supplied by manufacturer) were added and the reaction incubated at 37°C for 1 hour. This was then heated to 90°C for 10 minutes before PCR was carried out.

PCR Amplification of 1st Strand

10µl of first strand cDNA were mixed with 10µl PCR buffer, 10µl 25mM $MgCl_2$ 100ng each primer, 68µl dH_2O and 2.5 units AmpliTaq (Perkin Elmer). PCR was then carried out as in section 6.5.1.

6.6 Radiolabelling of DNA by Random Priming with ^{32}P-CTP

Table 6.5 Probes labelled by Random Priming

Fragment	Isolation technique	Use
Nt 1100 to 1516 *Dach1*	PCR with primers Q827 and Q829	C-terminal Genomic Southern blot
Nt 138 to 382 *Dach1*	PCR with primers P396 and P432	N-terminal Genomic Southern blot. Mouse genomic and zebrafish cDNA library screening
Human IMAGE clone 381801	digestion with SmaI and XhoI	Mouse Clontech cDNA Library screening
Human IMAGE clone 668097	digestion with EcoRI and NotI	Mouse Clontech cDNA library screening
Nt 372 to 636 *zfDachA*	PCR with primers S924 and S925	Zebrafish cDNA library screening

250-500ng of DNA in a total volume of 12µl dH_2O was denatured at 100°C for 10 minutes then cooled rapidly on ice. Labelling was then carried out using a Random Prime DNA labelling kit (Boehringer Mannheim). Denatured DNA was mixed with 3µl dNTP (1:1:1 G:A:T each at 0.5mM), 2µl 10x reaction buffer containing random primers, 3µl α^{32}P-dCTP (Amersham) and 1µl Klenow fragment, then incubated for 30 minutes at 37°C. Total volume was made up to 400µl with TE (pH8.0) then the sample was run through a Nick column (Pharmacia Biotech) according to manufacturers instructions to removed unincorporated nucleotides. Probe was eluted in 400µl TE, denatured at 80°C for 10 minutes and chilled on ice before use.

6.7 Library Screening

6.7.1 Libraries screened

mouse cDNA: oligo dT+ random primed mouse E11.5 embryonic 5' stretch plus cDNA library (Clontech) in λ GT11 host.

mouse genomic DNA: λ-GET library (a gift from Dr T.Boehm - Nehls et al., 1994)
zebrafish cDNA: zebrafish 15-19hour (28.5°C) polyA+ cDNA library (Obtained from Bruce Appel, University of Oregon). In Uni-ZAP XR lambda vector (Stratagene) with EcoR1 linkers 5' and XhoI linkers 3'.

6.7.2 Preparation of plating bacteria

XL 1-Blue MRF': Δ(mcrA) 183, Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac [F' proAB, $lacI^q$ ΔMI5, Tn 10(tet^r)] (Stratogene). Used as hosts for cDNA libraries in λZAP, ie the zebrafish library.

Y1090 : *Δ(lac) U169Δ(lon)? ara D139 strA supF mcrA trpC22::* Tn*10*(Tet^r) [pMC9Amp^r Tet^r](Stratagene). Used as hosts for the mouse cDNA λ-GT11 and gDNA λ-GET libraries.

XLOLR : *Δ(mcrA) 183* Δ(*mcrCB-hsdSMR-mrr*) 173 endA1 thi-1 recA1 gyrA96 relA1 lac [*F'proAB* $lacI^q$ *ZΔM 15* Tn*10* (Tet^r)]^c (Stratagene) . Used for excision of inserts from λ Uni-ZAP XR

ExAssist : (Stratagene) M13 helper phage. Used for the in-vitro excision of pBluescript SK phagemid from the λ Uni-ZAP XR

BNN132 : (Elledge et al., 1991) cre-recombinase expressing. Used for in-vitro excision and circularistion of plasmid from λ-GET library clones.

A single colony of plating bacteria was picked from a plate and grown overnight in 25ml L-Broth containing 10mM $MgSO_4$ and 0.2% maltose. This was then spun down at 3000 rpm in a Sorvall RT-6000B refrigerated centrifuge and re-suspended in 10mM $MgSO_4$ to an OD of 1.0. XLOLR, XL1-Blue and Y1090 were grown in the presence of tetracycline.

6.7.3 Titration of the library

A series of 10 fold dilutions of the library were set up in λ buffer and mixed with 100µl of plating bacteria. These were incubated at 37°C for 15 minutes then plated onto L-Agar plates in 3mls CY top agarose containing 10mM $MgSO_4$ at 50°C. Titre was calculated by counting the number of plaques after overnight growth (1 plaque = 1 plaque forming unit [pfu]).

6.7.4 Plating λ Libraries

Approximately 200 000 pfu of phage were plated onto each of four 22cm x 22cm square plates. Phage was mixed with 1ml of plating bacteria and incubated for 15 minutes at 37°C, to allow adsorption of phage to host then this was mixed well with 30ml CY-top containing 10mM $MgSO_4$ at 50°C. This was plated onto previously warmed and dried L-Agar plates. The library plates were then incubated overnight at 37°C. DNA was transferred onto nylon filters and hybridised as in section 6.7.5.

Positive plaques were excised from the plate using the large end of a sterile blue Gilson tip. The plug was then placed into 1ml λ buffer and eluted for at least 30 minutes at room temperature.

For secondary screening a 1:1000 dilution was made of this stock and 5µl, 10µl and 20µl of this dilution plated onto 90mm plates exactly as for library titration. Plaques were removed using the wide end of a sterile yellow Gilson tip and if necessary tertiary screening was carried out in the same way. Single plaques were isolated and stored at 4°C in λ-buffer with several drops of chloroform.

6.7.5 Pulling and Hybridising the Filters

Denature : 0.5M NaOH, 1.5M NaCl

Neutraliser : 1.5M NaCl, 0.5M Tris (pH7.5)

20x SSC : 44.1g Sodium citrate, 88.6g NaCl in 500ml dH_2O

Hybridisation (Hyb) mix: 4x SSC, 0.4% SDS, 0.2% NaPPi, 100µg/ml salmon sperm DNA and 2x Denhardts

Wash Solution : 2x SSC, 0.4% SDS and 0.2% NaPPi

50x Denhardts: 0.5g BSA, 0.5g polyvinylpyrrolidone, 0.5g Ficoll (Pharmacia) in 50mls dH_2O

Phage DNA was lifted onto nylon filters (Hybond-N, Amersham life sciences) or Protran discs (Schleicher and Schuell) for 90mm plates. The filters were laid onto the plate for 2 minutes (4 minutes for the duplicate filter) and orientation marks added by punching through the filter into the library plate with a syringe needle dipped in Indian ink. Filters were washed in denature for 4 minutes, neutraliser for 4 minutes and 2x SSC for at least 30 seconds. To cross link the DNA to the filter Hybond filters were UV crosslinked and Protran discs were baked for 1 hour at 80°C.

The filters were pre-hybridised in Hyb mix for 1 hour at 65°C in a rotary incubator (Hybaid). Hybridisation was carried out overnight at 65°C in the same solution with the addition of labelled probe (the product of one random prime reaction [section 6.6] was split between two hybridisation bottles). Filters were washed at 65°C, three washes of 20 minutes usually being sufficient to remove background radioactivity, then sealed into polythene. Auto-radiography was carried out overnight at -70°C using X-OMAT-AR film (Kodak).

6.7.6 Isolating DNA from λ-clones

To obtain DNA from the Clontech mouse cDNA library, isolated plaques were amplified by PCR using primers to the GT11 vector arms (CTCCTGGAGCCCGTCAGTATC and CTGGTAATGGTAGCGACCGGC). The PCR products were cloned into PCR2.1 using an Original TA cloning kit (InVitrogen) according to manufacturers instructions.

Excision of pBluescript SK+/- phagemid Uni-ZAP libraries.

200µl XL1-Blue MRF' cells (OD1.0) were mixed with 10^5 PFU pure λ ZAP library plaque and 10^9 PFU ExAssist helper phage (InVitrogen). This was incubated at 37°C for 15 minutes to allow adsoption, then 5ml L-Broth added and a further 2 hour incubation carried out at 37°C to allow excision of the phagemid. Remaining phage were killed by incubation at 72°C for 20 minutes, cellular debris spun down at

500xg for 10 minutes and the phagemid containing supernatant recovered. XLOLR cells could now be transfected with phagemid. A series of tenfold dilutions of phagemid were mixed with 200µl of fresh XLOLR cells (OD1.0), incubated for 15 minutes at 37°C and then plated onto Ampicillin containing plates and incubated overnight at 37°C.

Excision of phagemid from λ-GET libraries

Excision of plasmid from λ-GET library phage was carried out according to Nehls et al., 1994. E-coli strain BNN132, containing CRE recombinase, was infected with λ-GET phage as for library titration (section 6.7.3) then streaked onto Ampicillin containing plates and incubated overnight at 37°C.

6.8 Southern Blotting

6.8.1 Preparation of genomic DNA

TNES : 50mM Tris (pH7.5), 400mM NaCl, 100mM EDTA, 0.5% SDS.

Mouse tissue (either spleen, liver or kidney) was homogenised in 1.5ml TNES then washed out of the homogenizer into a 4ml tube with a further 1ml TNES. 20µl 50mg/ml proteinase K was added and the tissue digested overnight at 55°C. The sample was split into three eppendorf tubes filled with 2.6M NaCl. These were shaken vigorously for 15 seconds and spun for 10 minutes at 13000rpm to pellet debris. Samples were then pooled back into a large tube leaving the debris behind and 2 volumes of ethanol added for DNA precipitation. DNA was spooled onto a blue loop and re-suspended in 500µl of dH_2O.

6.8.2 Preparation of Genomic DNA digests for southern blot analysis

Genomic DNA, from livers of adult AKR/J and DBA/2J mice, was digested with Acc1, Apa1, EcoR1, Nde1 and Pst1 (BCL). 10µg of DNA was digested, each digest being set up in duplicate, once with AKR/J DNA and once with DBA/2J DNA. The digests were run out on 1% agarose alongside 10µl of 1kb marker (GibcoBRL) at 70v for 16 hours.

6.8.3 Southern transfer of DNA onto Nitrocellulose membranes

Gels were photographed alongside a ruler before blotting so that the size of hybridising bands could later be confirmed. Gels were washed in denature for 30 minutes, neutraliser for 30 minutes and 20x SSC for at least 30 seconds (solutions as in section 6.7.5) before DNA was transferred to membranes by Southern blotting, essentially as described in Sambrook et al 1987. The gel was placed on top of a 10MM paper (Whatman) wick laid over a glass plate with the edges dipping into 20x SSC. The membrane, cut just larger than the gel, was placed on top of the gel ensuring that no air bubbles were trapped. This was sealed around the edges with Saran wrap (Dow chemical company) to ensure that capillary flow could only occur through the gel and membrane. Four pieces of 3MM paper (Whatman) wetted with 20x SSC were placed on top of the membrane, then a stack of paper towels and a brick on top of this. This apparatus was left overnight for transfer to occur.

For genomic DNA blots Zeta-Probe GT membrane (Biorad) was used. For all other blots Hybond-N (Amersham) was used. After blotting, DNA was cross linked to the membrane by exposure to UV for Hybond-N and by baking at 80°C for 30 minutes for Zeta-Probe.

6.8.4 Filter Hybridisation

Hybond Hyb mix: : 6x SSC, 10% dextran sulphate, 4x Denhardts, 0.1% NaPPi, 0.5% SDS/SLS

Zeta-Probe GT blots were prehybridised in Hybond Hyb. mix at 65°C for 4 hours. Hybridisation was carried out overnight at 65°C in the same solution with the addition of ^{32}P-dCTP labelled probe. Two washes of 5 minutes were carried out at 30°C in 2x SSC then a further two for 30 minutes at 65°C in 2x SSC, 0.1% SDS. The blot was then sealed into plastic and exposed to X-OMAT-AR film (Kodak) overnight at -70°C.

Hybond-N blots were hybridised and washed exactly as for λ-library screening (section 6.7.5)

6.8. 5 Stripping radioactive probe from membranes

The same southern blot (or library filter) could be used more than once by removing the probe after use. This was done by pouring boiling 0.1% SDS solution directly on to the membrane which was then allowed to cool to room temperature. The membrane was then sealed into polythene to keep it damp until used again.

6.9 Sequencing

6.9.1 Manual sequencing

Glycerol tolerant buffer: 216g Tris base, 72g Taurine, 4g $Na_2EDTA.2H_2O$ and dH_2O to 1 litre.

Stop Mix: 95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF.

Manual sequencing was carried out using ^{33}P-ddNTP terminator cycle sequencing (Amersham life sciences) according to manufacturers instructions except that reaction volumes were reduced by half. Reaction mixtures were set up containing 1µl reaction buffer (supplied by manufacture), 1µl primer [15ng/µl], 1µl Thermo Sequenase (supplied by manufacturer), 50-500ng DNA template and dH_2O to 10µl. Termination mix containing 1µl of termination master mix and 0.25µl ^{33}P-ddNTP labelled terminator was set up for each terminator (ddATP, ddCTP, ddGTP, ddTTP). 2.25µl of reaction mix was then mixed with 1.25µl of each termination mix and the four samples overlaid with mineral oil and cycled 50 times as follows: 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute.

The resulting sequence was run out using Biorad apparatus using 6% GTG acrylamide (Amersham life sciences). 40ml acrylamide was mixed with 200µl 10% ammonium persulphate and 40µl TEMED for the main body of the gel. The apparatus had previously been sealed with a small amount of acrylamide according to manufacturers instructions. Gels were run at 90W (large 20cm gels) or 60W (small 40cm gels) in Glycerol tolerant buffer.

Before running, 2µl of stop mix was added to each sample which was then denatured at 80°C for 3 minutes. 2.5µl of reaction was run on the gel. After running,

gels were transferred to 3MM (Whatmann) paper, covered in Saran wrap and dried at 80°C using a vacuum gel drier (Hoefer). Sequencing was visualised by exposure to BioMax film (Kodak) at room temperature.

6.9.2 Automated fluorescent sequencing

Most plasmid sequencing was carried out using ABI automated fluorescent sequencing using an ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (P.E. Applied Biosystems) according to manufacturers instructions. 1-3μg of DNA template (Qiagen maxi-prep) to a total volume of 11μl in dH_2O was mixed with 8μl dRhodamine terminator ready reaction mix (supplied by manufacturer) and 1μl 3.2nM primer. This was topped with a drop of mineral oil before cycling 25 times in a Hybaid Omnigene PCR machine as follows: 94°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes. The reaction was then ethanol precipitated and re-suspended in loading buffer before electrophoresis on an ABI 377A machine. As each dideoxynucleotide terminator in the reaction mix is labelled with a different fluorescent dye the four termination reactions are carried out simultaneously in one tube

Sequences were initially analysed using Applied Biosystems 377A software then using GCG software (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, Wisconsin).

6.10 Histology

PBS: 0.85 NaCl, 0.02%KCl, 0.02% MPO_4 (pH 7.3)

6.10.1 Paraffin embedding/Sectioning

Embryo preparation - mouse embryos

Embryos were dissected into ice cold PBS before fixing overnight at 4°C in 4% paraformaldeyde (PFA)(Sigma) in PBS (When embryos were to be used for RNA *in-situ* hybridisation all solutions were kept RNAse free). Embryos were then washed in PBS, then in 30% ethanol, 50% ethanol and 70% ethanol in PBS. Length of time in each solution was dependent on embryonic stage, ie. 15, 30, 45, 60 and 75minutes for E9.5, E10.5, E11.5, E12.5/E13.5 and E14.5/15.5 respectively. Embryos of E11.5

and beyond, including the E12.5 chimeric mouse heads, were then processed using a VIP Tissue-Tek processor. The program used was as follows: 70% ethanol, 85% ethanol, 95% ethanol, 100% ethanol, 100% ethanol, 2 changes of ethanol, and four changes of wax (at 58°C). For E11.5 - E13.5 embryos each step was for 1 hour, and at E14.5-E15.5 each stage was 1.25 hour. Pressure and vacuum cycles were used at E13.5-E15.5.

The E9.5 and E10.5 embryos were processed by hand as follows. They were washed 3 times in 100% Ethanol for 15 minutes at E9.5 or 30 minutes at E10.5, then washed twice in Xylene, for 10 minutes at E9.5 or 20 minutes at E10.5. They were then taken through 3x 1 hour changes of molten (56°C) paraffin wax before being embedded in plastic moulds.

Alternatively embryos could be stored indefinitely in 70% Ethanol at -20°C before processing.

Embryo preparation - zebrafish embryos

Fish were fixed overnight in 4% PFA at 4°C before being taken through a series of alcohols in PBS: 50% ethanol, 70% ethanol, 85% ethanol, 95% ethanol twice and 3x 100% ethanol, each for 5 minutes. They were then cleared in 2 changes of Histoclear (National Diagnostics) for 20 minutes, the second at 56°C and taken through one 20 minute change of 1:1 Histoclear : molten wax (56°C) then two 20 minute changes of molten paraffin wax (56°C) before embedding in wax in plastic moulds.

Slide preparation (TESPA coating)

Slides were coated before mounting sections to prevent the sections from falling off during staining. They were rinsed for 20 seconds in each of the following solutions; 1) 10% HCL in 70% ethanol, 2) sterile distilled water, 3) 100% Acetone, then air dried before the final 3 rinses also for 20 seconds; 4) 2% Tespa in acetone, 5) 100% Acetone, 6) 100% Acetone, then air dried before use. These slides could be stored for up to 4 weeks with desiccant before use. For RNA *in-situ* work all these solutions were kept RNAse free.

Sectioning

The wax block containing the embryo was mounted on a wooden block in the orientation desired, before cutting using a microtome. The sections were cut at between 6 and 10μm and mounted on slides by floating sections on to slides in a water bath at 45-50°C. Again for RNA *in-situ* work everything was kept RNAse free. Slides were then baked at 60°C overnight before staining and mounting or *in-situ* hybridisation.

6.10.2 Vibratome Sectioning of mouse embryos

Aqueous mountant : 10g gelatin, 60mls distilled water, 70ml glycerine, 0.25g Phenol.
Embedding medium : 0.5% gelatin, 20% sucrose, 15.5% bovine serum albumin (BSA) in PBS

Prior to sectioning, the embryos were washed in PBS overnight, in 4% sucrose in PBS again overnight, then in 20% sucrose in PBS for several hours (6-7 hours), before finally washing overnight in embedding medium. Embryos were fixed in 25% gluteraldehyde (Sigma) for 20, 25 and 30mins for E10.5, E11.5 and E12.5 embryos respectively and dried on a tissue before being embedded in 6ml of embedding medium with 400μl of 25% gluteraldehyde added last to solidify the medium. The block was allowed to set then sectioned using a vibratome.

Sections were cut at 100μm and mounted in aqueous mountant (at 50°C.) These slides dry out after a month or two of mounting so were photographed immediately.

6.11 *In-Situ* Hybridisation

6.11.1 Radioactive RNA *In-Situ* Hybridisation

35S Labelling of Probes

Probes were transcribed from the appropriate promoter for each plasmid as detailed in table 6.6. The transcription reaction was set up as follows adding these substances in this order:

6μl 5 x transcription buffer (Boehringer Mannheim),

1µl each 10mM rATP, rCTP and rGTP (Pharmacia Biotech)

1µl 1M DTT (dithiothreitol - Melford),

3µl H_2O

12µl ^{35}S rUTP (1mCi/100µl) (Amersham),

5µl DNA template (total 0.5 - 1µg) - linearised

1.2µl RNase inhibitor (Boehringer Mannheim),

0.8µl T7 or T3 polymerase (Boehringer Mannheim).

The reaction was incubated for 25 minutes at 37°C then a further 0.8µl of polymerase added and the reaction incubated for a further 25 minutes. 2.0µl of 10mg/ml tRNA (Sigma) and 1.0µl DNAse (Boehringer Mannheim) was added to remove the DNA template before incubation for a further 10 minutes at 37°C. 2.0µl of 100mM EDTA was added to stop the reaction and the total volume made up to 200µl with TE pH8.0 containing 50mM DTT.

A Microcon 30 (Amicon - Millipore) column was used to purify the probe. The probe was put into the microcon tube following manufacturers instructions. This was spun at 13000rpm for 15 minutes. 50µl TE/50mM DTT was then added to the reservoir of the filter and the device spun again for 5 minutes. 25µl of TE/50mM DTT was then added to the filter and the microcon incubated on ice for 15 minutes. The column was inverted and inserted into the recovery tube and spun for 5 minutes to collect the clean probe. 1µl of this probe was diluted into 19µl of TE/50mM DTT for scintillation counting.

Table 6.6 : *In-situ* probe production summary

Plasmid	Vector	Cut with....	Transcribe with....	Orientation
murine probes				
***Dach1*- 5A**	PCR 2.1	Hind III	T7	antisense
***Dach1*-3'UTR**	PCR 2.1	Hind III	T7	antisense
***Dach1*- 500**	pBluescript SK +/-	BamHI/ HindIII	T7/T3	antisense/sense
***Dach1*- 3A**	PCR 2.1	Hind III	T7	antisense
mi	pBluescript SK +/-	BamHI	T7	antisense
Trp2	pBluescript SK +/-	HindIII	T7	antisense
Pax6	pBluescript SK +/-	XbaI	T7	antisense
zebrafish probes				
zfDachA	pBluescript SK +/-	EcoRI	T7	antisense
zfDachB	pBluescript SK +/-	PstI	T7	antisense
zfDachC	pBluescript SK +/-	EcoRI	T7	antisense
***zfDachA*/hr**	pBluescript SK +/-	PstI	T7	antisense
zfPax6	pBluescript SK +/-	SmaI	T7	antisense
zfRx1	pBluescript SK +/-	EcoRI	T7	antisence
zfEn1	pBluescript SK -	EcoRI	T7	antisense
zfEn3	pBluescript SK -	BamHI	T3	antisense

Quantifying probe produced by scintillation counting

10µl of the diluted probe was applied to the centres of two glass microfibre filters (Whatman) which were allowed to air dry before one of the pair was trichloroacetic acid (TCA) precipitated to remove unincorporated nucleotides. This was achieved by washing this filter 3 times with 15 ml 5%TCA then once with 10ml 100% ethanol. When dry the filters were placed into scintillation vials containing 5ml Scintillation fluid (ICN). Counts/µl were then ascertained using a Packard Tri-Carb 1500 liquid scintillation counter.

Probe was then mixed with hybridisation solution (see below) to a concentration of 2.2×10^6 counts/ml (1.1×10^5 per slide). Just before the probe was used for hybridisation 50mM DTT was added to this solution.

RINS method

Hybridisation solution: 50% formamide, 10% dextran sulphate, 1x Denhardts, 20mM Tris, 0.3M NaCl, 5mM EDTA, 10mM sodium pyrophosphate, 0.5mg/ml tRNA
Proteinase K buffer: 50mM Tris, 5mM EDTA
NTE: 0.5M NaCl, 10mM Tris, 5mM EDTA, pH7.5
HSW: 50%formamide, 2x SSC, 50mM DTT
20x SSC: 175.3g NaCl, 88.2g sodium citrate in 1 litre distilled water (pH7.0)

Pre-Hybridisation:

Sections were cleared using two 5 minute changes of Xylene, then rehydrated through an ethanol series in PBS, 100%, 100%, 90%, 70%, 50%, 30% each for 2 minutes. They were then washed in PBS for 2 minutes before being fixed in 4% PFA (pH7.2) for 10 minutes. A further 2 washes in PBS were carried out before treatment with 20 µg/ml proteinase K in proteinase K buffer. Slides were then washed for 1 minute in PBS before re-fixing for 2 minutes in 4% PFA. A 10 second wash was then carried out in distilled water and a 30 second wash in 0.1M TEA pH8.0 (Triethanolamine - Sigma) before treatment with 0.1M TEA, acetic anhydride (600µl in 200mls) twice for 5 minutes. Further washes were carried out in PBS for 2 minutes

and 0.85% NaCl for 2 minutes before dehydrating through an ethanol series, 30%, 50%, 70%, 90% each for 1 minute then 100% three times for 5 minutes. Slides were then air dried before hybridisation.

Hybridisation:

50µl of hybridisation solution containing probe was heated to 80°C for 2 minutes to denature the probe then cooled on ice before being added to the section. A glass coverslip was applied ensuring that no air bubbles were trapped and the slides put into a hybridisation box which also contained a tissue soaked in 5 ml 50% formamide, 5xSSC. The box was then sealed and incubated at 55°C overnight.

Post-hybridisation:

Slides were rinsed in 5x SSC then washed in 5x SSC, 10mM DTT for approximately 20 minutes at 55°C then a high stringency wash in HSW was carried out for 30 minutes at 65°C. The slides were washed 3 times at 37°C in NTE for 10 minutes, then for 30 minutes at 37°C in NTE with 2µl/ml of 10mg/ml RNAse A, and for a further 5 minutes in NTE also at 37°C. A second high stringency wash was carried out in HSW for 30 minutes at 65°C before washing four times in 2x SSC for 10 minutes and four times in 0.1x SSC for 5 minutes. Sections were then dehydrated through an ethanol series of 30%, 50%, 70% and 90% ethanol for 1 minute each and then rinsed twice for 5 minutes in 100% ethanol before air drying.

Autoradiography:

Slides were dipped in 1:1 water:autoradiographic emulsion (Ilford K5) heated to 40-42°C. Slides were dipped into the emulsion twice and drained before placing in a light tight box with silica gel. The slides were left to develop for 3 weeks or more, depending on the probe used. Test slides were always developed first to ascertain the length of time necessary for each batch of probe.

Developing:

Slides were placed in D19 (Kodak) solution for 4 minutes followed by a 10 second rinse in water before placing into 1:3 AMFIX (Champion) : water, twice for 5 minutes. Slides were washed twice in distilled water for 10 minutes and once in the distilled water the slides could be taken into the light.

Staining:

Sections were stained for morphology using 1% methyl green (Sigma). They were placed into the methyl green solution for approximately 10 seconds and then washed in running tap water. The backs of the slides were scraped to remove excess developing solutions and left to air dry before mounting using DePeX mounting medium (Boehringer Mannheim).

6.11.2 Whole Mount In-Situ Hybridisation with DIG Labelled Probes

Probe Preparation

Templates were digested and transcribed with appropriate enzymes as detailed in table 6.6.

DIG Labelling of Probes

Probe was labelled using a DIG labelling kit (Boehringer Mannheim), according to manufacturers instructions. Labelling was tested by running out a 10µl aliquot of the probe on a 1% agarose gel and by using DIG test and control strips (Boehringer Mannheim) according to manufacturers instructions.

Whole Mount *In-Situ* to Mouse Embryos

PBT: PBS, 0.1% Triton X-100 (Sigma)

Hybridisation solution: 50% formamide (Sigma), 5x SSC, 2% blocking powder (Boehringer), 0.1% Triton X-100 (Sigma), 0.5% CHAPS (Sigma), 5mM EDTA, 50µg/ml Heparin (Sigma), and 1mg/ml yeast RNA (Sigma)

TNT: 100mM TRIS (pH7.5), 150mM NaCl, and 0.1% Triton X-100

Blocking solution: TNT, 2%BSA (BDH), 15% heat inactivated sheep serum (Sigma)

NMT: 100mM NaCl, 50mM $MgCl_2$, 100mM Tris (pH9.5)

Collection and Preparation of Embryos

Embryos were dissected into ice cold PBS then immediately fixed overnight in 4% paraformaldehyde (Sigma) in PBS at 4°C. If genotyping was necessary then the yolk sac and membranes were kept for this purpose also in ice cold PBS. Embryos were dehydrated through a series of 25%, 50% and 75% methanol in PBT (PBS + 0.1% Tween 20 -Sigma) for 30 minutes each at E10.5 and 45 minutes each at E11.5 and E12.5. They could then be stored indefinitely in methanol at -20°C.

Whole mount In-Situ Method (Mouse)

The method used is based on several protocols (Hemmati-Brivanlou et al., 1990; Wilkinson, 1991; Izpisua-Belmonte et al., 1993; Hecksher-Sørensen et al., 1998).

Embryos were rehydrated through a series of 70%, 50% and 25% methanol in PBS for 5 minutes each, then washed 3 times for 5 minutes in PBT. Treatment in proteinase K (10μg/ml in PBT) was carried out dependant on the age of the embryo; E10.5, E11.5 and E12.5 were treated for 30, 40 and 50 minutes respectively. Embryos were then refixed in 4% PFA for 45 minutes at room temperature.

Embryos were washed twice in hybridisation solution then prehybridisation was carried out at 65°C in hybridisation solution for 1 hour then for 4 hours in fresh hybridisation solution. 1μg/ml DIG labelled RNA probe was denatured at 80°C for 3 minutes in 50μl hybridisation mix, which was then added to the embryos and hybridisation carried out overnight at 65°C.

Post-hybridisation washes were carried out in decreasing concentrations of hybridisation mix in 2x SSC (75%, 50%, 25%) at room temperature, followed by 2 washes in 2x SSC, 0.1% CHAPS and 2 washes in 0.2x SSC, 0.1% CHAPS all for 30 minutes at 55°C. Embryos were then washed twice in TNT, then blocked for 3-4 hours in blocking solution at 4°C. Fresh blocking solution containing anti-DIG-AP fragments (Boehringer) at a dilution of 1/2000 was added and incubation carried out overnight at 4°C.

Embryos were washed four times for 1 hour, once overnight then twice for 30 minutes in TNT, 0.1% BSA then three times for 10 minutes in NMT. Staining was carried out in NMT, 3.5μl/ml BCIP (Boehringer) (50mg/ml in 100%

dimethylformamide) and 4.5µl/ml NBT (Boehringer) (75mg/ml in 70% dimethylformamide). At this point it was important that the embryos should be in glass vials rather than plastic, to prevent the stain from precipitating. When developed, embryos were rinsed in PBT to stop the reaction then fixed overnight in 4% paraformaldehyde.

Whole Mount *In-Situ* Hybridisation to Zebrafish Embryos

Collection and preparation of Embryos

Eggs were collected as soon as possible after laying and sorted into synchronous groups of 50 in petri dishes filled with system water containing methylene blue. These were incubated at 28.5°C until the embryos reached the required stage. For analysis of embryos older than 24hpf (hours post fertilisation) wildtype embryos were treated with 0.0035% PTU (1-phenyl-2-thiourea, Sigma) in 10% Hank's solution (see section 6.10.2) to remove pigment. Alternatively *gol*$^{-/-}$ embryos, which have reduced pigment levels, were used.
Embryos were then transferred to 4% PFA in PBS and fixed overnight at 4°C. Chorions were removed after fixing from embryos up to 24hpf and before fixing for embryos of 24hpf. After fixing embryos were dehydrated through a series of 25%, 50%, 75% and 100% Methanol in PBS for 10 minutes in each. They could then be stored indefinitely at -20°C in 100% Methanol.

Whole Mount *In-Situ* Method (Zebrafish)

PBTw: PBS, 0.1% Tween (Sigma)
Fish hybridisation solution: 50% formamide (Sigma), 5x SSC, 500µg/ml tRNA (Sigma), 5µg/ml Heparin (Sigma), 0.1% Tween 20 (Sigma), 9.2mM Citric acid
Post hybridisation solution: 50% formamide, 5xSSC, 0.1% Tween 20
BCL buffer: 0.1M Tris/HCL (pH9.5), 50mM $MgCl_2$, 0.1M NaCl, 0.1% Tween

For all steps until the colour development reaction the embryos were treated in eppendorf tubes, at this point they were transferred to a 24 well culture dish.

Embryos were rehydrated through 75%, 50% and 25% methanol in PBS for 10 minutes each, then washed four times for 5 minutes in PBTw. Embryos older than 24 hours were incubated for 20 minutes in proteinase K (10μg/ml in PBTw) at room temperature. Younger embryos waited in PBTw or proceeded straight to refixation. All embryos were refixed at room temperature in 4% PFA for 20 minutes to maintain morphology. 5 rinses of three minutes in 1x PBTw were carried out before pre-hybridisation for 1-2 hours at 70°C in fish hybridisation solution. Just before hybridisation DIG labelled probe was mixed with 50μl of hybridisation solution and heated to 80°C for 3 minutes before being made up to 1μg/ml in hybridisation solution. Hybridisation was then carried out in 200μl of this solution per tube, at 70°C overnight.

Post-hybridisation washes were carried out in a series containing decreasing amounts of post-hybridisation solution [PH], 75% PH / 25% 2xSSC, 50% PH / 50% 2xSSC, 25% PH / 75% 2xSSC and 100% 2xSSC each for 10 minutes at 70°C. Two further washes in 0.2x SSC for 30 minutes were also carried out at 70°C followed by a series of 5 minute washes at room temperature in 75% 0.2xSSC / 25% PBTw, 50% 0.2xSSC / 50% PBTw, 25% 2xSSC / 75% PBTw. Three washes in PBTw for 10 minutes were performed before blocking at 4°C for 4 hours in PBTw, 2% sheep serum, 2mg/ml BSA (BDH). This solution was replaced with fresh blocking solution containing 1/1000 dilution of anti-DIG-AP fab fragments (Boehringer Mannheim) and left overnight at 4°C on a shaker.

Post antibody washes were carried out in PBTw, washing as many times as possible in 2 hours (5-6 washes). The embryos were then equilibrated in BCL buffer for 5 minutes and transferred to a 24 well culture dish. Staining was carried out in BCL buffer, 3.5μl/ml BCIP (Boehringer) (50mg/ml in 100% dimethylformamaide) and 4.5μl/ml NBT (Boehringer) (75mg/ml in 70% dimethylformamide). Once colour developed the reaction was stopped by washing in PBTw then fixing in 4% PFA. Embryos were stored in the dark in 4% PFA.

Clearing and Photography

For photography whole embryos were cleared to remove obscuring colour from the yolk, and then mounted using bridged coverslips. These were prepared by

building two stacks of small coverslips at either end of the slide, stuck down with mounting medium (either 3 or 4 were used depending on the nature of the picture to be taken and the size of the embryos). The embryo was then mounted between them and a large coverslip applied over the top.

Embryos were dehydrated through 50% ethanol in 1x PBS then 100% ethanol. Clearing was then carried out in 2:1 benzoyl alcohol : benzoyl benzoate (BBA); It was extremely important that embryos were properly dehydrated before placing in BBA or they blistered. The extent of clearing was monitored using a dissecting microscope and when sufficiently cleared embryos were mounted in DePeX mounting medium (Boehringer Mannheim) The slide was then stored in the dark until photographed as a light sensitive reaction darkens the embryo. For this reason slides were always prepared immediately before photography. If desired the yolk could be removed prior to clearing and the embryo mounted flat.

Photography was carried out using a Zeiss Axioplan II microscope using DIC optics.

6.12 Zebrafish Over Expression Techniques

Table 6.7 : plasmids used for capped RNA synthesis.

Plasmid	**Insert**	**Vector**	**Linearised with.**	**Transcribe with.**
BS-1C	*ZfDachA*	pBluescript SK+/-	Asp718	T3 polymerase
CS-1/hr	*zfDachA* HindIII/EcoRI fragment	pCS2+	NotI	SP6 polymerase
PCS2+nβgal	β-galactosidase	pCS2+	Not1	SP6 polymerase

6.12.1 Synthesis of capped RNA

A 5' methyl-guanylyl analogue cap which was added to the transcript is extremely important to allow translation to occur efficiently and the transcript must contain a polyA tail to prevent degradation. The pCS2+ vector includes a 5' globin flanking region together with a polyA tail which can be used to add these stabilisation sequences to templates which do not contain their own. Where the template does contain a polyA tail injection of transcripts made from other vectors such as pBluescript can be successfully used.

Transcription

It was very important that all reagents used were maintained in an RNAse free state (see section 6.5.2).

DNA template was linearised by restriction digestion with an appropriate enzyme (see table 6.7) and cleaned before use using a Geneclean II kit (see section 6.3.7). 5µg template DNA in 28.5µl dH_2O was mixed with 5µl transcription buffer, 5µl 0.1M DTT, 2µl rNTP mix (25mM rATP, 25mM rCTP, 25mM rUTP, 2.5mMGTP), 5µl 5mM $m^7G(5')ppp(5')G$ (P'-5'-(7-methyl)-ganosine-P^3-5'-guanosine-triphosphate, Boehringer Mannheim), 2µl RNAse inhibitor and 2.5µl of appropriate RNA polymerase. This was mixed and incubated for 30 minutes at 37°C before the addition of 2.5µl 10mM rGTP and incubation for a further 1 hour. 5µl of DNAse was added and the sample incubated for a further 30 minutes at 37°C. A 1µl aliquot was taken at this point for analysis by agarose gel electrophoresis (below).

RNA was now phenol / chloroform extracted twice, chloroform extracted once and ethanol precipitated as in section 6.3.4. RNA was dissolved in 50µl DEPC treated dH_2O and quantified by measuring the A_{260}. A 1µl aliquot of the final RNA preparation was taken and run out with the previous aliquot on an RNAse free 1% TBE gel to ensure that a single clean band was present.

RNA was stored in aliquots at -70°C.

6.12.2 Injecting RNA into zebrafish embryos

Hanks solution #1: 8.0g NaCl, 0.4g KCl in 100ml DEPC dH_2O

Hanks solution #2: 0.358g Na_2HPO_4, 0.6g KH_2PO_4 in 100ml DEPC dH_2O

Hanks solution #4: 0.72g $CaCl_2$ in 50ml DEPC dH_2O

Hanks solution #5: 1.23g $MgSO_4.7H_2O$ in 50ml DEPC dH_2O

Sodium bicarbonate : 0.35g $NaHCO_3$ in 10ml DEPC dH_2O

Hanks Premix : 10ml Hanks #1, 1ml Hanks #2, 1ml Hanks #4, 86ml DEPC dH_2O, 1ml Hanks #5 combined in this order.

Hanks Final : 9.9ml Hanks Premix, 0.1 ml fresh sodium bicarbonate solution.

A petri dish was prepared containing a layer of 1.5% agarose made with 10% Hanks final solution in DEPC dH_2O into which several parallel grooves, to hold the embryos while injecting, had been made.

2µl injection RNA diluted to the desired concentration (range 25ng/µl to 250ng/µl) with dH_2O was mixed with 1µl 1:5 1% phenol red (Sigma):distilled water and 1µl 500ng/ul pCS2+nβgal. The RNA was then taken up into a fine glass needle by capillary action. Injections were carried out using a 1M 300 Microinjector (Narishige) with nitrogen gas.

Embryos were collected at the 2-8 cell stage and placed into the pre-prepared petri dish (above) in 10% Hanks solution containing 1/1000 streptamycin to prevent infection of injected embryos. Embryos were injected at 2-16 cell stages into a single blastomere, the phenol red showing the site of injection. After injection embryos were transferred to a fresh petri dish containing 10% Hanks in DEPC dH_2O with 1/1000 Streptamycin and methylene blue (Sigma) to prevent fungus growth. The petri dish was then placed in a 28.5°C incubator until embryos were ready for analysis. Injected embryos were analysed at 24-30 hpf (hours post fertilisation).

6.12.3 Analysis

Methyl Cellulose Mounting :

Embryo Medium : 1.0ml Hank's #1, 0.1ml Hank's #2, 0.1ml Hanks #4, 95.9ml distilled water, 1.0ml Hank's #5 (pH 7.2) combined in that order (see section 6.12.2 for Hank's solutions)

Tricaine (3-amino benzoic acidethylester - Sigma) : 400mg tricaine powder, 97.9ml distilled water, 2.1ml 1M Tris (pH7) aliquoted and stored at -20°C. (4.2ml used in 100ml clean tank water for anaesthetisation.)

Live embryos could be observed under a compound microscope by mounting embryos in a drop of 3% methyl cellulose in embryo medium with a drop of undiluted tricaine using a depression slide as described in Westerfield 1993. The embryo could be carefully orientated using fine forceps and after coverslipping photography was possible. Removal of the embryo from the slide was achieved by sliding off the coverslip and dropping the whole slide into embryo medium until the embryo could be teased free.

Visualising β-Gal :

Staining buffer : 154µl 0.2M Na_2HPO_4, 46µl 0.2M NaH_2PO_4, 60µl 5MNaCl, 60µl 0.1M $K_4Fe_3(CN_6)_6$, 60µl 0.1M $K_3Fe_2(CN_6)_6$ and dH_2O to 2ml

Staining solution : Staining buffer plus 25µl 40mg/ml X-gal (Melford laboratories) in DMSO.

Embryos were dechorionated and fixed at 4°C for 40 minutes in 4% PFA (Sigma), 0.02% glutaraldehyde (Sigma), 2µl/ml NP40 (Sigma). They were then washed 5 times for 5 minutes in 1x PBS, 0.02% NP40 then once in staining buffer. Embryos were then transferred to staining solution in a 24 well plate, wrapped in aluminium foil and incubated at room temperature until colour development had occurred (usually approximately 30 minutes). To stop the reaction embryos were washed in 1x PBS, 0.02% NP40. If *in-situ* hybridisation was to be performed on

these same embryos they were then refixed in 4x PFA (pH 7.2) at 4°C for at least 2 hours but no more than 24 hours.

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Appendices

Appendix 1 : Oligonucleotides used for PCR and cloning.

Oligo no.	Sequence	Location	Use
Q800 (*Pax6*$^{sey\text{-}neu}$ F)	GCA ACA CTC CTA GTC ACA TTC C	*Pax6* nucleotide 1039 - 1060	Chimera genotyping for *Pax6*$^{sey\text{-}neu}$
Q801 (*Pax6*$^{sey\text{-}neu}$ R)	ATG GAA CCT GAT GTG AAG GAG G	*Pax6* nucleotide 1124 - 1125	Chimera genotyping for *Pax6*$^{sey\text{-}neu}$
Q802 (*Pax6*^{sey} R)	CTT TCT CCA GAG CCT CAA TCT G	*Pax6* nucleotide 865 – 886	Chimera genotyping for *Pax6*^{sey}
Q803 (*Pax6*^{sey} F)	GGC TGC CAG CAA CAG GAA GGA	*Pax6* nucleotide 730 – 750	Chimera genotyping for *Pax6*^{sey}
S305 (M13 reverse)	GGA AAC AGC TAT GAC CAT G	Cloning vectors: Bluescript SK, PCR2.1	Sequencing and colony PCR
U315 (T7)	TAA TAC GAC TCA CTA TAG GG	Cloning vectors: CS2+, Bluescript SK, PCR2.1	Sequencing and colony PCR
V469 (Asp718 - XhoI linker R)	TCG AGC AGA TAT CTG GAG GTA C		Cloning linker
V468 (Asp718 - XhoI linker F)	CTC CAG ATA TCT GC		Cloning linker
V471 (PstI – EcoRI linker R)	GAG CTC TAG AAG TG		Cloning linker
V470 (PstI – EcoRI linker F)	AAT TCA CTT CTA GAG CTC TGC A		Cloning linker
P890 (*Dach1* rt extra rev)	AGG AAG TTC CAG TCC ATT TGG	*Dach 1* nucloetide 773 – 793	RT-PCR primer
P889 (*Dach1* rt extra for)	CAT GAA TTC AAG TGT TGG CAG	*Dach 1* nucleotide 607 – 627	RT-PCR primer
N991 (*Dach1* rt for)	CTA TGA GCA ACT ATC ATG CCA G	*Dach 1* nucleotide 549 – 570	RT-PCR primer
P26 (*Dach1* rt rev)	TTG CCA TGG TGA CAG ATG CTG	*Dach 1* nucleotide 537 –557	RT-PCR primer
P396 (Dach-box N for)	GCT TTC GAC CTG TTC CTG AAG	*Dach 1* nucleotide 138 – 159	Southern blot and library screening
P432 (Dach-box N rev)	CTT TGA GTC CTC TTA GGA GGC	*Dach 1* nucleotide 369 – 382	Southern blot and library screening
Q827 (Dach-box C for)	AAT GGC CTG TCC ATG AAC CAG	*Dach1* nucleotide 1100 - 1120	Southern blotting
Q829 (Dach-box C rev)	CTT TTG TTC CAT GGC CAG CTG	*Dach 1* nucleotide 1496 – 1516	Southern blotting
R150 (Dach-500 for)	ATC TGT CAC CAT GGC AAT GAG	*Dach1* nucleotides 841-861	Subcloning to produce *in-situ* probe
R151 (Dach-500 rev)	CAT CAG GAA ACA GAA AGG GAG	*Dach 1* nucleotides 1317-1337	Subcloning to produce *in-situ* probe
N752 (λ-GT 11)	CTG GTA ATG GTA GCG ACC GGC	λ-GT 11 vector arm	Recovery of insert from λ-GT 11
N751 (λ-GT 11)	CTC CTG GAG CCC	λ-GT 11 vector	Recovery of insert

	GTC AGT ATC	arm	from λ-GT 11
S925 (*zfDach A*)	GTG AAA GTG GCC TCG TTC AC	*zfDachA* nucleotide 372 – 391	Screening zebrafish library
S924 (*zf Dach A*)	AAC TGG CAT TGG TGC AGT CG	*zfDachA* nucleotide 617 – 636	Screening zebrafish library

Appendix 2a : *Dachshund* sequence data

Mouse *Dachshund 1 (mDach1)*

```
   1  TTCCGGTGCT GCTGCTACTG CTGCTGCTGC TGCTACTGCT GCTGCTGCTG
  51  CCGGTGAGTG CAAAATGGTG GATCTGAGAG GGGCCAAAGT GGCTTCCTTT
 101  ACGGTGGAGG GCTGCGAGCT GATCTGCCTG CCCCAGGCTT TCGACCTGTT
 151  CCTGAAGCAC TTGGTGGGGG GCTTGCACAC CGTCTACACC AAGCTGAAGC
 201  GGTTGGAGAT CACGCCGGTG GTGTGCAATG TGGAACAGGT TCGCATCCTG
 251  AGGGGACTGG GGGCCATCCA GCCCGGAGTG AACCGCTGCA AACTCATCTC
 301  CAGGAAGGAC TTCGAGACCC TCTACAATGA CTGCACCAAC GCCAGTTCCA
 351  GACCTGGAAG GCCTCCTAAG AGGACTCAAA GTGTCACTTC CCCAGAGAAC
 401  TCTCACATCA TGCCGCATTC TGTCCCTGGC CTCATGTCTC CTGGAATCAT
 451  TCCACCAACA GGTCTGACTG CAGCTGCTGC AGCTGCTGCA GCTGCTACCA
 501  ATGCAGCTAT TGCTGAAGCA ATGAAGGTGA AAAAAATAAA ATTAGAAGCT
 551  ATGAGCAACT ATCATGCCAG TAACAACCAA CATGGAGCAG ATTCTGAAAA
 601  CGGGGACATG AATTCAAGTG TTGGCAGCAG TGGTGGTTCT TGGGATAAGG
 651  AAACACTGCA CTCTCCCCCA TCCCAGGGAT CCCAGGCTCC TGTTACACAT
 701  GCCCGCATGC CTGCAGCGTT TAGCCTTCCA GTTAGCCATC CTCTCAACCA
 751  TCTGCAGCAC AGCCACCTTC CGCCAAATGG ACTGGAACTT CCTTTTATGA
 801  TGATGCCCCA CCCTCTCATT CCTGTCAGCC TACCTCCAGC ATCTGTCACC
 851  ATGGCAATGA GTCAGATGAA CCACCTTAGC ACCATTGCAA ATATGGCGGC
 901  GGCAGCACAA GTTCAGAGTC CTCCATCCAG GGTGGAGACA TCTGTTATTA
 951  AGGAGCGTGT TCCCGACAGC CCCTCGCCTG CTCCATCTCT GGAGGAGGGC
1001  CGGAGGCCCG GCAGCCACCC ATCCTCACAC CGCAGCAGCA GTGTGTCCAG
1051  CTCCCCGGCG CGGACTGAGA GTTCTTCCGA CAGAATCCCT GTCCATCAGA
1101  ATGGCCTGTC CATGAACCAG ATGCTTATGG GTTTATCCCC AAATGTGCTT
1151  CCTGGGCCAA AGGAGGGGGA TTTGGCTGGT CATGACATGG GGCATGAGTC
1201  AAAACGGATC CACATTGAAA AAGATGAGAC CCCACTTTCC ACACCAACCG
1251  CAAGAGACAG CATCGACAAA CTTTCTCTAA CTGGGCATGG ACAACCGCTA
1301  CCTCCCGGCT TTCCATCTCC CTTTCTGTTT CCTGATGGCC TGTCTTCCAT
1351  AGAGACCCTT CTCACTAACA TACAGGGCCT CTTGAAAGTT GCCATAGACA
1401  ATGCCAGAGC TCAAGAAAAG CAGGTCCAAC TGGAAAAAAC AGAGCTGAAG
1451  ATGGATTTTT TAAGAGAAAG AGAACTAAGA GAAACACTGG AGAAGCAGCT
1501  GGCCATGGAA CAAAAGAACA GAGCCATAGT TCAAAAGAGG CTAAAGAAGG
1551  AAAAGAAAGC AAAGAGAAAA CTGCAGGAGG CACTAGAATT TGAGACAAAA
1601  CGCCGTGAGC AAGCAGAGCA GACACTGAAA CAGGCAGCTT CAGCGGACAG
1651  TCTCCGGGTC TTAAATGACT CCCTGACCCC TGAGATAGAA GCTGACCGCA
1701  GCGGAGGGAG AGCAGATGCT GAAAGGACAA TACAAGATGG AAGACTGTAT
1751  TTGAAAACTA CTGTCATGTA CTGAATATTC CTTGTTGAAG AAACACGTCA
1801  TCTACTACAG AACTCTGGAG GCAGACCTTC ATCGTCGGAA AGTTCAGGAA
1851  AAAAAACAAA GTCCATATAA AAGAACTTCT TCAATTTTGC GTGTTAGTGC
1901  TCTTTTTCAT TTAAGTATTC CACAAAGGCA GAAAAGTTTC CTCCATTGAT
1951  TTTTTTTCAC CCGTGGtTCC TACCnAGAGA CTGAGAATGT TTGTAAATGT
2001  ACACATATCA AAGTTCTTAC AGTTAATAAC TCCCCTGTGC TGCTGGACAC
2051  TTGTGTAGAG AGCTAAAGAC AGGTCTGAGG AAGACCGAGC TCTGGTTTGT
2101  TTTCTTTTTC TTCTTAATGG AATGAACTTA TTTCCCCTCT TCTGACAGTT
2151  CTGTAACTGA GCACATCAGC AGACTTTGTA GCAGCTCACC CAGACTTACA
2201  GAATTGTGTC CCACAGAAAC CAGCAAGAAT CTATGGGATA AACTTTGAAG
2251  GGTCATAGAG GATTCTGGGA GGGGGAAATG AGAGAAGTGA GAGTATTCTG
2301  TTACATACAA TTTTAAACTC TATAACTGCA GGTTTTGTCC TGAAGACCTT
2351  TTTCATATAC TTTCTCCGGA ATTCCAGCTG AGCGCCGGTC GCTACCATTA
2401  CCAGAAGCCG AATTCTGCAG ATATCCATCA CACTGGCGGC TGCTCGAGCA
2451  TGCGGCAGAG GAACACC
```

Appendix 2b: *Dachshund* sequence data

Zebrafish *Dachshund* A (*zDachA*)

```
   1  CTGCACGAGG GAAGGTACTA GCACATTACC GAACGTCAGC AGGCGCTACC
  51  GGAGGCGCAT GAGAAACGAC GAGTAAAAGC CGCGAAACAA TCCGAGGAGC
 101  CAGCAAAGGT GTGCTTGACT CAAAAGTACG GAAAGGAGGA AACTTCGCGT
 151  AAACTATGGC CGTATCTGCA ACTCCTCCGG TGCTTTCACC GACCTCCACT
 201  CCCGGGGGCG CGAGCCTGTT CCGTCCCGAC TCTCTGTACT CCAACCCAGC
 251  CGAGTCGCCG CGACTGACCA GCAGCATGAT CAACTCTTTC ATCACCGGCG
 301  GCGGCAGCAC GAACGGGAAC GGGCCCGGTG GTGTCCACAA TAACGAGTGT
 351  AAGATGGTGG AGGTGCACGG GGTGAAAGTG GCCTCGTTCA CTGTGGACGG
 401  TCAGGAGCTC ATCTGCCTCC CGCAGGTGTT CGACCTGTTC CTGAAGCACC
 451  TGGTCGGCGG ACTGCACACC GTGTATACCA AGCTGAAGCG GCTGGATATA
 501  TGTCCGGTGG TGTGCACGGT GGAGCAGGTG CGGATCCTGC GCGGACTCGG
 551  GGCCATTCAG CCGGGCGTGA ACCGGTGCAA ACTCATCACC CGAAAAGACT
 601  TCGAGACACT TTATAACGAC TGCACCAATG CCAGTTCTCG GCCTGGTCGT
 651  CCTCCTAAGC GCTCTCTCGG AGTGGCGATG CAGGACAGCT CTCGTTTGcT
 701  TCCTCACAGC GTTCATGGGc TGCTCTCTCC AGGTCTGCTG TCACCAACAG
 751  GACTGACAGC AGCAGCCATG GCTGAAGCTA TGAAACTCCA GAAAATGAAA
 801  CTGATGGCTA TGAACAACAT TCATGGAGCA GGAAGCCAGA ATGGTACAGA
 851  GTCTGAGAAC GAAGAGCTGA ACTCCAGTGC AGGCGGCAGT GAGTCGTCTT
 901  GGGATAAAGA GAAACTCCAG TCCCCCCCTT CCTCTGGAGC ACAGCATGGT
 951  CTGGCTCACG CCGCCCTCTC CGCTCAGCAT GGCCTATCAG GGTCTCATCT
1001  CTCTTCCCTA CAGCAGAGTC ATCTCCTGGC TAACCGGCTG GACCTGCCAT
1051  TTATGATGAT GCCCCACCCC CTGCTGCCCG TCAGTCTGCC GCCTGCCTCC
1101  GTTGCCATGG CAATGAACCA GATGAACCAC CTGAACACCA TCGCCAACAT
1151  GGCCGCAGCT GCTCAGATGC ACAGCCCCCT GTCCAGAGCA GGAGCGTCCG
1201  TCATCAAGGA GTGTGTACAG GACAGTCCAT CCCCTACTCC TTCTCTGGAG
1251  GAAGCTCCTC GTCCCGGATc ACAGCCCTCT TCCCATCCTA GCAGTAGTGT
1301  GTCTAGTTCT CCGAACCCGC ACACACAGAG TCCTGAACGC CTGGTTTTGA
1351  ACCCGACTGA TGGAGATCTG CCAGAACGAG ACACCGGCAT CAATATGAAG
1401  AAAATGCTTA AGGAGAAAGA TGAGGCACAG ATAACTTTGC CAATGCAGAA
1451  GCCAGGGTTC GAGAAGCTTC CTCTGGGCAC TCAGACTCTT CCTCCAGGCT
1501  TCCCTGCTCC CTTCCTGTTT GCAGATGGCC TCTCTTCTGT AGAGACATTG
1551  CTCACTAACA TCCAGGGTCT GCTGAAGGTG GCTGTGGATA ATGCCCGTGT
1601  GCAGGAGAAA CAGGTGCAGC AGGAACGCAA GGAGTTAAAG ATGGAGCTGT
1651  ACAGAGAAAG AGAGATGAGA GAGAGTCTGG AAAGACAACT CACCTCTGAA
1701  CTGCACAGCC GAGCCACCAT TCAGAAGCGT CTGAAGAAGG GAAAGAAGGC
1751  GAAAAGGAAA CTGCAGGAGG CTCTGGAGTT TGAATCCAAG AGGAGAGAGC
1801  AGGTGGaACA AGCTCTCAAA CAGGCCACTT CTCCTGAGAG TCTCCGCTTG
1851  AGTCTAAACG AAGCAATCAT ACCAGAAGGT GAATCTGAAC ATAATGGCAA
1901  CCAGCAGGAG AACTCATCTG TACAAGAAAA CAGACCCTAC TCCAAACCCC
1951  CCATCATGTA CTGAAGACTC TAACATCTTT AGCTCCACGA CCATGACAGA
2001  CATCTCAGCC CACAGGCAGA CGGAGCTTTT TCCACTAAGG AAAAGTGGTG
2051  TGGGTGCTGA AGAGCAGTCA CCTGAAACCC GTGTACAGGA CTTGAGTGTG
2101  TATGTACATT TATGTGTGTG GAGGTGCAGT GCGCTTACGG TCCTCGTGAG
2151  ACGCGTGTGT GCGTGTGAAT GTTTCTGGAG CACTGCTCGT CGACGACCTT
2201  TCCACACCAC ATGCACCGAC TTGTAAAAAT TGTCTTTCTC TCTTGTTTTG
2251  ACTACTTCTA CATCCCACTT TTTTGAGATT AAAGCAAGTG TTTGTTGTTT
2301  TTTTTCTGGA ttAAAATGTC ATTACCTAAT GAAATACCCG TCTTTATGTG
2351  AAGCACACAG CATCACCCTC CACTAATCTC CAGTTTGTTA TTATTATTAT
2401  TATTATTATT ATTATTACTG AGTGTTATCT GCTGATCCCC CATGATTCTG
2451  TGCACCAGTG ATTCTGAACA GCACAACAGT CTTGACGAGT CTGTATAGCA
2501  ATTTGTCGct ACACAAAGGG ACAGAAAAGA CATATTATaT GGACAGACTC
2551  TTAACTTAAG ATGTGTGCTT CTTTATTAAA CATTGTCTTT TTTGTTGTTT
2601  TGCCTTTAAA AAAGGGATTC TACAGTATAT CAATACTTTT TTTGTTTCTA
2651  ATTTGGTGTA TTTTTTTAGG AAGTGTAGAA ATATTTTCCT CATGTTGTCA
2701  TTTCTCTGCC GTTTGTGTTT ATTTCATTAT CTGGTTTGTC GTTTTCCTTT
2751  GTATTTTAAT CAGCATTTTA AAAACAGATG CTCAAGTGAT CTTGAAACAT
2801  GTTCTTTGTA TGTCTTTCTT TGACCCGCGA CAATGTTTTT CTCTATTGCT
2851  CCTTAACGTA AAAGTAAAAA AAAAAAAAAA AAA
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Appendix 2c : *Dachshund* sequence data

Zebrafish *Dachshund B (zDachB)*

```
   1  GCACGAGGCG CGCGGCtTCG CTGCGGACAC ATCGCTCTCT GGACGCTCTG
  51  CAcaTCACGG GAAAGCCTAG AACGGAGAGT TATGAGAAAG TAGACGATCT
 101  AAAAGAGCAC ATTAGGAATT TTAAGTTCAA TGACCACTAT GGCCGTTCCC
 151  GCGACTCTTC CGGTTCACTC TGGGACTTCT GCGGTGTCCG GTAAACTCTT
 201  CAGGACCGAG CCGTTCTTCT CGAGTCCCGa TGAGTCTCCG CGGATCATCA
 251  ACTCGCTGCC GAGCGGTGGG ACCACAAACG AGTGCAAGAT CGTGGAGGTT
 301  CACGGGGTTA AAGTGGCGTC TTTCAGTGTT GATGGGCAGG AGCTTATTTG
 351  TCTCCCTCAA GTATTTGACC TTTTCCTGAA GCACCTGGTG GGCGGACTGC
 401  ACACCGTGTA CACTAAACTC AAGCGCTTGG ATATAAACCC AGTGGTGTGC
 451  ACCGTGGAGC AGGTGCGCGT GCTGCGCGGA CTCGGTGCCA TCCAGCCCGG
 501  AGTGAACCGC TGCAAACTCA TTTCCAGAAA AGATTTCGAA GCGCTGTACA
 551  ACGACTGCAC CAACGCAAGC TCTCGTCCAG GACGCCCACC TAAGCGCTCC
 601  TATGGGGCCA GTGTTCAAGA AAGCCCCAGA ATCCTCCACC ACAGAGCAAA
 651  CCTCCTGTCT CCAGCCCTGC TTTCACCCAC AGGTTTAACA ACAGCAGCTA
 701  TGGCTGAAGC TTTAAAGATA CAGAAGATGA AGATGATGAT GAACCTGCAC
 751  AAGACTCACA ATGGCTCCGA ATTCGATTCA GATGAGCTGA ACTCTAACGC
 801  CGGTACAGTA TGCAGCACCC TGTCCTGGGA GAGAGAGAAA CATTCATCAC
 851  CTGCTTCAGA GAAAACCCAG CACAGCTTGA ACAACTCACA GCTCAACTCT
 901  CTCCAACACA CCCATCTACT GGCCAACAGA CTAGAGCTGC CCTTCATGAT
 951  GATGCCCCAC CCTCTGCTTC CTGTTGGACT TCCTCCTGCC TCTGTTGCCA
1001  TGGCAATGAA CCAGATGAAC CACCTTAATA CAATTGCCAA CATGGTTGCC
1051  AGCGCACAGG TGCACAGCCC CGTCTCCAGG CCGACATCTG CCATCAAGCA
1101  GGAACGTTTT GAAGAAAGCC CCTCTCTGAC TCCATCTGTA GAGGGGATTG
1151  TTTCTCAAAA AACCGAACCT TCACCACAAC AAAGCAGTTC GGTTCCCAGT
1201  AGCCCCACAC ACCCTTACAC ACATTCTCCT CTAAAAACAG CATATGACGC
1251  TCATGATGAA CAAAGGGAGA CTGATTCAGC TCTGCATGTG AACAGAcTTT
1301  CCAACGACAG GGTTGAGCAG AATGCAGTGA AGCCAGCATT GTTTGAGAAG
1351  GTTCCAGCTC AGACGTTTCC CTCAGGCTTT CCTGCTTCTC TCCTGTTCAC
1401  TGACGGCCTT TCCTCTGTCG AGACGCTGCT CACTAATGTT CAGGGTCTGC
1451  TGAAGGTGGC TCTGGAAAAC GCACGTCTGC AAGAGAAGCA GCTTCAGCAG
1501  GAGAGGAGAG AGCTCAAGAT GGAGCTGTAC AGAGAGAGAG AGATGAGAGA
1551  GAGTCTGGAG AGACAGCTCA CCTCAGAGCT ACGCACTCGA GCCACCATTC
1601  AGAGACGTCT GAAGAAGGAG AAGAAGGCAA AGAGGAGGTT ACAGGAGGCG
1651  CTGGAGTACG AGTCCAAGAG GAGAGGACAA ATAGAGCAGG CTTTACAGCA
1701  GGCCACTTCA TCAGACTCTC TAACACACGA TCCAATCAGT CTGGAGATGG
1751  AGACAGAGCG ATGCCGCAGC CCAGAGGACA ACTGCTTGTT ACAAGAAAGC
1801  AGAACATATA CGAAAAATCC AATCATATAC TAAGATGACG TGAACTCCCA
1851  GCGAGAGAAT AAGGAAATTT TCCATATCAT GAATGACCCC TACAATCAAC
1901  TTCCTGAATA AAGTTTTCAA TGAAGAGAGG ACTTGAACAT GAGCTTCAGA
1951  TCTTCAATCG AATGCTCCAA CCACTCTTTT TCTCATCCTC ACAACATTCC
2001  AGGTTCCTCA ACTGTTGTTT TTATTCACCT AGATTGTGCT TAATTCCCTG
2051  TTTTGCTGTG TGGAGAGCAC TGCTGGCATT CTGTAACTGG TTTTAAACAG
2101  TGTTTTTTTT TAAATGATGT CATGACTTTA TCCCTTCAGG ACAGTGACCT
2151  TCAGTCTAAA GCCCTCACGA AGGAGCTGAT GAAAATGTAA GAAAAAGAAA
2201  GGAAAAGAGA TTTGGCTTGT GTTTCATTCT TGTTGAAAGA CTCTGAAGAG
2251  TGTGGAATGT TACATTTTCT TGCTTTTCTC TCATATGACC ATCTTCTGAT
2301  TCTTCGGTCC ATTTATTAGC TCAGTTTAGC TTCTTGATAC TGTTCACCAG
2351  TTTGTATGTG GTAGTTTTCT TTTGTAA
```

Appendix 2d : *Dachshund* sequence data

Zebrafish *Dachshund C (zDachC)*

```
   1  CGGGCTGCAG GAATTCGTGC ACGAAGCGGC TGCGCTCCTA CCCGCGACAC
  51  CGATGGCCCA CGCGCCTCCG ATGTCGATCT CCGCGGTTGT CAACTCATCG
 101  ACCCCGGCGA CCCTCTCGCC TTCACCGTCC GTCGCCCCAG CCGGACCGAG
 151  CCTCTTCAGA ACGGAACTGC TCTCCTCCGC GAGCCCGGGC ATCCCCATCG
 201  GCAGTCTCCC GCACAAACCT GTGTACTCCA CACCATCTCC GGTGGAAAAC
 251  ACTCCGCAAA ACAACGAGTG TAAAATGGTC GAGGTCCGTG GCGCAAAGCT
 301  GGCGTCTTTC ACTGTCAACG GCAACGAGCT CATCTGTCTC CTGCAGGCGT
 351  TTGACCTCTT CCTCAAGCAC CTGGTCGGCG GATTGCACAC CGTGTACACG
 401  AAACTTAAAC GTCTGGAGAT AACGCCCGTG GTGTGCAACG TGGAGCAGGT
 451  TCGCATCCTC CGTGGACTGG GCGCGATTCA GCCTGGAGTC AATCGCTGCA
 501  AACTTATATC GAGAAAAGAC TTCGAGGTCC TGTATAACGA CTGCACCAAC
 551  GCAAGCTCCA GACCTGGCAG ACCTCCTAAA AGGACCCAGA ATGTGACATC
 601  ACCCGATAGC CCTCATGTCC TACCCCACTC CGTCTCTGGA CTCATGTCTT
 651  CAGGATTAAT GTCTCACACT GGCcTAACAG CTGCGACACT CGCTGAAGCC
 701  ATGAAGAAGA AGATCAAATT GGaGGTGATG AACAgTTACC ATGGCAATAA
 751  TAACCACAGT GCAGATTCTG AGAATGGTGA CATCACCTCC AgCATGGGTT
 801  TGGAGCTGCC ATTCATGATG ATGCCACATC CCTTGATTCC AGTCAGTCTG
 851  CCTCCTGCGT CGGTCACCAT GGCAATGAAC CAGATGAACC ATCTCAGCAC
 901  TATTGCCAGC ATGGCAGCTG CTGCTCAGGT CCAGAGTGTC CCGTCCAGAA
 951  TGGTGACTTC AGTAATAAAG GAGCGAGTTC CAGACAGTCC GTCACCTGTA
1001  CCCTCTTTGG ATGACGGTCG CAGGTCAGGG AGCCATTTAT CCTCCAGACA
1051  GAGCAGCAGC GTGTCCAGTT CTCCTGCCCA CACAGAGAGC TCTTCTGACA
1101  GGCCACATTT ACATCAgAAT GGTCTGTCTC TTGGCCATGC CTTGTTAGGC
1151  CTTTCTCCAA GTGCACCACC AGGACCAAAA GAGGGGGATC TGGCAACCCA
1201  TGACACAGTG CATGAAACAA AGAGGGCTTC TACAGAAAAA GAAGAAAATG
1251  TCCTGTGCAC CCcAACATCA CGGGACACCT ATGAGAGATT GTCTCATTCT
1301  GGGCCAACAT TACCTCCAGG ATTCCCTGCT CCATTGCTCT TTCCTGAAGG
1351  CCTGTCCTCC ATAGAAACTC TTCTCACCAA CATACAGGGT TTGTTAAAGg
1401  TGGCTATTGA TAATGCACGA GCGCAAGAGA AGCAGGTTCA GCTGGAGCGC
1451  ACAGAGCTGA AGATGGAGCT GTACAGGGAG AGAGAGCTGC GCGAGACACT
1501  AGAGAGACAG CTCTGTGTGG AGCAGAAAAA CAGAGCGCTC ATCCAGAAGC
1551  GACTGAAGAA GGAGAAGAAG ACCAAGAGAA AACTGCAGGA GGCTCTGGAG
1601  GTGGAGTCTA AGAGAAGAGA CCTGCATGAG CAGACGCTTC AGAGGACAAC
1651  TTCCTGTGAG AGATCACCCA TTCACAACGA CTCTCAACAG GAATTGGAGA
1701  CAATCCTTAT TACCAGCAAA ACGGACACTG AGGGAACAAT ACAAGATGGA
1751  AGGCTCTTTC TAAAGTCCAC AATGATGTAC TGAGGATTGA AGATGAGGAG
1801  TAAATGCATT CAAACTGTAC AGAAGAGCCT GTAGTCAATC GCCTGGTAAA
1851  TAACGTCTGC AATTCTGCTG GAGGACTGAC GGAAGACAAG GAGGCAGTTT
1901  TATAGGTTGA TGTCAGTGTT TTTGAGGCAC AAGGACTTGA CAAGATTATT
1951  ATGGCCATTG AAAGTCAGCT TTTAGTAAGA TAAAGCGTGA GCCGAGATGN
2001  CTGAAGAAAA TGCTATCACA ATGCACACCT GGTTGAAAAA CCATGTTTCC
2051  AGGGGACTTT TTGCAACACT GA
```

Appendix 3: Previously published work

ELSEVIER

Mechanisms of Development 74 (1998) 121–131

Mammalian and *Drosophila dachshund* genes are related to the *Ski* proto-oncogene and are expressed in eye and limb

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Abstract

We have isolated mammalian homologues of the *Drosophila dachshund* gene. Two domains of high conservation, one of which contains an α-helical, coiled-coil motif, show similarity to the *Ski* family of genes. We therefore propose that *Dachshund* belongs to a superfamily including these genes. Mouse *Dachshund* (*Dach*) is expressed in the eye and limb, structures affected by the *Drosophila* loss-of-function mutant, and rib primordia, CNS and genital eminence. *Pax6* and *Dach* show overlapping but non-identical expression patterns. *Dach* expression is unaffected in *smalleye* mouse brain, indicating that *Pax6* is not directly activating *Dach*. In *Drosophila* eye development *dachshund* is a component of an interacting network of proteins. Genes homologous to many of these exist in mammals; *Dach* joins this expanding group.

Keywords: Mouse *Dach*; Human *DACH*; *Drosophila dac; Ski; Sno; Pax6*; Eye; Neuroretina; Limb; Forebrain; Dorsal root ganglia; Rib; Genital eminence; Neural tube; Cranial ganglia; *Smalleye*

1. Introduction

The *Drosophila dachshund* (*dac*) gene is involved in both eye and leg development as demonstrated in loss-of-function mutants in which the legs are drastically shortened and the eyes are reduced or absent (Mardon et al., 1994). Shortening of the segmental leg structure is due exclusively to fusion and condensation of the intermediate segments; the proximal and distal segments are unaffected. In the eye, disruption of development occurs at the earliest stages affecting initiation of the morphogenetic furrow. Expression of *dac* is found in the imaginal discs of both the eye and leg in domains directly associated with the phenotype. Other tissues in which *dac* is expressed include antennal and wing imaginal discs and the central nervous system, including the optic lobe of the larval brain; however, there is no obvious phenotype in these areas.

Analysis of *dac* function has focused predominantly on the developing eye. A network of proteins interact during the early stages of *Drosophila* eye development and includes, in addition to *dac*, the putative transcription factors *eyeless* (*ey*), *eyes absent* (*eya*) and *sine oculis* (*so*). Like *dac*, these genes are fundamental to eye development, causing eyes to be absent or reduced when the gene is lost (Bonini et al., 1993; Cheyette et al., 1994; Quiring et al., 1994). Much attention has been paid to three of these genes, *dac*, *ey* and *eya*, as potential master genes in eye formation as each can induce the formation of ectopic eye structures when ectopically expressed in *Drosophila* (Halder et al., 1995; Bonini et al., 1997; Shen and Mardon, 1997).

The mammalian *Pax6* gene and members of the *Eya* and *Six* gene families are homologous to *ey*, *eya* and *so*, respectively and *Pax6*, *Eya1–3* and *Six3* are expressed during eye development (Walther and Gruss, 1991; Walther et al., 1991; Oliver et al., 1995; Xu et al., 1997). Furthermore *Pax6* and *Eya2* were shown to be functionally equivalent to their *Drosophila* counterparts; *Pax6*, like *ey*, induces ectopic eyes in *Drosophila* and *Eya2* rescues the *eya*

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[1] These authors contributed equally to this work.

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PII S0925-4773(98)00071-9

loss-of-function mutant (Halder et al., 1995., Bonini et al., 1997).

Initially it was suggested that *Drosophila ey* was at the top of a regulatory hierarchy of genes which together produce the eye (Halder et al., 1995). Indeed *ey* cannot induce ectopic retinal development in the absence of either *dac* or *eya*, and misexpression of *ey* strongly induces *dac* expression (Shen and Mardon, 1997), indicating that *ey* is upstream of *dac* and *eya*. Conflicting evidence, however, argues for a network of interactions in which *dac* and *eya* participate and is inconsistent with a simple linear hierarchy. For example ectopic expression of either *dac* or *eya*, like *ey*, induces ectopic retinal development although at a lower frequency. When *dac* and *eya* are expressed together, they act synergistically, dramatically increasing the penetrance of ectopic eyes (Chen et al., 1997). Furthermore ectopic expression of both *eya* and *dac* induces ectopic *ey*. Thus, a network has been postulated involving these genes and a complex series of positive feedback loops (Chen et al., 1997).

The mammalian homologues of the *Drosophila* genes described above are also likely to be involved in eye development given the conserved nature of their expression domains. Expression relationships between these genes also seem to be retained. *Pax6* is known to be fundamental to eye development; i.e. mutations disrupt eye formation in *smalleye* mice when homozygous and cause aniridia in humans when heterozygous (Hill et al., 1991; Ton et al., 1991; Glaser et al., 1992; Jordan et al., 1992. The relationship between *Eya* and *Pax6* also appears conserved; *Eya* has three mammalian homologues expressed in the developing eye which overlap *Pax6* expression domains. In *smalleye* mice no expression is found within these regions, suggesting that *Pax6* function is essential for normal *eya* expression (Xu et al., 1997). Similar molecular mechanisms to those found in *Drosophila* may therefore operate in mammals despite the vast differences in eye morphology and development.

We have isolated mouse and human genes coding for homologues of *dac*. The *Drosophila* and mammalian genes share two highly-conserved domains, the more C-terminal of which contains an unusual extended α-helical coiled-coil motif. On the basis of amino acid homology and structural comparisons, *dac* and its mammalian homologues are related to the *Ski* proto-oncogene and *Sno*, a *Ski*-like gene. We therefore suggest that *Dachshund* is a member of a gene superfamily which includes the *Ski* family of oncogene-related proteins.

Expression of mouse *Dachshund* (*Dach*) is found within the eye and limb, structures affected in *Drosophila* by loss-of-function mutations (Mardon et al., 1994). Expression is also found in the brain, neural tube, dorsal root ganglia, rib primordia and genital eminence. *Pax6* is also expressed in some of these areas, particularly the eye and the brain; however, the pattern of *Dach* expression in these structures, whilst overlapping with *Pax6*, is not identical. In addition, *Dach* expression is unaffected in the *smalleye* mouse forebrain, indicating that, at least in brain, *Pax6* is not directly regulating *Dach*. Thus *Dach* joins the group of homologous eye genes shared by *Drosophila* and mouse and supports the concept that a conserved genetic network operates in eye development in highly diverse organisms.

2. Results

2.1. Identification of the mouse and human homologues of dachshund

A human expressed sequence tag (EST) (accession number AA059243) was identified in a search of the EST database using the *Drosophila dachshund* amino acid sequence. This EST was derived from an IMAGE consortium human adult retina cDNA clone, ID 381801. End sequences were obtained and four additional overlapping human ESTs identified; together these formed a cDNA contig of 2.6-kb. The human cDNAs were used to screen an embryonic E11.5 mouse library, and overlapping clones were identified which formed a cDNA contig of 2.5 kb. The genes were designated *DACH* (human) and *Dach* (mouse). An alignment of the human and mouse protein sequences is shown in Fig. 1; the predicted coding regions are highly similar (99.3%) throughout. The human coding region is 517 amino-acids long; the mouse sequence contains an additional 52 amino acids. Analysis of this region by RT-PCR (data not shown) suggests that the additional coding region is a rare alternative splice form; the major mouse transcript is identical to the human transcript. The similarity between mouse and human *Dachshund* decreases 3′ and 5′ to the predicted coding regions. The region 5′ to the putative start methionine contains long stretches of trinucleotide repeats which differ between the mouse and human cDNAs (Fig. 1).

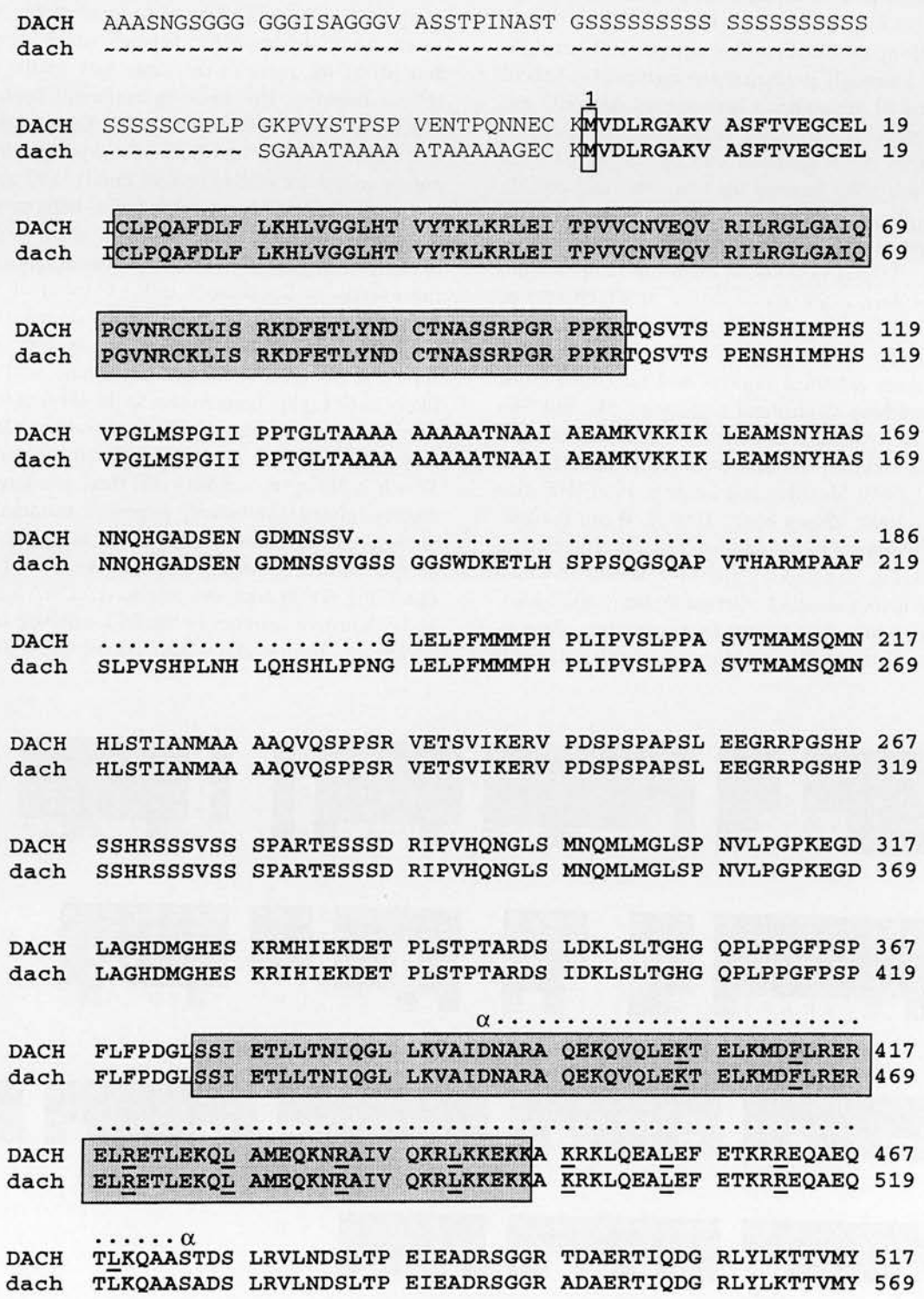
2.2. Two conserved domains in the Dach gene

Two highly-similar domains exist between *Drosophila dac* and the mouse and human genes (Figs. 1 and 2). The N-terminal domain (Fig. 2a) is referred to as Dachbox-N and is 83 amino acids in length with an overall similarity of 87% between the *Drosophila* and mammalian proteins. The *C. elegans* homologue of *dac* (identified in cosmid U80953, as described in Section 4) also contains Dachbox-N and shows 73% identity with the mammalian proteins (Fig. 2a). The C-terminal domain, Dachbox-C, is 72 amino acids long and the degree of similarity to *Drosophila* is 63% (Fig. 2b). These regions are therefore likely to have important conserved functions. Given that the conserved protein motifs are identical in *Dach* and DACH, we use the name *Dach* to indicate both mammalian homologues.

A search of the TREMBL protein sequence database with the entire predicted amino acid sequence of Dach detected weak but significant identity with Ski, a proto-oncogene

which normally functions during myogenesis and neurulation, and Sno, a Ski-related protein of unknown function (Nomura et al., 1989; Berk et al., 1997).

The homology between Dach and Ski/Sno was based around the two Dachboxes. Dachbox-N has 28% identity with the consensus sequence of all vertebrate Ski and Sno



g. 1. Amino acid alignment of the predicted protein products of DACH (human) and Dach (mouse). Numbering begins at the putative initiating methionine sidue (boxed). No upstream in-frame stop codon was identified in either cDNA, and therefore the upstream amino acids are shown (light face). The mouse otein has an insertion of 52 amino acids (residues 187–238) compared to the human protein which appears to correspond to the inclusion of an alternatively liced exon. Two regions of significant homology with *Drosophila* dachshund protein are shown as shaded boxes: Dachbox-N, from residues 21–103; and achbox-C, from residues 375–446 in the human protein and residues 427–498 in the mouse protein. A strongly-predicted α-helical coiled-coil domain is dicated by $\alpha....\alpha$. Within this helical region, the residues which comprise the basic-hydrophobic heptad repeat (helix face 'a' in Fig. 3) are underlined.

proteins (Fig. 2a); in Ski and Sno the homologous domain is also near the N-terminus. Dachbox-C has very weak identity with the C-terminal region of Ski and Sno, confined to the occasional alignment of basic and hydrophobic amino acids.

Secondary-structure analysis of the entire Dach open reading frame using the Garnier–Osguthorpe–Robson algorithm revealed a strongly predicted uninterrupted α-helical domain covering 81 amino acids between residues 393 and 474 (Garnier et al., 1996). This helical region starts 20 amino acids from the N-terminal end of Dachbox-C and extends 28 amino acids beyond the box, finishing outside the region of high conservation (Fig. 1). The helical domain is also predicted to have a very strong tendency to form a coiled-coil ($P = 1.0$, GCG Coilscan algorithm; Lupas et al., 1991; Lupas, 1996), a tertiary structure in which two or more α-helices coil round each other. This contrasts sharply with the remainder of the protein, which is predicted to contain only short α-helical regions and no coiled-coils. The weakly-matching C-terminal regions of Ski and Sno also contain an extended α-helical coiled-coil domain which mediates Ski/Ski, Ski/Sno or Sno/Sno dimerisation (Nagase et al., 1993; Sleeman and Laskey, 1993; Heyman and Stavnezer, 1994; Zheng et al., 1997a). When the predicted α-helical domain of mammalian Dach is projected on a helical wheel (Fig. 3) a striking motif is revealed, in which one face of the helix comprises alternating basic and hydrophobic residues while the adjacent face comprises alternating acidic and hydrophobic residues. A similar motif is found in the *Drosophila* dac protein: secondary structur algorithms also predict an extended α-helical coiled-co domain beginning in Dachbox-C. When visualised on helical wheel, residues 778–827 in the sequence of Mardo et al. (1994) contain eight basic–hyrophobic heptads an seven acidic–hydrophobic heptads which form faces (a and (d) of the helix in the same way as the mammalia helical domain. The basic-hydrophobic heptad of Dac and dac is similar to that described in *Xenopus* ski, (Sleema and Laskey, 1993), but the acidic–hydrophobic heptad i unique to the dachshund protein family, and may compris a new zipper motif involved in helix–helix interactions.

Dach and dac proteins therefore share two conserve domains with Ski and Sno; an N-terminal motif correspond ing exactly to Dachbox-N and a C-terminal motif whic corresponds to the helical coiled-coil domain beginning i Dachbox-C. This second motif, while very weakly con served at the level of the primary amino acid sequence, i likely to be highly homologous at the level of tertiary struc ture and may mediate Dach dimerisation. *Dach* and *da* therefore belong to a gene superfamily including *Ski* an *Sno*. It is, however, unlikely that there is a large number o closely-related *Dachshund* genes in mammals. Souther analysis of genomic mouse DNA using the highly-con served Dachbox-N as a probe, indicates a single hybridisin band (Fig. 4). In addition, extensive cDNA library screen and exhaustive searches of the EST database have failed t reveal any further *dachshund* homologues. This indicate

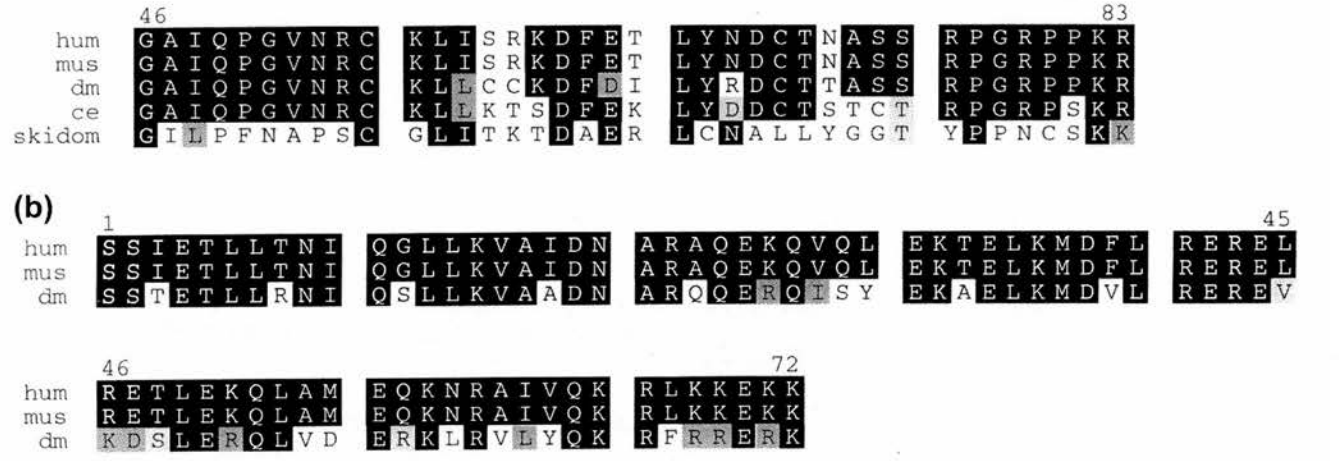


Fig. 2. Conserved amino acid sequences in homologues of dachshund. (A) Dachbox-N, which spans 83 amino acids near the N-terminus of all members of th Dach/Ski/Sno superfamily. Hum, human DACH; mus, mouse Dach; dm, *Drosophila* dachshund; ce, predicted *C. elegans* dachshund homologue encoded b cosmid B401; skidom, consensus amino acid sequence of the vertebrate Ski/Sno protein family (domain ID no. 4362 from ProDom (http://protein.toulou se.inra.fr/prodom.html)). The *Drosophila* protein sequence is from splice variant 4, accession number U19269 (Mardon et al., 1994). (B) Dachbox-C, whic spans 72 amino acids near the C-terminus of *Drosophila* dac and its mammalian homologues. hum, human DACH; mus, mouse Dach; dm, *Drosophil* dachshund. Sequence alignments were created using the program Pileup and highlighted using the program Prettybox, both from the GCG package.

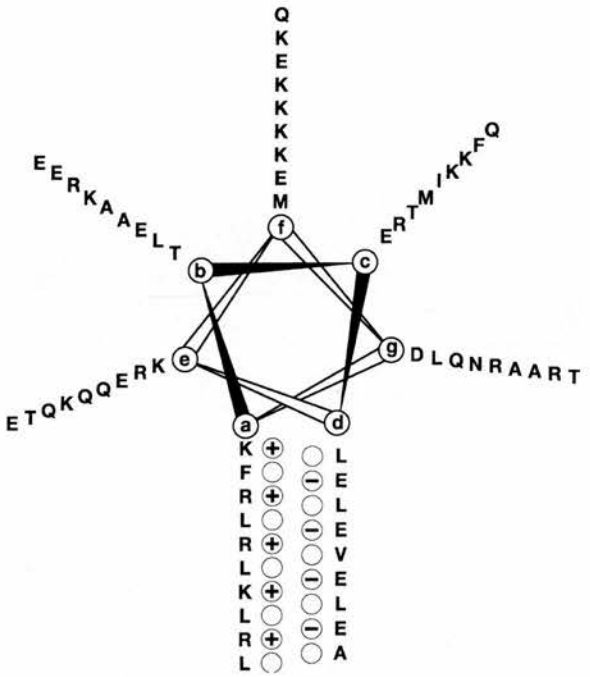


Fig. 3. Helical wheel projection of the predicted coiled-coil domain of mammalian Dach protein. Residues 406–469 of DACH or 468–521 of Dach are shown as a schematic α-helix in which the side chain of every seventh amino acids is aligned (Landschulz et al., 1988). Each 'spoke' represents a face of the cylindrical helix, which is viewed end-on with most N-terminal residues at the centre of the wheel. Spokes (a) and (d) are shown close together to highlight the novel basic-hydrophobic, acidic–hydrophobic heptad motif. The amino acids in spoke (a) are those underlined in Fig. 1.

that *Dach* is present in the mammalian genome in low abundance, perhaps as a single copy gene.

2.3. Expression of Dach in the mouse embryo

Dach expression was analysed by in situ hybridisation in E10.5–E12.5 whole mouse embryos and at an additional stage, E13.5, for the limbs. *Dach* transcripts were detected at all stages (Fig. 5a) with expression in the limbs, brain, eye, neural tube and dorsal root ganglia. There is also expression in the rib primordia located between the fore and hind limbs and in the trigeminal ganglia and glossopharangeal–vagal ganglion complex.

2.4. Limb expression

Dach is expressed in both the fore and hind limbs at all stages analysed, from E10.5 to E13.5 (Fig. 5c). At E10.5, expression can be seen in both the posterior and anterior of the limb bud, with the anterior region extending centrally into the core. At E11.5, *Dach* expression becomes increasingly peripheral, extending around the entire handplate in the mesenchyme beneath the apical ectodermal ridge. At E12.5, expression is entirely peripheral with no central expression remaining and by E13.5 *Dach* is localised to the mesenchyme at the distal tips of the digits.

2.5. Eye expression

At all three stages analysed (E10.5, E11.5 and E12.5) *Dach* is expressed in the mesenchyme surrounding the eye (Fig. 5g) which is predominantly neural-crest derived. *Dach* expression also occurs within the neural retina at these stages but not in the lens or the retinal pigmented epithelium (RPE). The expression pattern of *Dach* therefore overlaps but is not the same as that of *Pax6*, a putative regulator of *dac* (Fig. 5h) which is expressed within the lens, neuroretina, RPE and overlying ectoderm, but not within the mesenchyme.

2.6. Brain expression

Dach is expressed from E10.5 to E12.5 in the telencephalon including the olfactory bulbs (Fig. 5f), but not in the diencephalon. *Pax6* too is expressed in the brain, with strong expression in the telencephalon, again including the olfactory bulbs, but is also found in the diencephalon. These brain regions all show abnormalities in *smalleye* mice (Stoykova et al., 1996; Caric et al., 1997; Warren and Price, 1997). As in the eye, therefore, *Dach* brain expression overlaps but is not identical to that of *Pax6*.

Analysis of *Dach* forebrain expression in *smalleye* mice adds evidence that *Dach* does not need to be activated by *Pax6*. In the brain of E12.5 *smalleye* mice, *Dach* expression

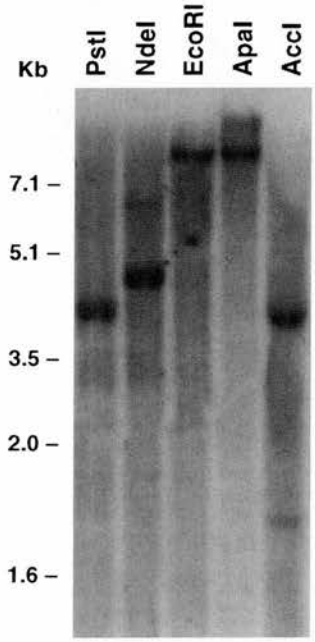


Fig. 4. Southern analysis using a probe to the highly-conserved Dachbox-N region of the *Dachshund* gene. This was hybridised to whole genomic mouse DNA digested with restriction endonucleases which do not cut within the probe, except for Acc1 which cuts once in the probe. The analysis was carried out on two separate DNA preparations from AKR/J and DBA/2J adult mouse livers, with the same result in both cases. The AKR/J blot is shown.

is found in exactly the same areas as in wildtype embryos (Fig. 6). The level of expression of *Dach* also appears to remain the same in the *smalleye* and the wildtype mice, leading to the conclusion that *Pax6* is not necessary for *Dach* expression within the embryonic brain.

2.7. Other regions of expression

Dach is expressed throughout the length of the neural tube from E10.5 to E12.5 (Fig. 5b). There is also expression of *Dach* within the dorsal root ganglia (Fig. 5i) extending to

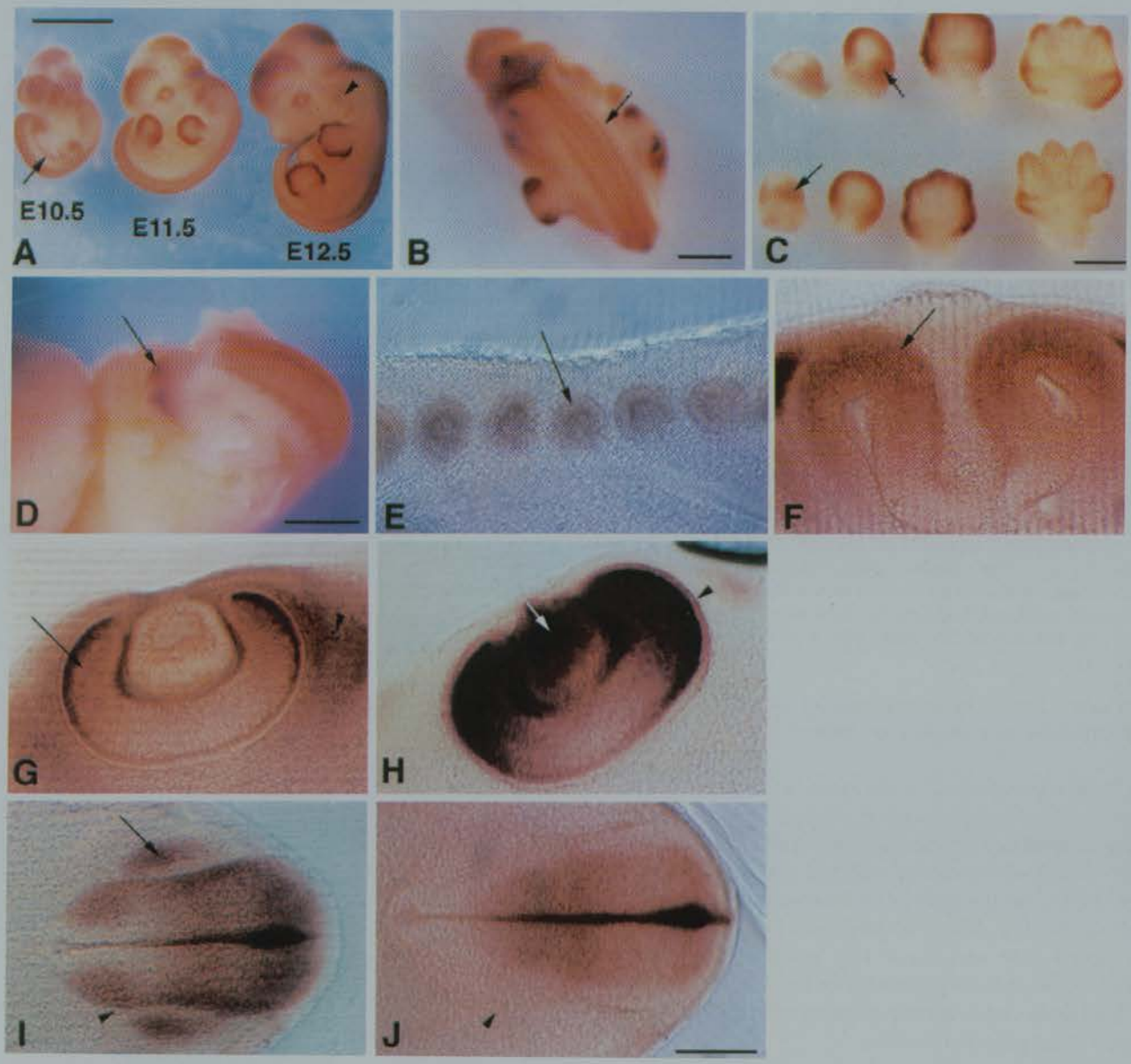
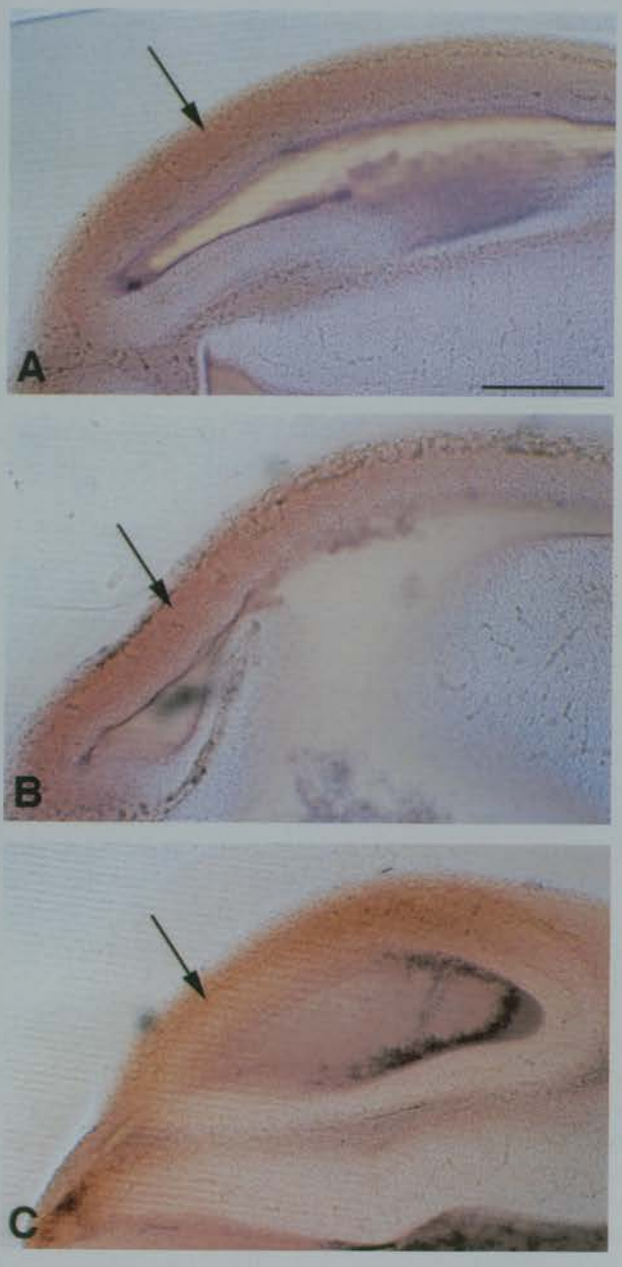


Fig. 5. *Dachshund* expression in midgestation embryos and comparison to *Pax6* expression. Whole-mount in-situ analysis, using *Dachshund* and *Pax6* probes. Vibratome sections are 100 μM thick. The scale bar represents 2 mm (A,B), 1 m (C,D) and all other plates have the same scale, with the bar representing 0.1 mm (shown in J). (A) *Dachshund* expression in E10.5, E11.5 and E12.5 embryos. Expression is seen at all stages in the eye, limb buds, neural tube, brain, and trigeminal and glossopharangeal–vagal ganglia (arrowhead). Rib primordia (arrow) expression is seen at E10.5 and E11.5 only. (B) Dorsal *Dachshund* expression in an E11.5 embryo. Note the expression in the dorsal root ganglia (arrow) either side of the neural tube. (C) *Dachshund* expression in E10.5–E13.5 limb buds. (E10.5 to the left, E11.5 and E12.5 centre, E13.5 right; forelimbs are at the bottom). At E10.5 there is an anterior region of expression which extends to the centre of the limb bud, and is beginning to disappear by E11.5 (arrows). Expression becomes peripheral by E11.5–E12.5 becoming localised to the distal tips of the digits by E13.5. (D) *Dachshund* expression within the genital eminence of an E11.5 embryo (arrow). (E) Vibratome section cut frontally between the limb buds showing *Dachshund* expression within the rib primordia (arrow). (F) Transverse vibratome section showing *Dachshund* expression within the olfactory bulbs (arrow) of an E11.5 forebrain. (G,H) Transverse vibratome sections of E11.5 eyes. (G) *Dachshund* expression within the mesenchyme surrounding the eye (arrowhead) and the neuroretina (arrow). (H) *Pax6* expression within the lens (arrow), retinal pigmented epithelium (arrowhead) and neuroretina. (I,J) Transverse vibratome sections of E11.5 neural tube. Arrowheads show equivalent regions on the outer edges of both sections, dorsal is to the right. (I) *Dachshund* expression within the dorsal, lateral and ventral mantle of the neural tube, within the dorsal ventricular zone and the dorsal root ganglia (arrow). (J) *Pax6* expression within the ventricular zone of the neural tube.

the region where the ganglia join the neural tube. The expression domain of *Dach* is mainly within the mantle of the neural tube, with expression in dorsal, lateral and ventral regions. There is also a dorsal region of expression within the ventricular zone. *Pax6* is expressed in the ventral ventricular zone of the neural tube, and not at all in the dorsal root ganglia in a complementary pattern to *Dach* expression (Fig. 5j). Again *Dach* expression does not correspond with *Pax6* expression (Fig. 5i,j).

At E10.5 and E11.5 *Dach* is expressed in a punctate pattern on the ventral side of the embryo between the fore and hind limbs, in the rib primordia (Fig. 5e). This expression has however disappeared by E12.5. At E11.5 and E12.5, Dach expression is also found within the genital eminence (Fig. 5d). Expression can also be seen in cranial ganglia in the trigeminal ganglion and the glossopharyngeal–vagal ganglion complex (Fig. 5a) at all three stages analysed.



ig. 6. *Dachshund* expression is unaffected by the lack of *Pax6* expression n a *smalleye* mouse brain. Whole-mount in-situ hybridisation carried out n E12.5 mouse brains using *Dachshund* and *Pax6* probes. Sections were ut transversely at 100-μM thickness using a vibratome. All three embryos re from the same experiment and were treated alike. The scale bar repreents 0.1 mm. (A) Wildtype mouse brain showing *Dachshund* expression ithin the forebrain (arrow). (B) *Smalleye* mouse brain showing *Dachsund* expression in the same forebrain region as in the wildtype embryo arrow). (C) Wildtype mouse brain showing *Pax6* expression within the orebrain (arrow).

3. Discussion

3.1. Dach and dac are members of a gene superfamily which contains an α-helical coiled-coil domain

We have isolated mouse and human genes which are related to *Drosophila dachshund*. When the mammalian (Dach) and *Drosophila* (dac) proteins are compared, two highly homologous regions (Dachbox-N and Dachbox-C) are identified, which are likely to be important functional domains. There appears to be a single *Dach*-like gene in mammals and we therefore suggest that the mouse and human genes are orthologues of *Drosophila dac*. Further sequence comparisons and structural analysis show that *dac* and *Dach* share two domains with the proto-oncogene *Ski* and the *Ski*-like gene *Sno*: an N-terminal domain which corresponds to Dachbox-N, and a C-terminal domain which is predicted to form an α-helical coiled-coil. We propose that *Dach*, *dac*, *Ski* and *Sno* are members of the same gene superfamily, and that structural and functional parallels can be drawn between *Ski* and *Dach*.

The shared C-terminal domain of the superfamily is defined by a structural motif rather than extensive sequence identity: dac, Dach, Ski and Sno proteins are all predicted from secondary structure algorithms to have an extended α-helical domain with a strong tendency to interact with other helices to form a coiled-coil-like tertiary structure. Coiled-coil proteins exhibit a characteristic heptad periodicity, with hydrophobic amino acids in every seventh position, so that in a schematic representation of an α-helix such as that shown in Fig. 3, face (a) is highly hydrophobic, with leucine predominant (Landschulz et al., 1988). To a lesser extent, hydrophobic residues are also common on face (d), and faces (a) and (d) together form the helical interface of the coiled-coil. Coiled-coils are found in a variety of proteins, including structural proteins such as myosin, in which there may be over 100 heptad repeats, and transcriptional regulators such as the leucine zipper DNA-binding proteins fos and jun in which the there are just four heptad repeats (Landschulz et al., 1988; Lupas et al., 1991; Lupas, 1996). The Ski protein superfamily falls between these extremes, with Ski, Dach and dac having 14, ten and eight heptads, respectively. The remarkable feature of these proteins is the unusual nature of the heptad repeat, which results in alter-

nating hydrophobic and basic amino acids aligned along face (a) of the extended helix (Fig. 3; Sleeman and Laskey, 1993). In Dach and dac there is also a striking complementary heptad repeat of acidic and hydrophobic residues which is offset by four amino acids from the basic–hydrophobic motif to form the adjacent face (d) of the helix. The positively- and negatively-charged side chains of faces (a) and (d) may interact intramolecularly to stabilise the α-helix or intermolecularly to stabilise protein dimers; in either case this is likely to be a new zipper motif. In the case of Ski, the α-helical coiled-coil domain is known to mediate Ski–Ski interactions, and the existence of a long rod-like structure has been confirmed by biophysical measurements of Ski helix dimers (Zheng et al., 1997a). Ski homodimerisation is known to be essential for the interaction of Ski with transcription factor NF1 (Tarapore et al., 1997) and we propose that dac and Dach may also form functionally important homodimers. Evidence from in vitro studies shows that *Drosophila* dac binds directly to the eyes absent (eya) protein, and that the interaction is mediated by a C-terminal fragment of dac which contains Dachbox-C and the coiled-coil motif (Chen et al., 1997). The eya protein and its vertebrate homologues have no coiled-coil potential ($P = 0$, GCG Coilscan algorithm) and consequently it seems unlikely that eya interacts with dac through a zipper mechanism involving the helical domain; instead the interaction may be mediated by the highly conserved amino acids immediately before the coiled-coil domain at the N-terminal end of Dachbox-C.

The shared N-terminal domain of the superfamily is defined by significant sequence identity between dac, Dach, Ski and Sno in a region of 83 amino acids which corresponds exactly to Dachbox-N. Dachbox-N has no strong homology with known motifs, but three cysteine residues which are absolutely conserved in all members of the superfamily may play a role in the structure of this domain, by forming disulphide bonds or coordinating metal ions (Fig. 2a). There is evidence that Dachbox-N may be involved in transcriptional activation: the *Drosophila* dac protein has been shown to have transcriptional activation activity within a region containing this domain (Chen et al., 1997). Dachbox-N is also contained within the exon 1 region of *v-Ski* which has been shown to harbour both transforming and myogenic activities (Zheng et al., 1997b). In addition, Dachbox-N is contained within an N-terminal domain of Ski protein which mediates DNA binding and transcriptional activation by interacting directly with the NF-1 family of transcription factors (Tarapore et al., 1997). Therefore the N-terminal domain of Dach/dac may function by interacting directly with proteins involved with the transcriptional apparatus.

It was suggested by Chen et al. (1997) that *Drosophila* dac functions within a complex to regulate transcription, and our protein sequence analysis suggests two domains of Dach/dac which may be involved in protein-protein interactions. No DNA binding motifs are evident within the protein, and this is consistent with the model of Chen et al. (1997) in which dac does not bind to DNA directly bu forms a protein complex with eya and other factors in orde to provide the specificity necessary for transcriptional acti vation of downstream genes during eye development. W provide further evidence that dac indeed acts as an 'adaptor in a multi-protein complex, and we propose that dac ma interact with itself through the coiled-coil domain, with cell specific factors such as eya through Dachbox-C and wit general transcriptional activators through Dachbox-N domain. We predict that these protein–protein interaction will be phylogenetically conserved.

3.2. Dach is expressed in eye, limb and CNS in mouse an Drosophila

Among other sites of expression, *Drosophila dac* is foun in the leg and eye primordia and CNS including the opti lobe of the larval brain. Similarly, mouse *Dach* expression i found in the embryonic eye, limb, and brain. Expression i the leg and eye is especially relevant, since these two struc tures are abnormal in *Drosophila* loss-of-function muta tions. The leg phenotype, in which the intermediate seg ments are abnormal, corresponds to larval dac expressio in a medial ring of cells in the imaginal disc. In the mouse *Dach* expression is found within the central mesenchyme o the limb bud at E10.5 (Fig. 5c). We speculate that thi central mesenchyme may be coincident with the regio that will form the long bones of the zeugopod or the tarsal and carpals of the footplate. Mesenchymal condensation indicative of chondrogenesis are initially detected aroun E11.5 (Wright et al., 1995), approximately 1 day after *Dac* is found in the central mesenchyme. By this stage, *Dac* expression has resolved into the distal mesenchyme of th footplate. Thus if *Dach* is performing an analogous role t its *Drosophila* counterpart, and effecting intermediate struc tures, it is likely to be specifying these elements rather tha regulating chondrogenesis. The later expression in the dista mesenchyme may be indicative of a role in outgrowth, par ticularly at the tips of the digits.

The eye in *Drosophila dac* mutants is either missing, o its size severely reduced, with head cuticle replacing al retinal structures. A roughened appearance in the smal eyes is caused by abnormal photoreceptor cells. Mutation in *Drosophila ey*, homologue of the mouse *Pax6* gene results in a similar phenotype and evidence suggests early eye development is mutually dependent on both *ey* and *dac* Based on the correlation with *Drosophila*, predictions fo the role of *Dach* in mouse is either at an early stage, i neuroretina formation or later, in photoreceptor cell dif ferentiation. *Dach* is expressed in the undifferentiated neu roretina in the same pattern as, and at similar stages to *Pax6*, which has a role at the earliest stages of neuroretina formation (Quinn et al., 1996). Thus, if the relationship between *Dach* and *Pax6* in mouse is similar to that in *Dro sophila* we would expect *Dach* to have a role in early retina formation.

In the vicinity of the eye *Dach* is expressed in the adjacent mesenchyme. This mesenchyme is neural-crest derived and is the source of many specialised eye structures such as the ocular muscles. The possibility exists that this mesenchyme is involved in inductive interactions specifying the layers of the early optic cup.

3.3. Dach expression is not directly controlled by Pax6

It was suggested (Halder et al., 1995) that since *ey* can induce ectopic eye structures in *Drosophila*, *Pax6* must be at the top of a genetic hierarchy controlling eye development. Other genes, *dac* and *eya*, have since been shown to produce ectopic retinal development (Bonini et al., 1997; Shen and Mardon, 1997) indicating that there is a complex network of interactions rather than a straightforward linear pathway. When any of the three genes, *dac*, *ey* or *eya*, is ectopically expressed, the remaining two are induced in the ectopic eye region, therefore positive feedback loops must exist. Both genetic and protein-protein interactions have also been shown between *dac* and *eya* (Chen et al., 1997). This suggests that complex formation and genetic interactions within a network are important for *Drosophila* eye formation.

Is *Dach* involved in a similar network in mammals? Many *Drosophila* eye developmental genes are conserved between mammals and *Drosophila*, and now we include *Dach* in this group. *Dach*, *Pax6*, *Six3* and three members of the *Eya* family are expressed in the embryonic mouse eye. Thus, several requisite members of the proposed network are present. Our data suggest that where both *Pax6* and *Dach* are expressed together, at least at the level of transcription, *Pax6* is not required for *Dach* production. The expression of *Dach* within the E12.5 *smalleye* telencephalon is the same as in the wildtype brain, showing that in this tissue *Dach* expression is either independent, or is upstream of *Pax6*. Alternatively, interactions may occur between these two gene products and it is at this level that these two operate in the genetic network. However, in the majority of the tissues in which *Pax6* and *Dach* are expressed the patterns do not overlap; for example a striking pattern is observed in the neural tube, where *Dach* and *Pax6* are expressed in complementary domains. Similarly, *Drosophila dac* has a function in the limb independent of *ey*. We suggest that *Dach* will prove to have a variety of functions within the developing mammal and that the relationship with *Pax6* may be important in specifying this function within a few of these tissues whereas in others *Dach* will act independently of *Pax6*.

4. Experimental procedures

4.1. Isolation of human and mouse dachshund-related cDNAs

The initial *dachshund*-related human cDNA clone was identified by a tBLASTn search of dbEST. The matching EST, accession number AA059243, was derived from an IMAGE consortium human adult retina cDNA clone, ID 381801, which was obtained from the UK HGMP Resource Centre, Hinxton. End sequences of 381801 were obtained with T3 and T7 primers and four overlapping IMAGE cDNAs were identified by database screening: 668097, accession numbers AA252130 (5′) and AA252079 (3′); 129969, accession numbers R11546 (5′) and R19271 (3′); 629976, accession numbers AA219450 (5′) and AA219327 (3′); and 132326, accession numbers R25458 (5′) and R26283 (3′). These cDNAs, and subclones of them, were sequenced in entirety on both strands using ABI Prism T3 and T7 dye primers (Perkin Elmer) according to the standard dye primer protocol, with the exception that the plasmid DNA was purified on a Centricon-100 column (Amicon) beforehand. Reactions were run on a ABI 373 automated sequencing machine and the data were processed using ABI Sequence Editor software. Sequence contigs were generated using the GCG program GelAssemble.

To screen a mouse cDNA library, a probe was produced from human IMAGE clone 381801 by digestion with SmaI and XhoI. The digest was run out on 1% agarose and the 1.8-kb band extracted using a Qiaquick gel extraction kit (Qiagen). The probe was labelled with [^{32}P]dCTP using a random-primed DNA-labelling kit (Boehringer Mannheim). Approximately 1×10^6 clones from an oligo dT plus random-primed mouse E11.5 embryonic 5′ stretch plus cDNA library (Clontech) were screened with this probe. Hybridisation was carried out at 65°C in 4× SSC, 0.4% SDS, 0.2% NaPPi, 100 μg/ml salmon sperm DNA and 2× Denhardt's. Washes were carried out at 65°C in 2× SSC, 0.4% SDS and 0.2% NaPPi. Positive clones were subjected to secondary and tertiary screening after which isolated plaques were amplified by PCR using primers to the GT11 vector arms (CTCCTGGAGCCCGTCAGTATC and CTGGTAATGGTAGCGACCGGC). The PCR products were cloned into PCR2.1 using an Original TA cloning kit (Invitrogen). Sequencing was then carried out using Sequenase 2.0 (Amersham life sciences) and dRhodamine terminator cycle sequencing (Applied Biosystems).

Two clones from the 5′ end of the gene (up to nt 1567) were obtained in this way. The 3′ end was obtained by rescreening the library using human IMAGE clone 668097.

4.2. Identification of a C. elegans homologue of dachshund

A search of the TREMBL protein database with the Dach amino acid sequence identified a match with B0412.1 (accession number P90985), a predicted protein from a *C. elegans* genomic cosmid, B0412 (accession number U80953; Wilson et al., 1994). Protein B0412.1 contains amino acids homologous to residues 21–24 of *Dach*,

directly adjacent to amino acids homologous to residues 59–103 of *Dach*, and is apparently lacking residues 25–58. Inspection of the nucleotide sequence of cosmid B0412 revealed a genomic exon of 102 bp (bases 29746–247 of U80953), flanked by consensus splice sites, encoding an amino acid sequence homologous to residues 25–58 of Dach.

4.3. Secondary structure prediction

All protein structure analysis was carried out using programs available from GCG (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, WI). Secondary structure prediction was done with the program Peptidestructure using the algorithms of Chou–Fasman and Garnier–Osguthorpe–Robson, and displayed using the program Plotstructure. Coiled-coil prediction was performed using the program Coilscan with the matrix mtidkcoils.dat (Lupas et al., 1991; Lupas, 1996). Screening was performed initially with a window of 28, which was reduced to 14 to refine the position of the ends of the coiled-coil region.

4.4. Southern blot analysis

Genomic DNA, from livers of adult AKR/J and DBA/2J mice, was digested with Acc1, Apa1, EcoR1, Nde1 and Pst1 (BCL). 10 μg of DNA was digested overnight in the appropriate buffer at 37°C, run out on 1% agarose and blotted onto Zeta-Probe GT membrane (Biorad). Probe was produced from the cloned cDNA by PCR using primers: GCTTTCGACCTGTTCCTGAAG and CTTTGAGTCCTCTTAGGAGGC (nt138–382). The band was extracted from a 1% low-melting-point agarose gel into 3× its weight in distilled H_2O, and boiled for 10 min before an aliquot was labelled by random priming. Hybridisation was carried out at 65°C in 6× SSC, 10% dextran sulphate, 4× Denhardt's, 0.1% NaPPi, 0.5% SDS/SLS. Two washes of 5 min were carried out at 30° in 2× SSC then two for 30 min at 65°C in 2× SSC, 0.1% SDS.

4.5. Whole-mount in situ hybridisation

The method used is based on several protocols (Hecksher-Sørensen et al., 1998; Hemmati-Brivanlou et al., 1990; Wilkinson, 1991; Izpisua-Belmonte et al., 1993).

Embryos were fixed overnight in 4% paraformaldehyde (Sigma, St. Louis, MO) in PBS, then dehydrated through a series of 25%, 50% and 75% methanol in PBT (PBS + 0.1% Tween 20, Sigma) and stored in methanol at −20°C. They were rehydrated through the same series in reverse, then washed three times in PBT. Treatment in proteinase K (10 μg/ml) was carried out, depending on the age of the embryo; E10.5, E11.5 and E12.5 were treated for 30, 40 and 50 min respectively. Embryos were then refixed in 4% paraformaldehyde for 45 min.

Embryos were washed twice in hybridisation solution (50% formamide (Sigma), 5 × SSC, 2% blocking powde (Boehringer), 0.1% Triton X-100 (Sigma), 0.5% CHAPS (Sigma), 5 mM EDTA, 50 μg/ml Heparin (Sigma), and mg/ml yeast RNA (Sigma). Prehybridisation was carried ou at 65°C in hybridisation solution for 1 h then 4 h in new solution. DIG-labelled RNA (1 μg/ml) probe was denatured at 80°C for 3 min in 50 μl hybridisation mix, which was then added to the embryos and hybridised overnight at 65°C Probe was produced using a DIG labelling kit (Boehringer) to transcribe from the T7 promoter of PCR 2.1 containing nucleotides 1674–810 of *Dach*. Sense probe and *Pax6* probe were also produced in this way and hybridisations carried out using all three.

Washes were carried out in decreasing concentrations of hybridisation mix in 2× SSC (75%, 50%, 25%) at room temperature, followed by two washes in 2× SSC, 0.1% CHAPS and two washes in 0.2× SSC, 0.1% CHAPS all for 30 min at 55°C. Embryos were washed twice in TNT (100 mM TRIS pH 7.5, 150 mM NaCl and 0.1% Triton X-100) then blocked for 3–4 h at 4°C (TNT, 2% BSA (BDH) 15% heat-inactivated sheep serum (Sigma)). Fresh blocking solution containing anti-DIG-AP fragments (Boehringer) at a dilution of 1/2000 was added and incubation carried out overnight at 4°C.

Embryos were washed for 1 h four times, once overnight then 30 min twice in TNT, 0.1% BSA and three times for 10 min in NMT (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5). Staining was carried out in NMT, 3.5 μl/ml BCIP (Boehringer) (50 mg/ml in 100% dimethylformamide) and 4.5 μl/ml NBT (Boehringer) (75 mg/ml in 70% dimethylformamide). When developed, embryos were rinsed in PBS to stop the reaction, then fixed overnight in 4% paraformaldehyde. Sections were cut, using a vibratome, at a thickness of 100 μM.

Prior to sectioning, the embryos were washed in PBS overnight, in 4% sucrose in PBS again overnight, then in 20% sucrose in PBS for several hours, before final washing overnight in 0.5% gelatin, 20% sucrose, 15.5% BSA in PBS. Embryos were fixed in 25% gluteraldehyde for 20, 25 and 30 min for E10.5, E11.5 and E12.5 embryos, respectively, and dried on a tissue, before being embedded in 6 ml of gelatin/sucrose/BSA/PBS solution with 400 μl of 25% gluteraldehyde to set it. The block was allowed to set, and then sectioned.

5. Note added in proof

The mouse *Dach* and human *Dach* cDNA sequences have been deposited in the EMBL database with accession numbers AJ005669 and AJ005670, respectively.

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BRIEF MAPPING REPORTS

Human (*DACH*) and Mouse (*Dach*) Homologues of *Drosophila dachshund* Map to Chromosomes 13q22 and 14E3, Respectively

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Functional gene description: *DACH* and *Dach* are the human and mouse homologues, respectively, of *Drosophila dachshund,* a gene involved in eye and leg development in the fruitfly (1, 3). The human and mouse cDNAs both contain a putative open reading frame of 517 amino acids, with just 3 amino acid differences between the two species (3). DACH, Dach, and dachshund proteins have extensive sequence and structural homology, sharing an N-terminal domain of 83 amino acids, a C-terminal domain of 72 amino acids, and a novel extended coiled-coil "zipper" domain (3). Like *dachshund,* mouse *Dach* is expressed in the developing limb and eye (3). The dachshund protein was recently shown to interact directly with the protein encoded by the *eyes absent* gene, another key regulator of *Drosophila* eye development (1). *eyes absent* also has highly conserved mammalian homologues; thus DACH and Dach may act to modulate transcriptional specificity during mammalian development by conserved protein–protein interactions (1, 3).

Name of clones or DNA source: Human *DACH* (Accession No. AJ005670) and mouse *Dach* (Accession No. AJ005669) cDNA clones have been described previously (3). Human FISH mapping was performed with the longest human *DACH* cDNA clone, IMAGE ID 381801. To obtain a probe for mouse FISH mapping, a mouse genomic library in the λget vector (5) was screened by hybridization with a PCR product corresponding to nucleotides 138–382 of the mouse *Dach* cDNA clone (3). Positive phage were recircularized as previously described (5), and one clone, designated pλDach, was used for mapping.

Description of clones or DNA: The human IMAGE cDNA clone 381801 has an insert of 1955 bp (bases 1–1955 of AJ005670) in a modified version of vector pT3T7. pλDach has an insert of 18 kb in the plasmid vector derived from λget.

Source: 381801 is from the Soares human retinal cDNA library and was obtained from the HGMP Resource Centre (Cambridge, UK). pλDach is from a mouse genomic library in the vector λget (5), a gift from Dr. T. Boehm.

Method to validate gene identity: Partial sequencing of the mouse genomic clone pλDach revealed an exact match with the 5′ end of the previously determined mouse *Dach* cDNA sequence (AJ005669).

Flanking markers used: To confirm the mouse FISH assignment (see below), the hybridization was repeated with a chromosome 14-specific paint (CamBio, Cambridge, UK).

Methods of mapping: The STS subset of GenBank was searched using the BLAST 2.0 algorithm (available from the National Center for Biotechnology Information (NCBI); http://www.ncbi.nlm.nih.gov/BLAST/) with the sequence of the human *DACH* 3′ untranslated region. An exact match was identified with a human STS, *WI-18453* (Accession No. G24265), which has previously been mapped to chromosome 13 as part of the genome-wide STS mapping project (4). The data vector for *WI-18453* is available at http://www-genome.wi.mit.edu/cgi-bin/contig/sts_info/669. *WI-18453* has been placed 191.5 cR from the top of the chromosome 13 linkage group, between the MIT framework markers *WI-5860* and *AFM350XA5* (lod score > 3.0). On the integrated map of chromosome 13 (available from NCBI at http://www.ncbi.nlm.nih.gov/Entrez/Genome/org.html), *WI-5860* and *AFM350XA5* are located 94–97 cM from the top of chromosome 13, in the distal portion of 13q21 near the boundary to 13q22.

To confirm the radiation hybrid assignment, the human *DACH* cDNA clone 381801 was labeled by nick-translation with bio-16–dUTP (Boehringer Mannheim) and hybridized to normal human metaphase chromosomes as previously described (2). Hybridization signals were detected with successive layers of avidin FITC, biotinylated anti-avidin, and avidin FITC. The chromosomes were counterstained with 4′,6-diamidin-2-phenylindol-dihydrochloride (1 μg/ml in Vectashield). Hybridization signals were visualized using a Zeiss Axioplan epifluorescence microscope, and images were captured using Digital Scientific Smartcapture software. Twenty-four cells were scored, 15 of which gave a specific localization to 13q22. This is in good agreement with the radiation hybrid data.

To determine the chromosomal map position of *Dach,* pλDach was labeled with bio-16–dUTP and hybridized to mouse metaphase chromosomes as previously described (2). Scoring was performed without prior knowledge of the location of the human gene. Twenty-eight cells were scored, and all gave a signal on chromosome 14, band E3. To confirm this assignment, the hybridization was repeated in the presence of an FITC-labeled chromosome 14-specific paint. Twenty cells were examined, and the pλDach signal always colocalized with the distal fifth of the painted chromosomes.

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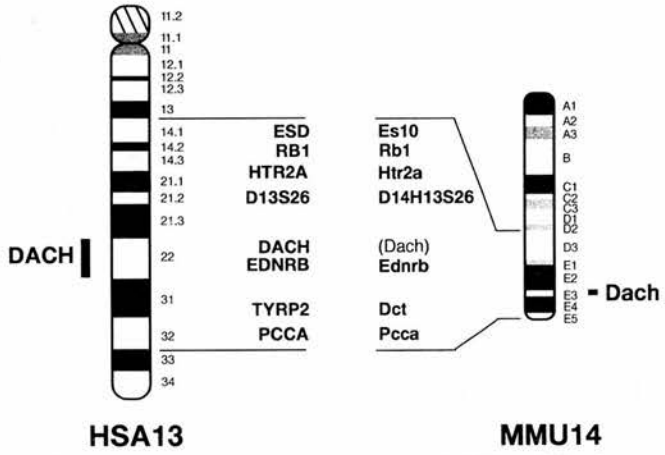

FIG. 1. Chromosomal localization of the *DACH* and *Dach* genes. Solid black bars show the position of *DACH* on human chromosome 13 (HSA13) and *Dach* on mouse chromosome 14 (MMU14) as determined by fluorescence *in situ* hybridization. Between the two idiograms are shown selected markers from the homology region between HSA13 and MMU14. Radiation hybrid mapping of human *DACH* (as STS *WI-18453*) places it just above the *EDNRB* gene; the most likely map position of the mouse gene is indicated by (Dach).

Results: The FISH results place *DACH* and *Dach* within a well-characterized region of extensive homology between human chromosome 13 and mouse chromosome 14 (Fig. 1). This region covers the distal portion of mouse chromosome 14 from 41 to 70 cM (the telomere) and two-thirds of the long arm of human chromosome 13 from 13q14–q32 (data taken from the human/mouse homology map, http://www.ncbi.nlm.nih.gov/Homology/ and the Mouse Chromosome 14 Committee Report, http://www.informatics.jax.org/bin/ccr/index). On the integrated map of human chromosome 13, the *DACH* STS *WI-5860* lies just distal of *EDNRB,* which is one of the markers included in the comparative map (Fig. 1). Therefore it is likely that mouse *Dach* maps close to *Ednrb,* which is located at 51 cM on mouse chromosome 14.

Additional comments: Two disease loci of interest have been mapped to 13q: *RIEG2* (Rieger syndrome type 2, OMIM 601499) and *PAP-A2* (postaxial polydactyly type A2, OMIM 602085). *DACH* lies outside the critical interval for *RIEG2,* but is located at the proximal extreme of the critical interval for *PAP-A2* (near *D13S800*). Given the expression pattern of mouse *Dach* in the developing limb (3), *DACH* could be a candidate for *PAP-A2.*

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Eye Development: Gene Control

Category:
Secondary

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Molecular analysis of developmental control genes is providing novel insights into evolutionary processes. A survey of a range of species examining the *Pax6* gene and its expression in the eye suggests a closer evolutionary relationship than expected for eyes as diverse as the planarian eyespot and the complex organ of the mouse. Analysis of other developmental genes, particularly in mouse and *Drosophila*, raises further questions about evolutionary mechanisms.

A Common Evolutionary Origin for Eyes?

An amazing variety of eyes exist throughout the animal kingdom, each type fine-tuned to the environment in which a particular organism lives. This diversity of eye type suggests that these very different structures evolved independently of one another. Indeed the eye has often been used as a textbook example of independent and convergent evolution. Recently, however, there has been much discussion of a common eye ancestry, as evidence has amassed that many regulatory genes are shared between eyes as different as those of *Drosophila* and mouse. Thus studies of developmental regulatory genes have acquired an evolutionary perspective, and have raised the prospect that undefined associations between distant organisms exist.

The case for the existence of independent and convergent evolution of the eye is persuasive. Throughout the animal kingdom at least 10 distinct eye types are found, which differ at all levels from the developmental mechanisms employed in eye formation to the final structure and method of function (reviewed in Land and Fernald, 1992). This is illustrated in a comparison of three diverse eye types as represented by *Drosophila*, mammalian and cephalopod (squid) eyes in Figure 1a (reviewed in Hill and Davidson, 1994; Halder *et al.*, 1995a). The fly eye, at a single glance vastly different from the eye of the mouse or the squid, is a compound eye composed of an array of approximately 750 facets or ommatidia, each capable of photodetection, which combine to interpret a complex image. Mammals and cephalopods have a single-lens arrangement which focuses a simple image on the retina for detection. All three of these ocular structures derive from developmental mechanisms that have little in common (Figure 1b). The fly eye develops from an imaginal disc within the larva, such that the photoreceptor cells develop in a wave of morphogenesis generated by the morphogenetic furrow spreading across the presumptive eye. The eyes of cephalopods and mammals, which superficially appear similar, are in fact an excellent example of convergent evolution. The developmental origins of the photoreceptors are very different, with those of the squid arising from an internalization of a surface epithelium-derived eye placode on the head. Mouse photoreceptors in contrast are neurepithelial derived and develop from an outpocketing of the fetal brain. In addition, cephalopod eyes have an everse retina, with the photoreceptors pointed towards incident light, while mammals have an inverted retina. This means that in mammals light must pass through the cell bodies of the photoreceptors before reaching the light-sensitive membranes. These two organisms have independently evolved a similar solution to the problem of vision using different evolutionary strategies and developmental mechanisms.

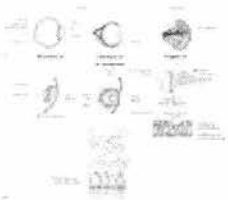

Figure 1
(a) Illustration of three diverse eye types based on a typical mammalian eye, a cephalopod (squid) eye and *Drosophila* eye. (b) The developmental processes which give rise to the mammalian eye and the *Drosophila* eye are very different. The expression of *Pax6* (eyeless in *Drosophila*) is shown in red. In the mammalian eye *Pax6* is expressed in the region of the forebrain which buds out to form the optic cup; the inner layer of this cup will become neural retina whereas the outer layer gives rise to the retinal pigmented epithelium (RPE). Invagination and differentiation within the optic cup is mediated by interactions with the head surface ectoderm which itself invaginates to form the lens. *Pax6* expression is downregulated first in the RPE and then in the neural retina. In *Drosophila* eye development *Pax6* is expressed in undifferentiated epithelium anterior to the morphogenetic furrow which sweeps across the eye imaginal disc in a posterior-to-anterior direction. The photoreceptors differentiate on the posterior side of the furrow.

Recent evidence that regulatory genes which control normal eye development are shared between eyes of vastly different organisms raises doubts of independent evolution and suggest an underlying commonality. For example, the *Pax6* gene in the homozygous mutant form results in the loss of eye structures in both flies and mammals (Quiring *et al.*, 1994). The conservation of *Pax6* between species and the fundamental role this gene plays raises the possibility that there is an evolutionary connection that links the ancestry of these eye types. The conundrum lies in the fact that these eye structures are clearly different, arise from diverse developmental mechanisms, and thus do not fit easily into an evolutionary scenario based on ancestral lineage. This raises the persistent question – is a *Pax6* function in both fly and mouse eyes fortuitous, since *Pax6* is broadly expressed in the central nervous system of both animals and may simply overlap in the eye? This now seems unlikely since *Pax6* homologues are also found expressed in the eyes of other species such as the squid, ribbonworm and planaria which represent quite diverse phyla (discussed below). Also there are more and more regulatory genes that have been found shared by the eye. Thus, this dichotomy between the molecular and the anatomical data has led to a re-examination of the relationship between these structures, which are linked by no apparent evolutionary continuity.

The Role of *Pax6* in Eye Development

Pax6 encodes a transcription factor containing a conserved paired box and paired like homeodomain which are 94% identical at the amino acid level between mammals and flies. Splice sites within the conserved regions of the gene are also conserved (Quiring *et al.*, 1994). Loss of *Pax6* in the mouse and in *Drosophila* leads to the smalleye and eyeless phenotypes respectively (Quiring *et al.*, 1994); both are characterized in the homozygous form by a complete loss of eye structures. The homozygous Pax^{sey}/Pax^{sey} mouse has no eyes or nasal cavities at birth and has brain abnormalities. Death occurs shortly after birth. Earlier in development, by 12.5 days postcoitum, the optic vesicle has become distorted and degenerates, and lens placode formation has not occurred (Hogan *et al.*, 1988). *Pax6* must therefore be necessary for the early stages of eye development. The most obvious phenotype in heterozygous mice is the small size of the eyes. They also have small vacuolated lens and cataracts may form about three weeks after birth (Hogan *et al.*, 1988). This phenotype is reminiscent of the human

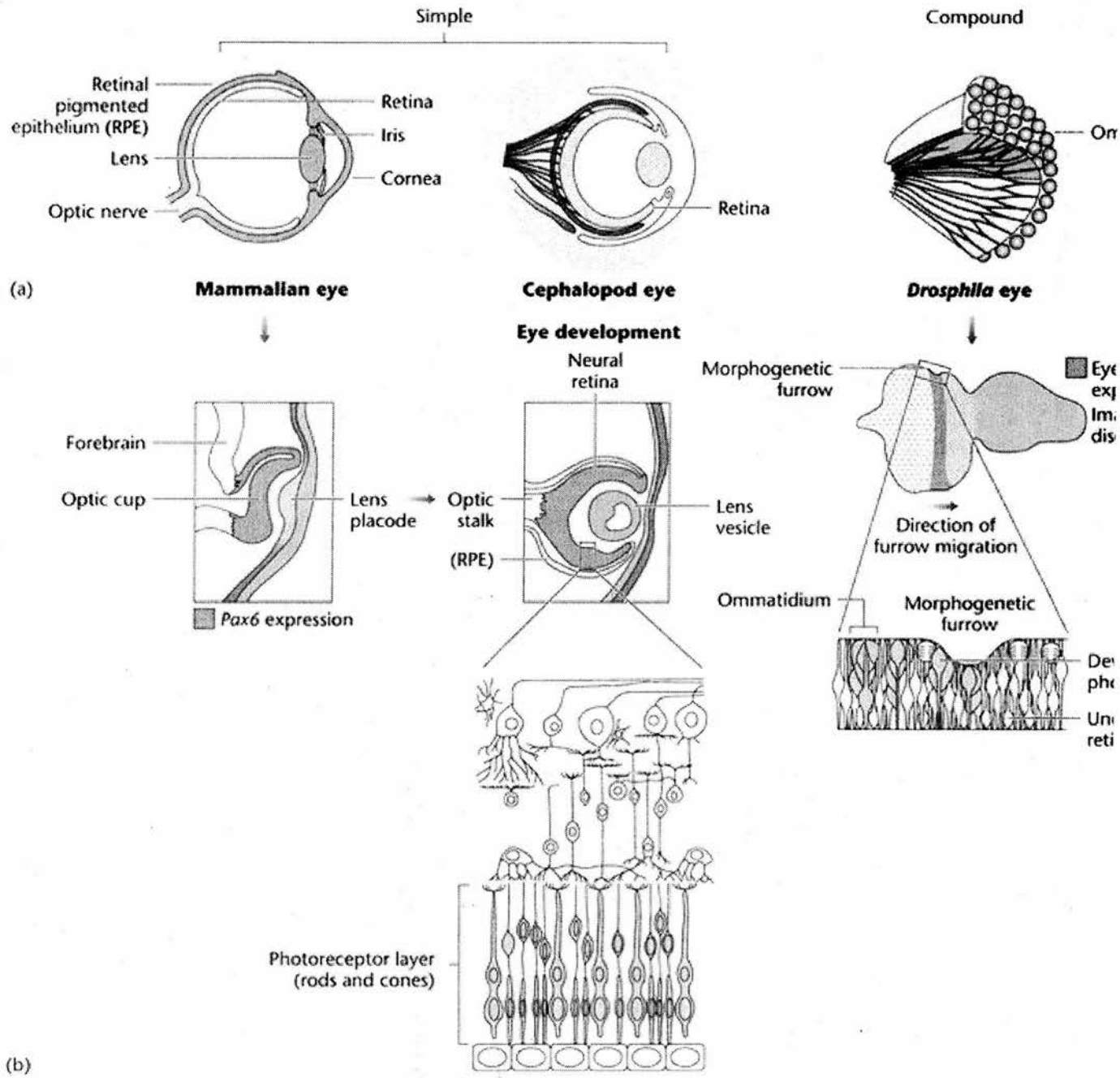

Figure 1 (a) Illustration of three diverse eye types based on a typical mammalian eye, a cephalopod (squid) eye and *Drosophila* eye. (b) The developmental processes which give rise to the mammalian eye and the *Drosophila* eye are very different. The expression of *Pax6* (eyeless in *Drosophila*) is shown in red. In the mammalian eye *Pax6* is expressed in the region of the forebrain which buds out to form the optic cup; the inner layer of this cup will become neural retina whereas the outer layer gives rise to the retinal pigmented epithelium (RPE). Invagination and differentiation within the optic cup is mediated by interactions with the head surface ectoderm which itself invaginates to form the lens. *Pax6* expression is downregulated first in the RPE and then in the neural retina. In *Drosophila* eye development *Pax6* is expressed in undifferentiated epithelium anterior to the morphogenetic furrow which sweeps across the eye imaginal disc in a posterior-to-anterior direction. The photoreceptors differentiate on the posterior side of the furrow.

diseases associated with loss of a single copy of *Pax6*. These include aniridia, characterized by a reduced or absent iris, and Peter's anomaly (reviewed in Graw, 1996). Thus, the paired family of genes originally identified in *Drosophila* has taken on clinical importance and is relevant to human disease.

Given then that *Pax6* is essential for eye development, what is its role within the eye? Is the only function at an early stage or does it persist and play a part in later development too? Investigating the expression patterns of the gene within both flies and mammals has, along with functional studies, provided some clues; however, the precise functions are still not clear.

In *Drosophila*, eyeless (*ey*) can be detected early in the embryonic eye anlagen and later, during larval stages, in undifferentiated cells of the eye disc anterior to the morphogenetic furrow (see Figure 1b). Investigation of eyeless function in *Drosophila* has focused mainly on the early role in eye specification. Indeed, it has been shown that *ey* is both necessary and sufficient for eye formation in the imaginal discs of *Drosophila* (Halder *et al.*, 1995b). *Ey* was ectopically expressed throughout the developing fly, resulting in the production of eyes from the wing, leg and antennal discs in positions corresponding to the sites of *ey* expression. Halder *et al.* (1995b) suggested that *ey* is a master control gene for eye formation, providing a switch which turns on the cascade of morphogenetic events for eye production. Interestingly, mouse *Pax6* can substitute for *ey* in this assay and induce ectopic eyes in *Drosophila*, demonstrating the homology of these genes (Halder *et al.*, 1995b).

In mice, *Pax6* is detectable in all layers of the eye from the earliest stages of development. The mammalian eye forms from an outpocketing of the brain which evaginates forming the optic cup which contacts the head ectoderm. Both the optic cup, which will form the retina, and the ectoderm, which will form the lens, express *Pax6* strongly at this stage (see Figure 1b). Again this is consistent with an early role for *Pax6*. As development proceeds, the neuroretina, the retinal pigmented epithelium (RPE) and the lens all express *Pax6* (Grindley *et al.*, 1995). RPE expression turns off just over half way through gestation but the neuroretinal and lens expression remain throughout development, the neuroretinal expression becoming localized to the ganglion cell layer. In the adult eye this expression pattern is maintained, suggesting an ongoing need for *Pax6* in various tissues as well as the early determinative role. *Pax6* is not only expressed in the eye of both mouse and fly but also in the central nervous system, and also, in the mouse only, in the nasal epithelium, pituitary (reviewed in Freund *et al.*, 1996), pineal gland and pancreas.

At the earliest stages it has been speculated that the optic vesicle may induce the formation of the lens placode from the ectoderm via *Pax6*. It was shown, however, that the optic vesicle cannot, as was initially thought, induce lens from all regions of ectoderm which it happens to contact. Large regions of the head ectoderm are competent to form lens early in development, but this region gradually reduces in size until only the presumptive lens area is competent (Grainger, 1992). *Pax6* is initially detectable throughout large regions of the head ectoderm, this region reducing as development proceeds, until eventually it is only detectable in the presumptive lens area. This pattern correlates well with the lens competent regions, suggesting that the presence of *Pax6* in the ectoderm is important for lens induction. Consistent with this suggestion it has been found using transplantation techniques, that a wild-type optic cup cannot induce lens formation from smalleye head ectoderm (reviewed in Freund *et al.*, 1996).

Various downstream targets for *Pax6* have been suggested, based on possession of *Pax6*-binding sites by genes that are affected by the presence of *Pax6*. In a variety of species some of the crystallins, which are vertebrate lens proteins, have been shown to be stimulated by *Pax6*. For example, *Pax6* binds to sites on aA-crystallin of mice and chickens, thus stimulating promoter activity (reviewed in Freund *et al.*, 1996). Since the crystallins evolved from proteins which were already present in the animal, it is possible that the acquisition of *Pax6*-binding sites allowed them to be tested out as lens proteins. It is also thought that L1-CAM, a cell adhesion molecule, may be targeted by *Pax6* as it contains binding sites and has been shown to bind to *Pax6 in vitro* (Freund *et al.*, 1996).

Functional evidence for the multiple roles of *Pax6* within the eye has come from the analysis of

chimaeric mouse embryos containing a mixture of wild-type and *Pax6*^{sey}/*Pax6*^{sey} mutant cells (Quinn *et al.*, 1996). These experiments clearly showed a cell autonomous role for *Pax6* within the lens, neuroretina and retinal pigmented epithelium (RPE). A threshold number of wild-type cells were also needed to produce a lens. If the embryo contained more than 60% mutant cells no lens formed, suggesting that *Pax6* may be important for interactions between the lens cells. In the optic cup absolute segregation of mutant and wild-type cells suggest that *Pax6* effects cell surface mediated cell–cell adhesion. In addition, the absence of pigmentation in the chimaeric RPE suggests that this tissue is not being properly specified. It seems then that in the mammalian eye *Pax6* has a multifunctional role to play controlling distinct aspects in each layer of the early eye.

Pax6 and Evolutionary Considerations

Pax6 has been isolated from a variety of organisms (Callaerts *et al.*, 1997) including a nemertean (the ribbonworm *Lineus sanguineus*), a cephalopod (the squid *Loligo opalescens*), a roundworm (the nematode *Caenorhabditis elegans*), a flatworm (the planarian *Dugesia tigrina*), two species of chordates (a urochordate *Phallusia mammillata* and a cephalochordate *Branchiostoma floridae* (amphioxus)) and representatives from each class of vertebrates.

In vertebrates *Pax6* is not only highly conserved at the gene sequence level, but has the same expression pattern found in the mouse. Zebrafish *Pax6*, for instance, has 97% identity to the mouse gene at the amino acid level, even having the same two alternative splice forms, and is found in identical domains of the embryonic eye and CNS. In the more distantly related ribbonworm *L. sanguineus* and planarian *D. tigrina*, both of which have considerable regenerative capacity, *Pax6* is expressed in the central nervous system and eye region of the regenerating head. *Pax6* was found in the region of the pigmented spots and photoreceptor cells which represent the earliest sign of regenerating eyes. It is hence likely that *Pax6* was present in primitive metazoa, before the separation of the chordate and arthropod lineages. Amphioxus (Glardon *et al.*, 1998), which represents the invertebrate chordates most closely related to vertebrates, shows *Pax6* expression in the lamellar organ and the frontal eye which are the presumed homologues of the vertebrate pineal eye and paired eyes, respectively. The underlying embryonic plan from which mammalian eyes develop may therefore have been in place since the lower Cambrian period.

From this growing survey of organisms does the analysis of *Pax6* reveal an evolutionary relationship between organisms? So far in all animals examined (with one exception the eyeless nematode *C. elegans*) *Pax6* is found expressed in the eyes. If basic eye development and structure argues against structural homology perhaps a rudimentary unit exists which is key to all eye designs. All known visual systems have specialized photoreceptor cells which express light-sensitive proteins called opsins. Opsins are members of the seven-transmembrane class of receptors and are all coupled to a vitamin A-derived chromophore. Studies based on the structure of photoreceptor cells, and the homology shown among the opsins from at least 12 species, suggest a monophyletic origin of this cell type (Land and Fernald, 1992). Photoreceptor cells are fundamental to light detection in every eye type and in the animals examined, *Pax6* is expressed either in these cells types or in their precursors. This leads to the model that ancestral *Pax6* became associated with the regulation of a target gene fundamental to photodetection, this relationship acting as the cohesive force during evolutionary change. Can the simplistic assumption that photoreceptor cells are the fundamental evolutionary building blocks account for the various eye types that have arisen? Nilsson and Pelger (1994) surmised that, beginning with a light-sensitive tissue, eyes could have evolved separately more than 1000 times since the first fossil evidence of eyes, about 550 million years ago. Independent evolution of all eye types therefore seems at least plausible.

Pax6 has roles in other ocular tissues, such as lens and RPE, as is known from studies in the mouse (described above). Further explanations are required to accommodate *Pax6* as a generalized eye factor into the evolutionary equation. It has been suggested that once instituted into a role in eye development *Pax6* acquired other gene targets, broadening the role of this regulator. However this by necessity follows if the model for eye evolution is accurate. The mere presence of *Pax6* in the ancestral photorecepter cell

does not provide a satisfactory mechanistic explanation for the expansion of *Pax6* regulatory roles. Perhaps further elucidation of the role of *Pax6* will shed more light on the coevolution of the eye and this regulatory gene.

Other Genes Involved in Eye Development

Other essential developmental regulators, in addition to *Pax6*, are shared by mouse and *Drosophila* eyes (see Table 1). In fact initial identification of these genes in *Drosophila* has provided a ready source for identifying the homologues in mammals. Three genes in particular are key to *Drosophila* eye development, homozygous loss of function mutations leading to severe reduction or loss of eye structures. These genes are sine-oculis (*so*), eyes-absent (*eya*) and dachshund (*dac*) (reviewed in Fini *et al.*, 1997). All three are expressed in the early stages of the eye anlagen, *eya* and *so* being involved in events anterior to the morphogenetic furrow, while *dac* is expressed at the posterior of the disc, prior to furrow initiation. The mammalian homologues of *so*, *eya* and *dac*; are *Six3*, *Eya1–3* (Fini *et al.*, 1997) and *Dach* (Hammond *et al.*, 1998) respectively, and are also expressed in the early eye. *Six3* is found in the neuroretina and lens, *Dach* in the neuroretina and the *Eya* genes between them are expressed in all the layers of the developing eye. A role in early eye development for these mammalian genes appears likely. Indeed *Eya2* can rescue *Drosophila eya* mutants, demonstrating that *Eya2* is the homologue of the *Drosophila* gene (Xu *et al.*, 1997). In the killifish medaka, ectopic expression of *Six3* can stimulate ectopic lens and retina formation, demonstrating the fundamental role that this gene may play in mammalian eye development (Loosli *et al.*, 1999). An examination of these genes in other species has not been performed so we are limited in understanding their evolutionary history. The existence of these homologues in the two species suggests that these regulatory genes were at least present in a common ancestor, before the divergence of the arthropods and vertebrates.

Table 1 *Drosophila* eye development genes and their respective mammalian homologues

Drosophila gene	*Drosophila* eye expression pattern	Mammalian gene	Mammalian eye expression pattern
eyeless (*ey*)	Embryonic eye anlagen and later, anterior to morphogenetic furrow	*Pax6*	Neuroretina, RPE, lens, cornea
eyes-absent (*eya*)	Anterior to morphogenetic furrow	*Eya3*	Neuroretina, lens vesicle
		Eya2	Neuroretina, sclera
		Eya1	Lens placode, iris, RPE, optic nerve, ciliary region
sine-oculis (*so*)	Anterior to mophogenetic furrow	*Six3*	Neuroretina, lens, optic stalk
dachshund (*dac*)	Posterior of disc prior to furrow initiation	*Dach*	Neuroretina
enhancer of split/ hairy (*E[spl]/h*)	Neural precursors	*Hes1*	Dividing neuroretina cells
prospero (*pros*)	Lens-secreting cone cells	*Prox1*	Dividing lens cells and fibres
sparkling (*spa*)	Cone cells, primary pigment cells, bristle cells	*Pax2*	Optic cup, glial cells of optic stalk, optic nerve

Recent investigations examine the interactions of these genes with *ey* and with each other. Mutations in *so* and *eya* do not affect *ey* expression, suggesting that *ey* does have a very early role previous to *so* and *eya* functions. Surprisingly, both *eya* and *dac* can induce ectopic eye structures, in a similar way to *ey*, when ectopically expressed in *Drosophila* imaginal discs (Bonini *et al.*, 1997; Shen and Mardon, 1997). *Ey* cannot therefore be the sole master regulator of eye development and it is likely that a network of interacting factors (including *so*, *eya*, *dac* and *ey*) must be present for eye formation to occur. It has been proposed, however, that *ey* is at the top of a cascade which then activates the remaining genes. Consistent with this, *dac* and *eya* do not produce ectopic eyes as often or to the same extent as *ey*, and in the absence of either *dac* or *eya*, *ey* cannot itself produce ectopic eyes. However, when any of the three genes are ectopically expressed, activation of the remaining two can be detected. Furthermore, *dac* and *eya* act synergistically, together producing ectopic eye structures with a greater penetrance than either can alone (Chen *et al.*, 1997). They have also been shown, using a yeast two hybrid assay, to interact physically *in vitro*. This suggests a model consisting of a self-reinforcing network of interacting genes rather than a linear hierarchy.

Similar mechanisms to those found in *Drosophila* may operate in mammals although there is no direct evidence for this as yet. The expression domains of the mammalian homologues are surprisingly conserved with respect to the fly, similar relationships being found between the genes. For instance the domains of the *Eya* genes overlap the *Pax6* expression domain within the eye in mammals as they do in *Drosophila*. In smalleye mutant mice no *Eya* expression is found within the lens placode where it is found in the wild-type situation. This suggests that *Pax6* is essential for *Eya* expression in mammals as it is in the fly (Xu *et al.*, 1997). *Dach* too is expressed in domains which overlap the *Pax6* expression domain, although *Dach* expression does not appear to be affected in the smalleye mouse (Hammond *et*

al., 1998). These patterns are therefore consistent with the possibility of interactions between *Pax6*, *Eya* and *Dach* constituting a conserved network in flies and mammals.

In mammals many other genes have a regulatory role in eye development. This can be inferred from their expression pattern and in some cases from the phenotype of mutants. Included within this group are microophthalmia (*mi*), *Chx10*, *msx1* and *2*, *Gli3* and *Dlx1* (reviewed in Freund *et al.*, 1996 and Graw, 1996). All are expressed within the developing eye, *msx2*, *Chx10*, *Gli3* and *Dlx1* being detected early in the presumptive neuroretina and *mi* in the RPE. *Gli3* is also found within the lens and mutations in this gene lead to small or missing eyes. The relationship of these genes to each other in eye development is unknown, however some of these genes also have homologues in the fly. For example, *Dlx* and *Gli* are the mammalian homologues of Distal-less (*Dll*) and cubitus-interuptus (*ci*) respectively (Freund *et al.*, 1996; Graw, 1996).

It might be expected that regulatory genes shared by mammals and flies would act early in the process of making an eye. Structures that appear later in eye development are often characteristic to a single class of animals and therefore late-acting genes would not be expected to be shared. Examples of homologous genes, performing analogous roles, during the later stages of eye development are, however, found. These include *Pax2* (Freund *et al.*, 1996) and the *Drosophila* homologue sparkling. Both of these genes are found in non-neuronal cells within the eye, *Pax2* in the optic stalk glial cells and sparkling in nonphotoreceptor accessory cells. In a similar way the mammalian gene *Prox1* is found in the central nervous system and the developing lens, while its *Drosophila* counterpart prospero is found in the central nervous system and in the lens-secreting cone cells (Freund *et al.*, 1996). One further example involves the mammalian gene *Hes1*, which is similar to both enhancer of split and hairy. The two fly genes are involved in neurogenesis in the eye, and in other regions of the body, where they suppress activity of the proneural genes of the acheate-scute complex. Similarly *Hes1* has been shown to suppress differentiation within the retina. Overexpression of *Hes1* results in an undifferentiated retina and lack of *Hes1* shows premature differentiation of the retina (Freund *et al.*, 1996).

There are a variety of possible explanations for these findings. It seems more than mere coincidence that a large variety of homologous genes are shared. The simplest explanation is that these genes were present in a common ancestral eye; however, further analysis in other species is required before persuasive arguments can be made. An alternative is that these genes control key developmental processes outside the eye that are coopted by the eye as modification and specialization occurs during evolution.

Summary

Evidence has accumulated that certain fundamental eye development genes are shared by diverse evolutionary lineages. It therefore seems likely that a common ancestral photosensitive organ existed requiring the presence of at least some of these genes. On the practical side, due to the relative ease of identifying key developmental genes, *Drosophila* has been instrumental in the discovery of relevant eye genes in mammals. In addition, *Drosophila* has proven to be a useful model system for understanding the molecular and biochemical properties of these gene products and intriguingly may provide an understanding of gene function in highly diverse organisms such as the mouse. Thus the study of eye development genes in a number of organisms should provide further unforeseen insights into the evolutionary process as well as a fundamental understanding of the development of an intriguing organ system.

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