ISOLATION AND CHARACTERISATION OF THE *DROSOPHILA DROR2* GENE

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ABSTRACT

Receptor tyrosine kinases (RTKs) are a family of cell-surface receptors that have an important role in an array of cellular responses including cell migration, proliferation and differentiation (Fantl *et al.*, 1993). RTKs and their ligands are important components in the determination of cell fate through signalling pathways that are activated during both invertebrate and vertebrate development (Pawson and Bernstein, 1990).

The Ror subfamily of RTKs are thought to be important for the development of the nervous system as they are expressed highly in the nervous system in the developing embryo, but expression is minimal in adults. Three receptors in this subfamily have been identified, Ror1 and Ror2 from humans (Masiakowski and Carroll, 1992) and Dror from *Drosophila* (Wilson *et al.*, 1993). This thesis involved the isolation and characterisation of the fourth gene in this family *Dror2* from *Drosophila melanogaster*.

Degenerate oligonucleotides to conserved regions of the tyrosine kinase domain of RTKs were used to PCR amplify a 200 bp fragment from genomic DNA. A λ genomic library was screened with the labelled fragment in order to isolate the gene. The resulting clone was subcloned and sequenced to obtain the complete sequence of *Dror2*. The 3' end of the gene was determined by RT-PCR. The transcriptional start point was identified by using 5' RACE and sequencing of the amplification product. Expression of *Dror2* was examined using Northern Blot hybridisation and *in situ* hybridisation to whole mount embryos.

The 725 amino acid mature Dror2 protein comprises an extracellular domain containing the signal peptide, cysteine-rich region and kringle domain, a hydrophobic transmembrane domain and the intracellular domain containing a catalytic kinase domain. Three introns were identified, one in the middle of the cysteine-rich region and two flanking the kringle domain.

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ABBREVIATIONS

α	alpha
β	beta
Δ	delta
λ	lambda
aa	amino acids
bp	base pairs
BSA	bovine serum albumin
CIP	calf intestine phosphatase
CNS	central nervous system
cDNA	complementary DNA
°C	degrees Celsius
dNTP	deoxynucleotide triphosphate
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
DEPC	diethyl pyrocarbonate
DTT	dithiothreitol
dsDNA	double stranded DNA
f	fico
g	grams
kb	kilobase pairs
L	litre
mRNA	messenger RNA
μCi	micro Curies
μ	micro
m	milli
M	molar
n	nano

nm	nanometres
nt	nucleotide
PNS	peripheral nervous system
pfu	plaque forming units
poly A^+ RNA	polyadenylated RNA
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase PCR
rpm	revolutions per minute
RNase	ribonuclease
RNA	ribonucleic acid
ssDNA	single stranded DNA
SDS	sodium dodecyl sulphate
UV	ultraviolet
U	units
UTR	untranslated region
V	volts
v/v	volume per volume
W	watts
w/v	weight per volume

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1. INTRODUCTION

1.1 RECEPTOR TYROSINE KINASES (RTKS)

Receptor tyrosine kinases (RTKs) are a family of cell-surface receptors that possess an intrinsic, ligand sensitive protein tyrosine kinase activity (Yarden and Ullrich, 1988). RTKs and their ligands are important components in the determination of cell fate through signalling pathways that are activated during both invertebrate and vertebrate development (Pawson and Bernstein, 1990). The receptors have an important role in an array of cellular responses including cell migration, proliferation and differentiation (Fantl *et al.*, 1993).

RTKs are Type I transmembrane proteins that have their N-termini outside the cell and single membrane-spanning regions (Van der Geer *et al.*, 1994). There are several structural features that are conserved among all known RTKs. At the N-terminus is a signal peptide that targets the protein to the secretory pathway. This is followed by an extracellular domain of several hundred amino acids that contains a distinctive pattern of cysteine residues and often a characteristic array of structural motifs. This ligand-binding domain is the most distinguishing feature from other RTKs. The transmembrane domain follows, which consists of a stretch of hydrophobic residues that are followed by several basic residues functioning as a stop-transfer signal. A juxtamembrane region on the cytoplasmic side of the membrane precedes the catalytic domain. All RTKs kinases share a conserved catalytic domain that contains kinase residues that are involved in receptor function. The C-terminal tail is typically very hydrophilic and rich in small amino acids and appears to have varying functions (Yarden and Ullrich, 1988).

RTKs have been classed into 14 subfamilies depending on the structure of each RTK as seen in Figure 1.1. Members of a given subfamily share common structural features that are distinct from those found in other subfamilies (Fantl *et al.*, 1993). Each subfamily contains different combinations of recognisable sequence motifs in their ligand-binding domain but all catalytic domains are similar.

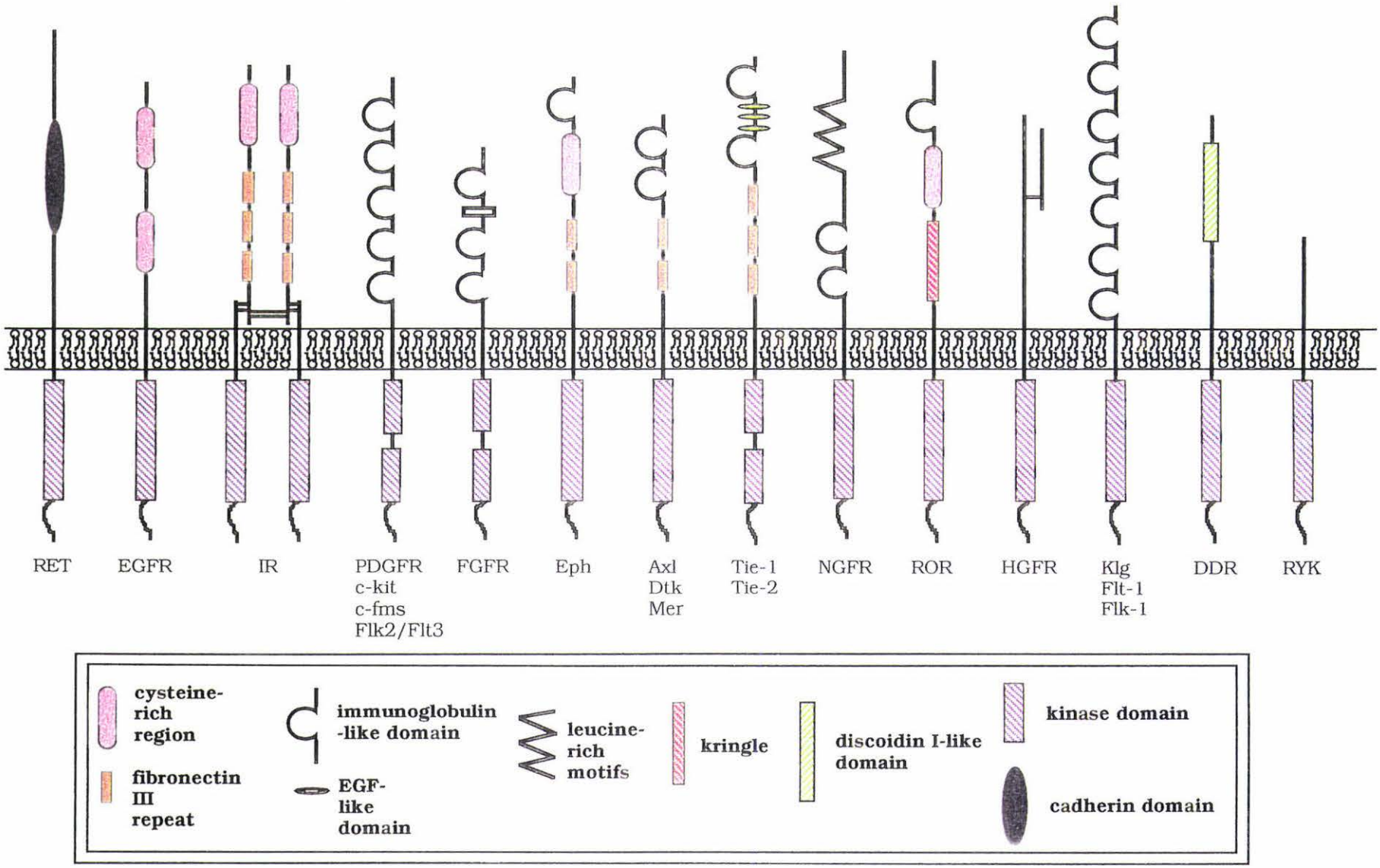
Despite the diversity of RTKs there is a great degree of commonality in the types of intracellular signalling pathways initiated by these proteins. In *Drosophila*, biochemical and molecular genetic analyses have shown that for all RTKs the binding of ligand to the extracellular domain activates the tyrosine kinase in the cytoplasmic domain. This leads to downstream activation of a number of common signalling molecules. The activation of signalling pathways involving these molecules leads to changes in gene expression and a change in the phenotypic state of the cell. A single type of RTK can elicit very different biological responses in different cell types (Fantl *et al.*, 1993). It is not clear how this is accomplished.

1.2 RTKs IN *DROSOPHILA*

The activation of similar signalling molecules has been confirmed through genetic analyses in two model systems, *Caenorhabditis elegans* and *Drosophila melanogaster*. These studies have proven that RTK molecules are important for the developmental specification of cell types and that RTKs in these organisms initiate signalling pathways that are very similar to those found in mammalian systems (Fantl *et al.*, 1993).

Figure 1. 1 Structure of the fourteen RTK subfamilies.

Abbreviations are as follows: EGFR: epidermal growth factor, IR: insulin receptor, PDGFR: platelet derived growth factor, *Flk*-2: foetal liver kinase-2, *Flt*-3: *fms*-like tyrosine kinase-3, FGFR: fibroblast growth factor receptor, NGFR: nerve growth factor receptor, HGFR: hepatocyte growth factor receptor, DDR: discoid domain receptor. Adapted from Van der Geer *et al.* (1994) by Dr Phil Crosier, University of Auckland.


RET
EGFR
IR
PDGFR
c-kit
c-fms
Flk2/Flt3
FGFR
Eph
Axl
Dtk
Mer
Tie-1
Tie-2
NGFR
ROR
HGFR
Klg
Flt-1
Flk-1
DDR
RYK
cysteine-rich region
fibronectin III repeat
immunoglobulin -like domain
EGF-like domain
leucine-rich motifs
kringle
discoidin I-like domain
kinase domain
cadherin domain

In *Drosophila*, the signalling cascades from three RTKs have been studied genetically. In the embryo, the torso (tor) RTK is present throughout the embryo and is required for the specification of terminal structures (Brunner *et al.*, 1994). In normal development an extracellular ligand activates tor at both poles of the embryo. There are no terminal structures in mutants that do not express tor. Tor gain-of-function mutants have a receptor that is active throughout the embryo, leading to the generalised expression of the tor target gene *tailless* (*tll*) (Perrimon and Desplan, 1994).

Another RTK important in development in *Drosophila* is the EGF receptor homolog named DER. DER has both maternal and zygotic functions. In the mother DER is required for the establishment of the dorsoventral polarity of the egg shell and the embryo (Schupbach, 1987). In the absence of a DER signal, a ventral instead of dorsal phenotype is induced (Pawson and Bernstein, 1990). In the zygote, the absence of DER activity at Stages 8 and 9 of embryonic development results in cells adopting an altered cell fate (Raz and Shilo, 1993).

In contrast to DER, the *sevenless* (*sev*) gene encodes a transmembrane RTK whose only known biological function is the specification of a single photoreceptor cell of the ommatidial clusters that comprise the *Drosophila* compound eye (Zipursky and Rubin, 1994). In the developing eye, specification of the R7 photoreceptor cell in each ommatidium depends on the local activation of the sev RTK in the R7 precursor cell by the membrane bound bride-of-sevenless (boss) protein on the neighbouring R8 cell (Hafen *et al.*, 1987).

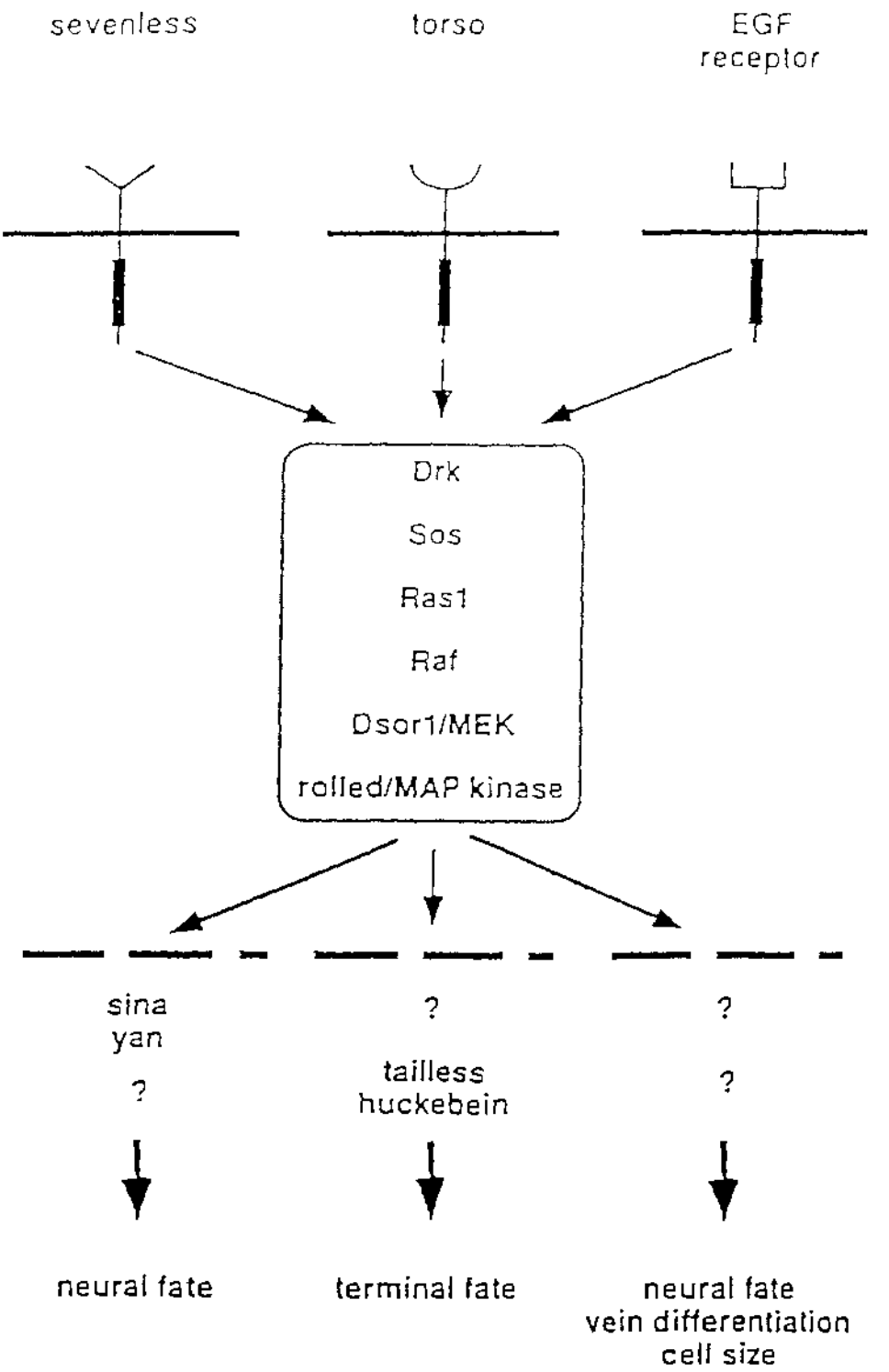
The sevenless, torso and DER RTKs are prototypes for tyrosine kinases that convey positional and developmental information by interpreting cues provided by nearby cells (Pawson and Bernstein, 1990). This type of local cellular interaction is likely to have vast significance in defining the developmental fate of cells in *Drosophila* embryogenesis.

These three RTKs have been shown to utilise a similar signalling cascade (Brunner *et al.*, 1994) as seen in Figure 1.2. Binding of a ligand to an RTK leads to activation of the cytoplasmic tyrosine kinase domain. This results in autophosphorylation of tyrosine residues allowing the receptor to interact with Drk. Following this, the Drk protein binds to the son-of-sevenless (sos) guanine nucleotide exchange factor activating the protein. Sos can now act to convert the inactive Ras-GDP to the active Ras-GTP form. The Ras guanine nucleotide binding protein has been identified as a critical determinant of cellular differentiation (Avruch *et al.*, 1994). The immediate target of Ras is Raf, a serine/threonine kinase that is the next member of the signal transduction cascade. Raf then binds and activates the MAP kinase activator MEK, which in turn activates MAP kinase. MAP kinase is the final component whose activation is both necessary and sufficient for signal transduction in the pathway.

From this point, transcription factors are phosphorylated by the pathway and subsequent genes are activated. In the torso pathway the transcription factor(s) involved is unknown, but both *tailless* (*tll*) and *huckebein* (*hkb*) are activated in the terminal regions in response to tor signalling (Perrimon and Desplan, 1994). In the sevenless pathway the expression of *sina* and *yan* are not dependent on sev activity, but may be required to mediate regulated transcription in response to sev activity (Brunner *et al.*, 1994). In the DER pathway neither the transcription factors nor genes involved in instructing dorsal follicle cell fate have yet been identified.

Figure 1. 2 Different receptors utilize a similar signalling cascade

Components common to the tor, sev and DER pathways are shown (Brunner *et al.*, 1994). See Section 1.2 for details.


sevenless
torso
EGF receptor
Drk
Sos
Ras1
Raf
Dsor1/MEK
rolled/MAP kinase
sina
yan
?
?
tailless
huckebein
?
?
neural fate
terminal fate
neural fate
vein differentiation
cell size

Along with the three major RTKs, a number of other *Drosophila* RTKs have been identified.

Dtrk is highly related to the trk family of mammalian neurotrophin receptors (Pulido *et al.*, 1992). The product of *Dtrk* is expressed in several areas of the developing nervous system. The gene is thought to promote cell adhesion that activates its tyrosine kinase activity. The trk family of receptors are discussed further in Section 1.3.3.

Dror encodes a gene that is homologous to the vertebrate Ror family of Trk-related RTKs (Wilson *et al.*, 1993). The neurotrophic receptor functions during the early nervous system development in flies. This family of receptors are discussed more fully in Section 1.3.4 and 1.4.

Dret is a homolog of the human proto-oncogene *ret*. It is transiently expressed in embryonic neuronal precursor cells including neuroblasts and CNS cells (Sugaya *et al.*, 1994). The structure of *Dret* and expression is highly related to that of human *ret*, so it is feasible that both genes are involved in the same developmental process in neuronal tissues.

The *Drosophila derailed* (*drl*) gene is involved in key aspects of neuronal pathway recognition (Callahan *et al.*, 1995). *Drl* is expressed in only a small subset of embryonic interneurons that share common pathways.

Lio encodes a putative RTK that is unusual as it contains a short extracellular domain and modified catalytic domain (Dura *et al.*, 1995). *Lio*, though not vital, is important for learning and memory in flies. Lio mutants have weak learning and memory capabilities with reduced shock reactivities and olfactory associative memory. Both *drl* and *lio* genes are homologous to the vertebrate gene *RYK*. The

function of *RYK* is unknown but evidence from *Drosophila* suggests the genes may be involved in a new, undetermined signal transduction pathway.

1.3 RTKs OF INTEREST

1.3.1 DTK

Dtk is a novel membrane bound developmental tyrosine kinase receptor that has been cloned recently in mice, zebra fish and humans (Crosier *et al.*, 1994a and b, Walshe *et al.*, 1995). Dtk is a well-conserved receptor tyrosine kinase in vertebrates, suggesting an important biological function. It is expressed in many tissues during embryogenesis and is strongly expressed in the adult brain and other tissues to a lesser extent. The 850 amino acid mature receptor protein comprises an extracellular domain with two immunoglobulin-like motifs and two fibronectin type III modules, a 25 amino acid transmembrane domain and a cytoplasmic region with a catalytic kinase domain (Crosier *et al.*, 1994a).

There are two repeating protein motifs within the domain. One of these contains two C-type immunoglobulin like motifs, the first domain has similar structure to a C1-like domain, while the second is more related to the C2-like domain. Also in the domain are two fibronectin type III motifs. Like Dtk, Axl (Ufo, Ark) also contains two immmunoglobulin domains followed by two fibronectin type III repeats in the extracellular domain (Crosier *et al.*, 1994a).

The mouse *Dtk* gene was first isolated from embryonic stem (ES) cells (Crosier *et al.*, 1994a). Using RNA isolated from differentiating ES cells, a number of RTKs were isolated that were potentially involved in haematopoietic development. Degenerate oligonucleotides derived from conserved domains within the catalytic kinase domain of protein tyrosine kinases were constructed and used in PCR

reactions to isolate the RTKs. A number of sequences from putative and known RTKs were found, one of which was designated *Dtk*.

In embryonic stem cells and embryonic bodies, *Dtk* is expressed almost uniformly from days 0-18. Within the embryonic tissues expression was detected in total RNA from a range of tissues including the brain, eye, thymus, lung, intestine, forelimb, hindlimb, and testis. There was limited expression in the heart and unfractionated liver. In the adult tissues the pattern of expression becomes restricted. Transcripts were greatest in the brain, oesophagus, bladder, testis and ovary. In the brain the expression of *Dtk* was more abundant in the adult than in embryonic tissue. There were also traces of *Dtk* in the lung, stomach and in the intestines (Crosier *et al.*, 1994a).

The human *Dtk* gene was isolated using the murine Dtk cDNA as a probe to screen a human brain cDNA library by Crosier *et al.* (1994b). This gene is identical to the human *Tyro3* gene cloned by Polvi *et al.* (1993). The protein has identical predicted structural features to those of the murine Dtk, and there is an 89 % amino acid identity between the two genes.

To analyse the expression of *Dtk* in humans, Northern blot hybridisation was carried out. *Dtk* was shown to be expressed in foetal brain and kidney, as well as low levels in foetal lung and heart (Crosier *et al.*, 1994b). In adult tissues *Dtk* expression was found in the brain, kidney, testis and ovary. Limited expression was seen in the other adult tissues with no expression in the peripheral blood leucocytes and liver. Thus, the pattern of expression was very similar to murine *Dtk*, with the notable exception that there is little expression in the adult kidney (Crosier *et al.*, 1994b).

1.3.2 EPH

The Eph subfamily is the largest of the RTKs with 13 receptors and 7 of their ligands identified in mammals to date (Muller *et al.*, 1996). The Eph subfamily of receptors comprise an extracellular domain that includes one immunoglobulin-like motif, two fibronectin type III repeats and a cysteine rich region, a hydrophobic region comprising the transmembrane domain and a cytoplasmic region with a catalytic kinase domain (Van der Geer *et al.*, 1994).

The first receptor isolated in the Eph subfamily was identified by Hirai *et al.* (1987) in a human genomic DNA library during a search for sequences homologous to the tyrosine kinase domain of *v-fps* (a viral oncogene). Following this, the remainder members of the family were identified from various vertebrate species including humans, mouse, rat, *Xenopus* and zebrafish (Pandey *et al.*, 1995).

Most of these receptors are specifically expressed in neurons, but the biological functions of many of the Eph-type RTKs are unknown. Ligand binding to an Eph RTK appears to lead to activation of a novel signal transduction pathway. For example, activation of the ectopically expressed receptors does not lead (as expected) to a biological response from the cell (Muller *et al.*, 1996).

Another unique feature of this subfamily is that only the membrane-bound forms of ligands are active, leading to speculation that these receptors play roles in axon guidance, neuronal bundling or angiogenesis (Stein *et al.*, 1996). More specifically it has been suggested that these receptors and ligands may play a significant role in forming topographic projections along the anteroposterior axis of the tectum (Tessier-Lavigne, 1995). The tectum is the structure in the brain that transmits the retinal image to the brain by rectifying the back-to-front, upside-down image. To do this, the anterior and posterior ganglion cells map to

the posterior and anterior tectum, and the dorsal and ventral ganglion cells map to the ventral and dorsal tectum (Orike and Pini. 1996). Studies performed on the chicken showed the presence of a retinotectal projection in which retinal ganglion cell axons project in a topographic manner onto the optic tectum, the target area. It is thought that complementary gradients of receptors in the retina and of corresponding ligands in the optic tectum are crucial for the formation of this projection (Muller *et al.*, 1996).

As well as this, expression of some Eph-related molecules in migratory cells suggests a further role of these receptors in the process of cell migration (Muller *et al.*, 1996). Thus it appears that Eph-type RTKs play important roles in maintaining and developing many cell and tissue types.

1.3.3 TRK

The Trk receptors belong to the Nerve Growth Factor (NGF) receptor subfamily of RTKs. The NGF receptors comprise an extracellular domain that includes a leucine rich region and two immunoglobulin-like motifs, a transmembrane domain and a cytoplasmic region with a catalytic kinase domain (Van der Geer *et al.*, 1994).

The original member of this family, proto-*trk* (Martin-Zanca *et al.*, 1986), was named after the human oncogenic product *TRK*, a cytosolic non-muscle tropomyosin receptor kinase fusion protein. This was renamed *TrkA* when two other members of this group were identified, *TrkB* (Klein *et al.*, 1990) and *TrkC* (Lamballe *et al.*, 1991). The Trk receptors share structure and sequence similarities and have been shown to bind similar ligands.

All three *Trk* genes are almost exclusively expressed in neurons and are the receptors for neurotrophins. Each neurotrophin activates specific receptors by binding to their extracellular domains. TrkA is a receptor for NGF the best

characterised neurotrophin, TrkB for BDNF and NT-4/5 and TrkC for NT-3 (Grimes *et al.*, 1993). These neurotrophins induce neurite outgrowth and rescue subpopulations of neurons from programmed cell death (Glass and Yancopoulos, 1993). In addition to the Trks, neurotrophins all bind to another cell surface receptor known as p75 or the low-affinity NGF receptor, whose physical role remains to be determined (Barbacid, 1993).

1.3.4 ROR

Ror1 and *Ror2* are two novel developmentally regulated receptor tyrosine kinases cloned from humans and rats (Masiakowski and Carroll, 1992). Both genes are highly expressed in the rat in the brain and body in early embryogenesis, suggesting a role in the development of the embryo. The Ror RTKs are thought to be involved in a network of regulatory interactions, including a possible role in signal transduction pathways and in cell-cell interactions. Ror1 and Ror2 contain one strongly homologous region to the Trk family of nerve growth factor receptors, the tyrosine kinase domain, but because of a unique extracellular region it appears they represent a new subfamily of RTKs (Van der Geer *et al.*, 1994).

The 937 (Ror1) and 943 (Ror2) amino acid mature receptor proteins comprise an extracellular domain with one immunoglobulin-like motif, a cysteine rich region and a kringle domain, a hydrophobic region comprising the transmembrane domain and a cytoplasmic region with a catalytic kinase domain (Masiakowski and Carroll, 1992). The Ror1 and Ror2 proteins are highly homologous, displaying an overall amino acid identity of 58 %.

A kringle domain is a cysteine-rich structure thought to participate in protein-protein interactions (Patthy *et al.*, 1984). Kringle domains have been identified in multiple copies in certain proteins involved in blood clotting, (Kanalas and Makker, 1991; Patthy, 1985), apolipoprotein(a) (McLean *et al.*, 1987) and

hepatocyte growth factor (Nakamura *et al.*, 1989; Seki *et al.*, 1991). Kringle domains have also been found in one muscle-specific RTK. The electric ray *Torpedo californica* possesses a kringle domain in the extracellular domain of the muscle-specific RTK (Jennings *et al.*, 1993). This RTK is thought to be part of the family of mammalian MuSKs (muscle-specific kinases), but is the only one to contain this kringle domain (Valenzuela *et al.*, 1995).

The cytoplasmic catalytic domains of Ror1 and Ror2 are very similar to the Trk kinase domain. For example, the kinase domains of Ror1 and Trk are 47 % identical (Masiakowski and Carroll, 1992).

Cloning of *Ror1* and *Ror2* in humans and rats was undertaken using PCR with degenerate oligonucleotide primers designed to conserved regions of Trk specific clusters of amino acids. Trk and TrkB sequences were eliminated by treating the PCR products with restriction enzymes expected to cut these genes. The remaining fragments were reamplified and cloned. These bacterial colonies were screened by PCR followed by direct sequencing, a cDNA library was screened with these fragments and the longest cDNA inserts sequenced.

To verify the enzymatic activity of Ror1 and Ror2, *in vitro* autophosphorylation of the immunoprecipitated kinase was undertaken. Data indicated that the protein kinase activity is specifically associated with the tyrosine kinase domain of Ror2, but results from Ror1 were inconclusive.

To analyse the expression of *Ror1* and *Ror2* in rats, Northern blot analysis was performed. Both *Ror1* and *Ror2* are highly expressed in the head and body during early embryonic development with the peak of expression at embryonic day 12. The level of expression drops drastically at embryonic day 16 and expression remains very low in adult rats. In contrast expression of *trkB* increases in the later stages of embryonic development (Klein *et al.*, 1990).

1.4 CLONING ROR IN *DROSOPHILA*

The *Drosophila* homologue of *Ror* (*Dror*) was cloned by Wilson *et al.* (1993) by screening a late third instar larval brain cDNA library using a PCR-based approach. Degenerate oligonucleotide primers designed to two conserved regions in the tyrosine kinase domain. Resulting clones were subcloned and sequenced, one of which was designated *Dror*.

Dror encodes a 685 amino acid mature receptor protein comprising an extracellular domain containing a cysteine rich region and a kringle domain, a hydrophobic transmembrane domain and a cytoplasmic region with a catalytic kinase domain (Wilson *et al.*, 1993). The Dror receptor demonstrates a 44 % identity with human Ror1, 40 % identity to human Ror2 and 32 % identity to human TrkB. Extensive sequence similarity between the Ror proteins and Dror is seen throughout the length. However, Dror lacks the immunoglobulin-like domain found in the extracellular domain of Ror1 and Ror2. Dror also lacks the extensive carboxyl terminus tail found in Ror1 and Ror2.

The tyrosine kinase domain of Dror shows significant similarity to the Ror1, Ror2 and Trk proteins of between 53 % and 61 %. A number of the shared amino acids in this domain are not found in many other RTKs suggesting the occurrence of a Trk-like superfamily of RTKs. Although *Dror* demonstrates the highest identity to Ror1 in the tyrosine kinase region (61 %), *Dror* does not share some of the features that distinguished the *Ror* genes from the *Trk* genes in this region.

Within the extracellular domain, Dror shares a 36 % identity with Ror1 and 34 % with Ror2, but no significant similarities to the Trk family. Like Ror1 and Ror2, Dror contains a cysteine-rich region and a kringle domain, but within the cysteine-rich region is a unique 55 amino acid lysine-rich insert. When these three proteins are aligned all 16 cysteines in this region in Ror 1 and Ror2 have

equivalent positions in the Dror protein. The immunoglobulin-like domain seen in Ror1 and Ror2 is not present in Dror. hence the cysteine rich region is positioned close to the amino terminus of Dror.

In situ hybridisation of whole mount embryos was performed to examine the expression of *Dror*. There was no expression of *Dror* in early embryos (Stage 1-10, 1-8 hours), but was seen at Stages 11 (extended germ-band stage) to Stage 15 (8-12 hours). Most of the neurons within the brain and ventral cord expressed *Dror*, as well as some cells in the head and trunk representing organs of the peripheral nervous system. No expression of *Dror* was seen outside the nervous system suggesting an important role of the Ror proteins in early neural differentiation and less important involvement in neuronal cell survival.

1.5 Aims of this Thesis

The original aim of the project was to clone the *Drosophila melanogaster* homologue of the human *Dtk* gene. This was to be accomplished by using the polymerase chain reaction (PCR) with degenerate oligonucleotides derived from conserved amino acid motifs within the tyrosine kinase domain.

Clones from four novel putative RTKs were isolated, but none were similar to Dtk. Thus, we were required to change the original aim of this thesis. The new aim was to characterise the gene for one of the novel RTKs, we subsequently called *Dror2*. The specific objectives were to first determine the sequence and structure of the *Dror2* gene. The latter included mapping intron-exon boundaries, transcription start point and poly A^+ site. The second objective was to determine the temporal and spatial pattern of expression of the *Dror2* gene. The former would be accomplished by using both RT-PCR and Northern blots on RNA isolated from various stages of *Drosophila* development. The pattern of expression would be determined by *in situ* hybridisation.