Investigations into the function of structural isoforms of the Platelet Derived Growth Factor A-chain.

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To My Sister, Louise.

Abstract.

Platelet derived growth factor (PDGF) is a covalent dimer of A and B chains with the structure AB, AA or BB depending on its source. The A and B chains are related but distinct polypeptides encoded by separate genes. PDGF-A can be differentially spliced, allowing production of two mature proteins one of which (PDGF-A$_L$) is longer than the other (PDGF-A$_S$) by 15 amino acids at its carboxyl terminus. PDGF-A homodimers are thought to be important in early development and, later, for regulating glial cell development in the central nervous system (CNS). In order to gain a clearer understanding of the role of PDGF *in vivo* it is important to define the functional significance of the different isoforms and their cellular sources and targets. This thesis describes my attempts to elucidate the functional differences between PDGF-A$_S$ and PDGF-A$_L$ and to determine whether they are differentially expressed in CNS derived tissues.

To investigate the functional differences between PDGF-A$_S$ and PDGF-A$_L$ I introduced plasmid vectors directing the expression of the two isoforms into cultured Cos cells and subjected the expressed molecules to biochemical and immunocytochemical analysis. These studies provide evidence that PDGF-A$_S$ is secreted as a freely diffusible molecule while PDGF-A$_L$ is sequestered near the cells that produce it by binding to heparin-like molecules on the cell surface and in the extracellular matrix (ECM). The implications of these findings are discussed with reference to other factors that exist in different isoforms that are either freely diffusible or immobilised at the cell surface and within the ECM.

I attempted to analyse the expression pattern in the CNS of the two isoforms by a combination of techniques, including S1 nuclease protection, PCR amplification on reverse transcribed RNA, cDNA cloning and sequencing. These studies suggest that PDGF-A$_S$ predominates in the tissues and cell types I have examined in agreement with other studies of a similar nature.

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Finally I would like to thank my grand parents, my sister and my mother and father whose support (financial and moral) and tireless encouragement throughout this, as with everything, has been beyond value. Thank you.

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Abbreviations

$\alpha\alpha$	Amino acid
bp	Base pair
BSA	Bovine Serum Albumen
CIP	Calf Intestinal Phosphatase
CNS	Central Nervous System
dH_2O	Deionised water
DNase	Deoxyribonuclease
EDTA	Ethylene Diamine Tertaric Acid
ECM	Extra Cellular Matrix
FGF	Fibroblast Growth Factor
FITC	Fluorescein iso-thiocyanate
GAG	Glycosaminoglycan
hnRNA	unspliced heterogeneous nuclear RNA
HPG	Heparin or heparan sulphate proteoglycan
Ig	Immunoglobulin
IPTG	Isopropyl β-D-thiogalactopyranoside
kb	kilobase
kDa	Kilodalton
Klenow	large fragment of E.coli DNA polymerase I
M	Molar
mRNA	Messenger ribonucleic acid

MW	Molecular weight
nt	nucleotide(s)
NTS	Nuclear targeting signal
P#	Postnatal day #
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
p.f.u	Plaque forming units
Poly (A)$^-$	non polyadenylated
Poly (A)$^+$	polyadenylated
PNS	Peripheral Nervous System
RNA	Ribonucleic acid
RNase	Ribonuclease
RNasin	Human placental RNase inhibitor
SMCs	Smooth muscle cells
TAE	Tris acetate agarose running Buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinases
TK	Tyrosine kinase
Tris	2-amino-2(hydroxymethyl)-1,3-propandiol

☿ Chapter One ☿

"One fine morning in the month of May an elegant young
horsewoman might have been seen riding a handsome sorrel
mare along the flowery avenues of the Bois de Boulogne."

Chapter 1. Introduction.

The developmental progression from single cell embryo to multicellular organism depends on a variety of processes, including cell proliferation, differentiation and cell migration, to produce an elaborate array of specialised cell types and tissues. In the mature organism these processes are normally restricted to tissues that undergo continual or periodic cell turnover, such as epithelia and hemopoietic stem cells, and tissues undergoing wound repair. Co-ordination of these activities relies on a diverse group of extracellular polypeptides, known collectively as growth factors. Aberrant growth factor activity may, however, lead to developmental abnormalities and neoplasia. How the delicate balance of stimulatory and inhibitory signals mediated by polypeptide growth factors is established and maintained is a fundamental problem in biology.

1.1 Growth factors act by binding to cell surface receptors.

Growth factors are secreted proteins that act in an autocrine or paracrine manner by high affinity binding to cell surface receptors. Possession of the appropriate receptor type determines the ability of a cell to respond to a particular growth factor though the precise nature of the response may depend on the type of cell and the modifying effects of a variety of different factors including extracellular matrix (ECM). Binding of a growth factor to its receptor initiates a cascade of intracellular signalling events that ultimately lead to an altered program of gene expression that can result in changes in cell shape and mitosis. Receptors typically consist of a glycosylàted extracellular ligand binding domain, a hydrophobic transmembrane domain and an intracellular signalling domain which is often, but not always, a tyrosine kinase and may include sites for autophosphorylation. There are four major sub-classes of tyrosine kinase receptors (See Figure 1 and discussion in following sections). Ligand-induced receptor dimerisation, or dimer stabilisation, has been suggested as the initial step towards intracellular signalling for a number of receptors including the epidermal growth factor receptor (EGFr) {Cochet *et al.*, 1988; Schlessinger, 1988} and the platelet derived growth factor receptor (PDGFr) {Williams, 1989}. However, though some of the intracellular signalling events have been characterised, it is still far from clear how they bring about their effects on proliferation, differentiation or migration of the target cells. A number of receptors have

been identified as the proto-oncogene counterparts of viral oncogenes such as v-*erb* B, v-*kit* or v-*fms*. In addition, mutations affecting receptors such as c-*kit* and its ligand (KL), respectively encoded by the *white spotting* locus and the *steel* locus of mouse, are known to cause embryonic lethality when homozygous, while heterozygotes display a range of phenotypes with defects in the development of the hemopoietic, gonadal and pigment cell lineages {Witte, 1990}. Furthermore overexpression of otherwise normal receptors combined with autocrine stimulation is commonly implicated in cell transformation. This not only illustrates the importance of these receptors and their associated factors in a wide range of activities but emphasises the need for careful regulation of their respective activities {Ullrich & Schlessinger, 1990 for review of tyrosine kinase receptors}.

1.2 Growth factors can be grouped into families based on sequence similarity.

The now relatively large and still increasing collection of known polypeptide growth factors can be organised into families on the basis of structural and functional similarities suggesting common evolutionary origins. Certain proteins not previously associated with growth factor activity, such as the *Drosophila decapentaplegic* gene product, which is involved in dorsoventral orientation during embryogenesis, have been identified as relatives of known growth factors. There is considerable overlap in terms of target cell range among members of different families. Moreover, it is clear that the effect of many growth factors can be modified by the simultaneous action of other unrelated growth factors, as shall be described in subsequent sections.

A) Insulin and the insulin like growth factors (IGFs).

The observation that high concentrations of insulin are mitogenic for certain cells, suggesting that it acts by low affinity binding to a receptor for a related but distinct protein, lead to the identification of the insulin-like growth factors (IGF-1 and IGF-2). Their high degree of amino acid and structural similarity to human pro-insulin and the fact that the IGF-II gene is contiguous with the insulin gene serve to emphasise their likely common evolutionary origins {Bell *et al.*, 1985}. A third member of the family, relaxin, is also highly homologous to insulin and both IGFs (Table 1). Insulin effects a

similar range of responses to some growth factors but is not considered a typical growth factor because it acts in an endocrine manner like growth hormone. IGF-I is expressed mainly in the adult while IGF-II is a predominantly fetal mitogen except for adult neural tissues that display high levels of IGF-II protein where it is thought to be associated with neural outgrowth and survival {Raff, 1992 for review}. Both IGF-I and II collaborate with platelet derived growth factor (PDGF) and epidermal growth factor (EGF) to stimulate proliferation of connective tissue cells especially adipocytes, while relaxin is a peptide involved in maturation of the female reproductive tract {Mercola & Stiles, 1988 for review}.

B) The epidermal growth factor (EGF) family.

The EGF family includes transforming growth factor-alpha (TGF-α) which shares sequence similarity with EGF and the virus-encoded vaccinia growth factor (VGF) (Table 1). All three factors are thought to be released from cells by proteolytic cleavage of a transmembrane precursor {Rall *et al.*, 1985; Gentry *et al.*, 1987; Teixido *et al.*, 1987} and to elicit mitogenic responses in a variety of cell types by binding to a common receptor, the product of the c-*erb* B proto-oncogene. In some cell types TGF-α is more potent than EGF leading to speculation that they either interact differently with the same receptor or there is an additional TGF-α receptor not bound by EGF. An early event associated with stimulation of certain normal cell types by TGF-α and EGF is the production of more TGF-α. In common with a number of other growth factors, including PDGF {Paulsson *et al.*, 1987} and TGF-β {Barnard *et al.*, 1989} this sort of positive autocrine signal (or autoinduction) is thought to serve as an amplifier of the initial mitogenic response.

TGF-α, which gained its name from the observation that it could induce reversible transformation of rat fibroblasts in conjunction with a second growth factor TGF-β, has been detected at the RNA level in a variety of tumor cell lines {Nistér *et al.*, 1988b} and directed overexpression has been shown to lead to pleiotropic effects including formation of carcinomas and various developmental lesions {Jhappan *et al.*, 1990; Sandgren *et al.*, 1990}, emphasising the role of TGF-α in cellular proliferation, organogenesis, and neoplastic transformation. Other studies have shown that it is also expressed in a number of normal cell types in embryonic {Rappolee *et al.*, 1988a} and adult tissues {Derynck, 1988 for review} indicating possible roles in both

development and homeostasis. The occurrence of regions with EGF amino acid sequence homology in the homeotic gene *lin-12* in *C.elegans* and the neurogenic gene *Notch* in *Drosophila*, both of which are also thought to encode transmembrane proteins {Greenwald, 1985; Wharton *et al.*, 1985; Knust *et al.*, 1987}, implies that EGF-like molecules may be involved in cell-contact mediated signalling events. Indeed, the membrane-bound pro-TGF-α has been shown to allow cell-cell adhesion while simultaneously promoting cell proliferation in associated cells, a combination of activities which has been termed "juxtacrine stimulation" {Anklesaria *et al.*, 1990}. It has been proposed that this type of receptor-ligand interaction may involve reciprocal signalling by the highly conserved intracellular domain of the transmembrane growth factor precursor, however, this region has no known sequence similarity with established receptors and has, as yet, shown no signs of receptor-like activity {Mercola & Stiles, 1988; Massague, 1990 for reviews of TGF-α}.

C) The transforming growth factor beta (TGF-β) family.

TGF-β belongs to a much larger group of factors including several closely related TGF-β like molecules (TGF-β1-4), the more distantly related Mullerian inhibiting substance (MIS), various bone morphogenetic proteins (BMPs), inhibins and activins (notably *Xenopus* XTC-MIF), the *Xenopus* Vg-1 gene product and the *Drosophila decapentaplegic* gene product (Table 1). Members of this family are renowned for the diversity of their regulatory activities and in particular their ability to potentiate or inhibit the effects of many other growth factors. TGF-β is produced as an inactive dimer by almost all cell types *in vitro*, while all normal cells and most neoplastic cells *in vitro* possess TGF-β receptors suggesting that its activity may be regulated by selective activation. A number of different physiochemical treatments, mimicking conditions thought to exist within wounds or during bone remodelling, are known to activate latent TGF-β. However, the most physiologically likely candidate is the serine protease plasmin, which is regulated in its turn by plasmin activators and inhibitors.

There are 3 known TGF-β receptors. Types I & II bind each of the closely related TGF-β forms with approximately equivalent affinity. Both are required for signal transduction and it is thought that they may dimerise {Laiho *et al.*, 1990b}. The type III receptor, a betaglycan with nearly 50% of its molecular

mass consisting of heparan sulphate and condroitin sulphate, exists in both soluble and membrane-bound forms. Some type III receptors have also been observed in association with the ECM where it is thought they may represent a site for TGF-β storage or titration since it seems that they are not required for TGF-β activity {Cheifetz et al., 1989; Andres et al., 1989}. The intracellular signalling events are only poorly understood. It seems that, for epithelial cells at least, TGF-β mediated growth inhibition probably involves suppression of retinoblastoma protein phosphorylation, which in turn down regulates c-myc expression {Laiho et al., 1990a}. It appears that retinoblastoma cell lines have no identifiable TGF-β receptors and are uninhibited by TGF-β although loss of TGF-β receptors seems to be a relatively unusual route to neoplastic cell growth {Barnard et al., 1990; Mercola & Stiles, 1988; Moses et al., 1990 for TGF-β reviews}.

TGF-β is an inhibitor of proliferation for a wide range of cell types, including nearly all epithelial, lymphoid and endothelial cells, though it is able to elicit an indirect mitogenic response in certain mesenchymal cells by inducing PDGF autocrine stimulation. Moreover TGF-β stimulates or inhibits differentiation and migration in a cell-type-dependent manner as well as promoting changes in extracellular matrix (ECM) formation. Indeed it is thought that many of the growth regulatory effects of TGF-β may be mediated, at least in part, by alterations in the ECM {Newton et al., 1990}. TGF-β has also been shown to be a potent inducer of angiogenesis and is known to be released from activated macrophages, indicating its likely involvement in wound repair and inflammation. In situ hybridisation studies have revealed the complex embryonic expression patterns for each of the closely related TGF-β types indicating their involvement during development {Millan et al., 1991; Schmid et al., 1991}. In vitro, TGF-β1 has been shown to induce the formation of Xenopus mesoderm in conjunction with basic fibroblast growth factor (FGF-2: see next section){Kimelman & Kirschner, 1987}, TGF-β2, on the other hand, seems to have a limited effect on its own {Rosa et al., 1988}. Subsequent studies have indicated that other members of the TGF-β family may be responsible for mesoderm induction in vivo: activin-A can induce mesoderm on its own and can be purified from the appropriate regions of the embryo although it can not be detected at the RNA level {Smith et al., 1988} while VG-1 mRNA shows correct localisation in the embryo but has not been shown to have MIF like activity {Weeks & Melton, 1987} more recently activin-B has been shown to

have mesoderm-inducing activity and to be present in the embryo at the right time {Thomsen *et al.*, 1990; Mitrani *et al.*, 1990}. Studies using dominant-negative FGF receptor mutants have led to the suggestion that an FGF-like activity induces posterior mesoderm structures while the *Xenopus* activin activity may be responsible for anterior mesoderm structures {Amaya *et al.*, 1991}. At least one other member of this family is known to play a role in normal development: MIS is produced by the testis and is responsible for causing regression, possibly by inducing cell death, of the Mullerian duct, which would otherwise go on to form fallopian tubes {Cate *et al.*, 1986}. It will be interesting to discover whether MIS has any homology with the *ced* (3, 4 or 9) genes, which have been shown to regulate programmed cell death in *C.elegans*, or indeed the mammalian *bcl*-2 gene product, overexpression of which is thought to allow oncogenesis by preventing programmed cell death {Williams, 1991; Hengartner *et al.*, 1992; Raff, 1992}.

D) The fibroblast growth factor (FGF) family.

The fibroblast growth factor family currently includes seven related proteins (FGF-1 to 7), which share a 30-50% amino acid identity, a 3 exon gene structure and the ability to bind to the glycosaminoglycan heparin. The original two members, aFGF (FGF-1) and bFGF (FGF-2), have been shown to modulate proliferation and differentiation of a broadly similar range of mesodermal and neuroectodermal cell types *in vitro*. In addition both factors are chemotactic for endothelial cells, fibroblasts and astroglial cells and are potent inducers of angiogenesis. Both are expressed in a wide variety of cell types though FGF-1 is particularly abundant in brain tissue where it is thought to play a significant role in nervous system development by modulating neuronal cell proliferation, differentiation and survival {Walicke, 1988a; Walicke & Baird, 1988a; Haynes, 1988 for a review}. However, neither factor has a secretion signal and it is still far from clear that they are released other than as a result of cell death {Flaumenhaft *et al.*, 1989}. For this reason, despite their pleiotropic effects *in vitro*, it seems possible that their normal function *in vivo* may be limited to wound repair during which both angiogenesis and chemotaxis are key processes. Certainly many of the cells expressing these two forms, such as endothelial cells, are known to be FGF responsive but normally remain quiescent. Moreover, the presence of FGF within neurons {Janet *et al.*, 1988} coupled with the survival effects of FGF 1

and 2 on the neurons, both normal and damaged {Anderson et al., 1988; Schubert et al., 1987}, provides further circumstantial evidence for their role in wound repair.

The other members of the FGF family, which were mostly identified by their association with cell transformation, all possess a functional secretion signal. Although FGF-2 has been isolated from tumor cells and cultured tumor cell lines {Lobb et al., 1986} where it is thought to be involved in tumor progression by effecting angiogenesis {Folkman a n d Klagsbrun, 1987}, neither FGF-1 nor FGF-2 expression is commonly associated with neoplastic transformation. Indeed it has been demonstrated that native FGF-2 does not exhibit transforming activity, but acquires this ability after fusion with an immunoglobulin secretory-signal sequence {Rogelj et al., 1988}. Moreover a recent study has implicated secreted FGFs in the mesoderm induction events discussed above (see section 1.5) rather than the nonsecreted FGF-2 {Paterno et al., 1989}. It therefore seems likely that most normal and neoplastic in vitro activities described for FGF-1 & 2 are actually carried out by the secreted factors in vivo. FGF-3 (Int-2) was identified as a proto-oncogene activated by mouse mammary tumor virus (MMTV) proviral integration {Peters et al., 1983; Dickson et al., 1984} and is thought to be involved in the associated mammary tumor formation. Although widespread expression during embryogenesis has been demonstrated as well as low level expression in adult brain and testis, its normal in vivo role remains unclear. The FGF-4 (kFGF/hst) gene was identified on a genomic DNA fragment, derived from a human stomach tumor, that was capable of transforming 3T3 cells. Subsequently transforming activity that can be ascribed to FGF-4 has been detected in DNA from a variety of tumor cell types. Like the other FGFs its normal expression pattern and function are not yet clear. FGF-5 and FGF-6 were identified by transfection of tumor cell DNA and library screening respectively and are probably the least well studied of the FGF family while FGF-7 (keratinocyte growth factor or KGF) was isolated from the conditioned medium of an embryonic lung fibroblast cell line and displays some notably different characteristics to the other members of the family. First, FGF-7 has a relatively lower affinity for heparin, eluting at 0.6 M NaCl while the other members of the family require 1 M NaCl or higher. Second, in contrast to FGFs 1 and 2 which are apparently capable of activating a broad range of cell types via interaction with several different FGF receptors, FGF-7 is not mitogenic for fibroblasts or endothelial cells but is

highly mitogenic for certain epithelial cell types {Rubin *et al.*, 1989 for reviews of FGF see; Burgess and Maciag, 1989; Gospodarowicz *et al.*, 1986}.

The fibroblast growth factor receptor (FGFr) family.

At least four genes encoding signal transducing high affinity FGF receptors (FGFr 1 to 4) have been identified to date. The predicted protein products share a common structural format: 2 or 3 immunoglobulin (Ig)-like extracellular ligand-binding domains with a region of acidic residues located between the first and second domains; an interrupted tyrosine kinase intracellular signalling domain, analogous to that found in members of the PDGF receptor family (See Figure 1 and PDGF section 1-2F), but with a relatively longer juxtamembrane region which sets them apart from the PDGF receptor family {Lee *et al.*, 1989}. The FGFr 1 and 2 genes appear to encode multiple forms of receptor protein by way of alternative mRNA splicing, giving rise to variation in both the number of Ig-like extracellular domains and the nature of the tyrosine kinase domains {Johnson *et al.*, 1990; Reid *et al.*, 1990; Hou *et al.*, 1991}. However, no differences have been observed to date in either the ligand binding or the receptor signalling of FGFrs 1, 2 or 3. By contrast FGFr 4 seems to have differential ligand-binding properties, binding FGF-1 but not FGF-2 {Partanen *et al.*, 1991}. This receptor, along with the recently cloned murine FGF-7 receptor (a potential fifth member of the FGFr family) which also binds FGF-1 but not FGF-2 {Miki *et al.*, 1991}, differs from the others by having a shorter (or absent) acidic region located between the first and second Ig-like domains. Surprisingly then, although there are many variations in the structure of FGF receptors, only a few differences in ligand specificity or receptor activity have been observed so far.
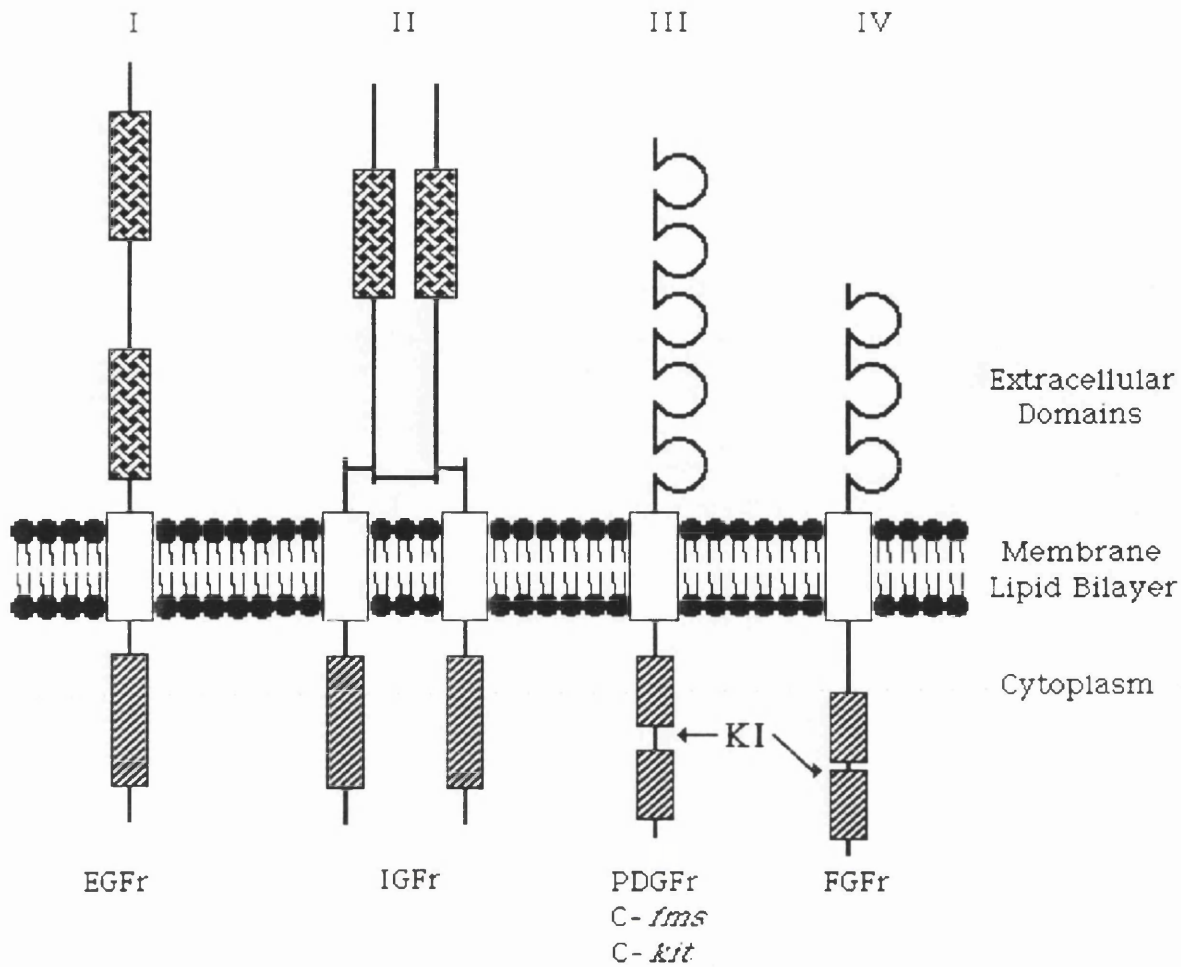
Figure 1.

Schematic representation of the four major growth factor tyrosine kinase ▨ receptor sub classes with examples of members adapted from Ullrich & Schlessinger (1990).

Type I receptors have two cysteine-rich repeat sequences ⊠ in the extracellular domain.

Type II receptors exist as disulphide linked heterotetramers ($\alpha_2\beta_2$) with a single cysteine rich region like that of the type I receptors in each of the two extracellular ligand binding domains.

Type III receptors have 5 immunoglobulin like domains in their extracellular domains.

Type IV receptors have 3 (or sometimes only 2) immunoglobulin like domains in the extracellular domains. The kinase regions of both **Type III** and **Type IV** receptors are interrupted by a hydrophilic region of varying length (**KI**).

Fibroblast growth factors and heparin binding.

It has been known for some time that FGFs also bind to the negatively charged glycosaminoglycan (GAG) heparin under physiological conditions (K_d 2 to 10 nM compared to 10 to 200 pM for binding to the FGFrs) and can be eluted at salt concentrations ranging from 0.6 M NaCl to 1.8 M NaCl. FGFs from a variety of different tissues have been purified on the basis of this affinity[*] and identified provisionally according to their salt elution profile. However, the physiological significance of this interaction and the role of the highly polymorphic proteoglycans, with which heparin and the closely related heparan sulphate are associated *in vivo,* has only recently started to become clear. The GAG heparin alone has been shown to protect FGF-1 and 2 from acid, heat and proteolytic inactivation {Saksela *et al.,* 1988; Sommer & Rifkin, 1989} thereby prolonging their biological half lives and potentiating their mitogenic effects {Gospodarowicz & Cheng, 1986; Damon *et al.,* 1989; Vlodavsky *et al.,* 1991 for review}.

Proteoglycans are generally composed of a protein core with associated GAG side chains that bind together ECM components, and mediate interactions between cells and ECM {Ruoslahti, 1989 for review}. Heparin and heparan sulphate proteoglycans (HPGs) exist in membrane-bound, ECM bound and secreted forms {Saksela *et al.,* 1988; Gallagher, 1989 for review} and have been shown to bind FGFs {Baird & Ling, 1987; Vlodavsky *et al.,* 1987a; Folkman *et al.,* 1988; Vigny *et al.,* 1988}. HPGs in the ECM are thought to act as a physiologic buffer, binding FGF when concentrations are high (e.g. when released from dying cells) and releasing it later when concentrations are low {Moscatelli, 1988; Flaumenhaft *et al.,* 1989} (See chapter 6 for discussion on why growth factors may be bound to ECM). It has been proposed that ECM sequestration may also restrict target cells' access to FGF {Folkman *et al.,* 1988}. FGF availability could then be regulated by mechanisms controlling its release from the matrix, such as the heparin-degrading endoglycosidases heparanase and heparitinase which have been shown to release FGFs from ECM *in vitro* {Bashkin *et al.,* 1989; Presta *et al.,* 1989; Vlodavsky *et al.,* 1991}. In support of this theory, degranulating mast cells have been shown to produce a heparin-

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[*] Purification of FGFs by heparin affinity :- {Besner *et al.,* 1990; Brigstock *et al.,* 1990; Hauschka *et al.,* 1986; Klagsbrun & Shing, 1985; Lobb & Fett, 1984; Lobb *et al.,* 1986; Shing *et al.,* 1984; Sullivan & Klagsbrun, 1985}.

degrading activity that releases FGF, in complex with a partial degradation product of heparin, a complex which may participate in mast cell-mediated neovascularisation {Bashkin et al., 1990}. The proteolytic enzyme plasmin, under the regulation of plasminogen activator, has also been shown to release FGF, complexed with a fragment of heparan sulphate, by proteolysis of the HPG core protein {Saksela & Rifkin, 1990}. In addition there is evidence that soluble heparin, which is able to compete for FGF binding with ECM HPGs {Flaumenhaft et al., 1990}, can raise the levels of circulating FGF activity when administered intravenously {Thompson et al., 1990; Whalen et al., 1989}. Each of these mechanisms involves a continuing FGF association with some form of heparin or heparan sulphate, providing indirect evidence that this may represent an active complex.

Two studies have provided more direct evidence that heparin or HPG binding is a requirement for FGF-2 activation of high affinity FGF receptors. FGF-2 activity was seen to be dramatically reduced when displaced from heparin by competition with heparin-binding polycationic molecules {Dauchel et al., 1989} indicating that simply releasing bound FGF is not sufficient for activation. More recently it has been shown that mutant cells lacking cell surface HPGs are unable to bind FGF-2 to their high affinity receptors {Yayon et al., 1991}. Binding could, however, be restored by adding soluble heparin or heparan sulphate to the culture medium, leading the authors to suggest that the GAGs may alter the conformation of FGF to allow receptor binding, which fits well with the established stabilisation effects of heparin on FGF (Figure 2). Alternatively it has been proposed that GAG binding may facilitate FGF-2 oligomerisation and thereby effect receptor dimerisation which is thought to be associated with receptor signalling (see section 1.1) {Ruoslahti & Yamaguchi, 1991} or that the GAG alters the structure of the receptor to allow FGF-2 binding {Klagsbrun & Baird, 1991}. Although the precise nature of this interaction remains to be elucidated it seems clear that heparin, whether present as part of a cell surface proteoglycan or in a freely diffusible form, plays an important role in regulating the interaction between FGF-2 and its receptors.

Several interesting questions are raised by these findings: since it seems likely that the other members of the FGF family use a similar mechanism of GAG-assisted receptor binding it will be interesting to discover

whether specific GAGs modulate interaction with specific FGF receptors. So far, the only significant receptor specificity identified involves FGF-7, which has a much lower affinity for heparin than any of the other FGFs (as described above), suggesting the intriguing possibility that it may require a different form of HPG for receptor binding. Alternatively the lack of Acidic sequence in the FGF-7 receptor (FGFr-4) may indicate an inability of receptor to associate with heparin although there is currently no evidence for a receptor/heparin association. Finally, it will be interesting to discover whether the ECM-bound FGFs can interact with cell surface receptors in the same way as FGFs bound to HPGs on the cell surface and those complexed with soluble GAGs fragments (Figure 2). If the ECM-bound FGFs are not available for receptor interaction it will be interesting to determine whether, how and when they are released from the ECM *in vivo* .
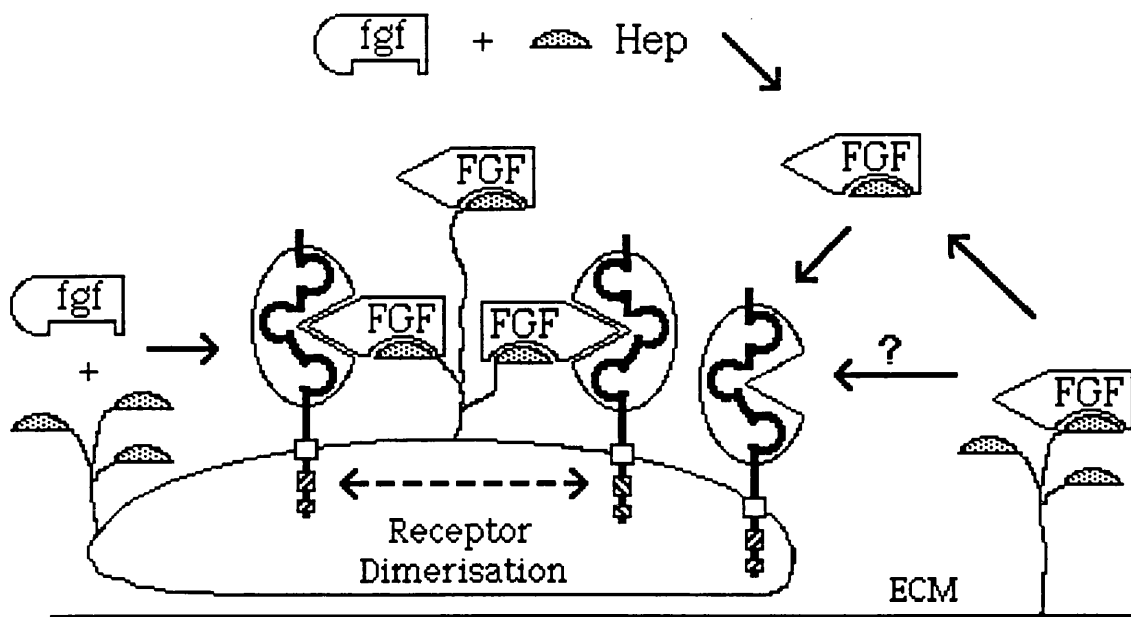
Figure 2.

Showing the induced fit model for both soluble and membrane-bound HPG assisted FGF/receptor activation systems and the possibility (denoted by "?") of ECM bound HPG regulating FGF/receptor interaction either directly or via proteolytic release of FGF/ heparin complexes. Also indicating the putative HPG involvement in receptor dimerisation. Adapted from Yayon *et al.*, (1991).

E) The hemopoietic and neuropoietic growth factors.

The processes of hemopoiesis and neurogenesis both depend on strict regulation of multipotential stem cells to generate a wide variety of cell types. *In vitro* several different hemopoietic growth factors (or cytokines) can influence the production, maturation and functional activity of each hemopoietic cell type. Moreover, each factor can invoke different responses in a cell type dependent manner. The explanation for this characteristic redundancy and pleiotropy probably lies in the nature of the receptors, which are quite different to those activated by the growth factors described above (see Section 1.1 & Figure 1). In general the hemopoietic receptors consist of an α-subunit that binds the ligand with low affinity and associates with a common β-subunit with intracellular signalling activity, to form a high affinity receptor. In this way several factors may invoke a similar response by activating the same β-subunit (Figure 3). Similarly it has been proposed that the ability to induce different effects may represent the association of the α-subunit/ligand complex with different β-subunits that have distinct signalling properties (Figure 3). However, although different β-subunits have been identified, it is still not clear whether this actually happens *in vivo* {Nicola & Metcalf, 1991 for review}.

Functional similarities have been observed between the cytokines LIF, IL-6 and ciliary neurotrophic factor (CNTF), which was originally identified as a survival factor for neurons {Manthorpe *et al.*, 1986}. Further analysis revealed sequence similarities between a number of these cytokines and the recently discovered growth regulator oncostatin M (ONC) {Bazan, 1991} (see Table 1). Clones of receptor components for both LIF and CNTF indicate a close structural similarity and predict a promiscuous receptor subunit system similar to that described for the hemopoietic cytokines (Figure 3) with the predicted redundancy being emphasised by the observation that ONC binds to the high affinity LIF receptor {Gearing and Bruce, 1991}. LIF mRNA has been detected in both the hemopoietic and nervous systems {Patterson and Fann, 1992; Yamamori, 1991} while IL-6 is expressed by astrocytes and has direct effects on both hypothalamic and CNS neurons {Van Snick, 1990}. It therefore seems possible that many of the factors identified for their role in regulating the cells of the hemopoietic system may also be involved in stem cell regulation during the development of the nervous system. The identification

of these structural and functional similarities has led to the definition of a new family of neuropoietic growth factors (see Table 1) {Patterson, 1992}.

In addition to having a remarkably diverse range of effects and an increasingly apparent functional redundancy, LIF is produced in distinct freely diffusible and ECM associated forms {Hilton, 1992 for review}. Although the nature of this association with ECM is unknown as yet, the mechanism giving rise to the two forms has been established: LIF hnRNA transcribed from alternative promoters and spliced differentially produces mRNAs with distinct first exons that encode different amino terminal signal sequences {Rathjen *et al.*, 1990}. Intriguingly, whereas other signalling systems such as that directing nuclear targeting {Kalderon *et al.*, 1984, Richardson *et al.*, 1986} retain the signal in the mature protein, those directing secretion and ECM association of LIF are proteolytically removed to produce apparently identical mature polypeptides. An understanding of this surprising mechanism awaits analysis of the ability of the LIF matrix binding signal to direct ECM retention of heterologous proteins. Nevertheless, it is clear that this mechanism provides an opportunity for further variation in the activity of LIF. It will be interesting to discover whether any other growth factors use a similar system.
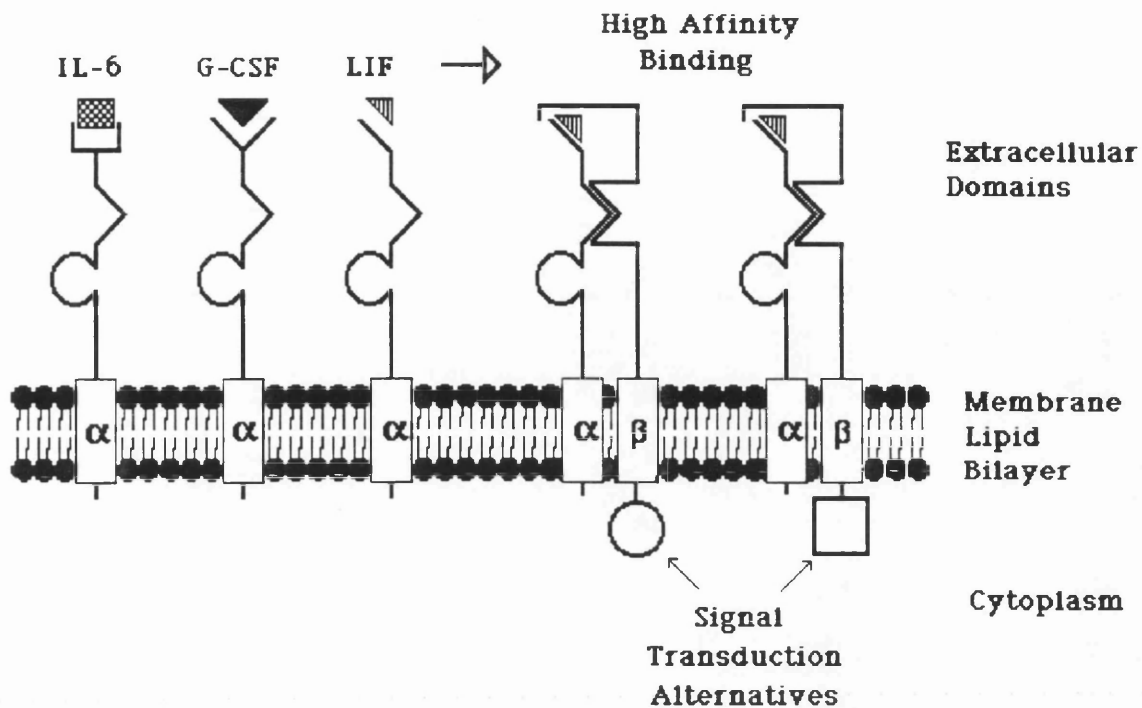
Figure 3.

Showing how heterodimeric receptors, such as those identified for cytokines and neuropoietic cytokines, may allow several different factors (e.g. Il-6, G-CSF and LIF) to transmit an identical signal by forming a high affinity complex with a unique α-subunit and a common signalling β-subunit. The pleiotropic effects of these same factors may be explained by proposing that α-subunits are able to associate with different β-subunits that activate distinct signalling cascades. Adapted from figures by Nicola & Metcalf (1991) and Hilton (1992).

Table 1. Some of the polypeptide growth factor families.

Groups	Members	Full and/or Alternative Nomenclatures.
Insulin-like growth factors	IGF-I	
	IGF-II / MSA	Somatomedin-C or Multiplication stimulating activity
	Relaxin	
Epidermal growth factor	EGF	
	TGF-α	Transforming growth factor-α
	VGF	Vaccinia growth factor
	Notch	*Drosophila*
	Lin-12	*C.elegans*
Transforming growth factor-β	TGF-βs 1-6	
	Activin-A (XTC-MIF)	Xenopus tissue culture - mesoderm inducing factor
	Activin-AB	
	Inhibins-A & B	
	MIS	Mullerian Inhibiting substance
	BMP-3 & 2A	Bone morphogenetic proteins 3&2A
	DPP	Decapentaplegic
Fibroblast growth factors	FGF	General term
	FGF-1	Acidic FGF (aFGF)
	FGF-2	Basic FGF (bFGF)
	FGF-3	Integration tumor-2 (Int-2)
	FGF-4	Human stomach cancer / Kaposi's sarcoma (hst / KS/ kFGF)
	FGF-5	Fibroblast growth factor-5
	FGF-6	Fibroblast growth factor-6
	FGF-7	Keratinocyte growth factor (KGF)

Neuropoietic cytokine factors

* Denotes a sub-family with a distinctive region of αα sequence.	CDF/LIF*	Cholinergic Differentiation or Leukemia Inhibitory Factor.
	CNTF*	Ciliary neurotrophic factor
	ONC*	Oncostatin M
	IL-6	Interleukin-6
	IL-11	Interleukin-11
	G-CSF	Granulocyte - colony stimulating factor
	MGF	Myelomonocytic growth factor
Platelet derived growth factor	PDGF	General term ¥
(¥) Dimer type and A-chain splice form only specified where known.	PDGF-AB	A-chain¥ / B-chain heterodimer
	PDGF-A	A-chain homodimer ¥
	PDGF-B	B-chain homodimer
	PDGF-A$_S$	Short A-chain homodimer
	PDGF-A$_L$	Long A-chain homodimer
	VEGF/VPF (VPF$_L$ & VPF$_S$)	Vascular endothelial cell growth factor or vascular permeability factor

N.B. Factors such as colony stimulating factor-1 (CSF-1 or M-CSF), kit-ligand (KL) and tumor necrosis factor alpha (TNF-α) are synthesised as transmembrane precursors in the same way as members of the EGF family but have no EGF homology thus preventing their allocation of family membership {Massague, 1990}.

F) The platelet derived growth factor (PDGF) family.

PDGF was originally identified as one of the principal mitogens in serum that directs the proliferation of a wide range of serum-dependent cells such as vascular smooth muscle cells, fibroblasts and certain glial cells. *In vivo*, PDGF is synthesised by a variety of cell types (see Table 2) and is thought to participate in a diverse range of cellular processes from wound healing and development to proliferative diseases such as atherosclerosis and cancer (see below). PDGF is a 30 kDa disulphide-linked, glycosylated, cationic dimer composed of two related peptides termed A and B that are 60% identical at the amino acid level and are encoded by separate genes on different chromosomes {Betsholtz *et al.*, 1986; Johnsson *et al.*, 1984; Heldin and Westermark, 1989 for review}. Both hetero- and homodimeric forms have been observed *in vivo*: PDGF from human platelets is predominantly a heterodimer {Hammacher *et al.*, 1988a; Hart *et al.*, 1990}, whereas porcine platelet PDGF consists entirely of BB homodimers {Stroobant & Waterfield, 1984} and a number of transformed human cell lines produce PDGF-AA homodimers *in vitro* {Heldin *et al.*, 1986; Hammacher *et al.*, 1988b}. A comparison of the receptor binding properties of each form indicated that the different PDGF dimers have overlapping activities mediated by receptors with different ligand specificities {Nistér *et al.*, 1988a}. Subsequently two independently regulated receptor subunits were identified: the alpha receptor subunit (PDGF-αR), which binds both A and B chains of PDGF, and the beta receptor subunit (PDGF-βR), which binds only the B chain {Östman *et al.*, 1989}. Both subunits have a typical transmembrane receptor structure (Figure 1: Type III) and are thought to exist as inactive monomers until ligand binding induces receptor dimerisation which activates their tyrosine kinase domains. The response of a cell to PDGF therefore depends on the relative numbers of α and β subunits present on its surface and the nature of the PDGF dimer(s) it encounters {Seifert *et al.*, 1989; Ferns *et al.*, 1990}. However, it is still not clear which of the many biological activities are mediated by each of these three main forms of PDGF {Ross *et al.*, 1986 for review of PDGF; Heldin and Westermark, 1989 for review of PDGF receptors; Williams, 1989 for review of receptor signalling}.

The role of PDGF in the vascular system.

Phylogenetic analysis indicating that PDGF-like activity only occurs in organisms with a pressurised vascular system led to the suggestion that it

functions in some aspect of vascular system regulation {Ross et al., 1974; Stiles, 1983}. There is certainly evidence that PDGF plays a role in vascular system damage repair: it is mitogenic for many connective tissue cell types and chemotactic for a number of cells involved in wound repair such as fibroblasts, monocytes and neutrophils, indicating a possible role in attracting these cells to the site of injury. PDGF-synthesising megakaryocytes (Table 2) give rise to the circulating non-nucleated platelets with the PDGF stored in specialised intracellular compartments known as α-granules. In the early stages of clot formation the stored PDGF is released in response to the coagulation agent thrombin, without recourse to de novo protein synthesis {Ross et al., 1986}. Endothelial cells lining the vascular system can also produce PDGF in response to thrombin and in the absence of protein synthesis, although unlike platelets they do not store PDGF intracellularly {Harlan et al., 1986}. This observation has led to the suggestion that thrombin may activate an inactive form of PDGF {Harlan et al., 1986; Ross et al., 1986}. In the later stages of vascular repair, monocytes, which are initially drafted in by the platelet released PDGF, infiltrate damaged vascular tissue where they differentiate into macrophages (Table 2) that produce PDGF in a protein synthesis dependent manner {Dohlman et al., 1984; Leslie et al., 1984}. In addition to directing migration and proliferation of cells PDGF is a highly potent vasoconstrictor and as such may play a role in damage limitation by reducing blood flow at the site of injury {Berk et al., 1986}. Thus, during vascular repair PDGF has a range of activities, and can be released from cells in at least three different ways. Although it has not yet been established, it remains an interesting possibility that the different release mechanisms and activities of PDGF may reflect the production of distinct dimeric PDGF isoforms {Ross et al., 1986 for review}.

Abnormal PDGF activity and proliferative disease.

Aberrant PDGF expression by cells in the vascular system is thought to be one of the principal factors causing the proliferative disease atherosclerosis. Characterised by arterial lesions which include proliferating smooth muscle cells and connective tissue associated with the inner lining of the vascular endothelium, atherosclerosis can lead to arterial wall thickening and eventual blockage. A number of possible explanations exist for the initial formation of these lesions. The primary risk factor for atherosclerosis is

chronic hypercholesterolemia, which causes monocytes to penetrate the vascular endothelium and differentiate into PDGF-secreting macrophages {Gerrity, 1981a & 1981b; Faggiotto et al., 1984}. It may be that these wayward macrophages can produce sufficient PDGF to induce the formation of lesions without further assistance. Alternatively, invasion of the arterial wall by macrophages may provoke the endothelial cells to secrete PDGF. However, inhibiting platelet function prevents formation of atherosclerotic lesions, indicating that the initial formation of the lesion may require input from several sources. Although it is not clear whether PDGF plays a significant role in the initial stages of the disease process, it seems likely that both the mitogenic activity and the vasoconstricting action of PDGF play a role in progression of the disease {Ross, 1986}.

A second proliferative disease in which PDGF is thought to play an important part is bone marrow fibrosis. This myeloproliferative disease is characterised by replacement of normal tissue by fibroblasts which produce an inappropriate collagen matrix. Just as chronic hypercholesterolemia induces inappropriate vascular macrophage production in atherosclerotic lesions, chronic conditions such as tuberculosis are associated with excess macrophage and megakaryocyte production which leads to fibrosis. The fibroblasts involved in the abnormal matrix production are polyclonally derived, indicating that they are not derived from a primary transformation event and are probably proliferating in response to an excess of a normal signal, possibly PDGF. Further evidence for the involvement of PDGF comes from analysis of cells causing fibrosis during myelogenous leukemia, many of which have a chromosomal translocation which is thought to activate the PDGF-B chain gene {Francis et al., 1983}. Thus the progression of these diseases probably involves excessive or inappropriate PDGF expression, which in turn stimulates fibroblasts to produce excess matrix {Ross et al., 1986 for review}.

PDGF and Neoplasia.

The association between growth factors and oncogenesis was firmly established by the observation that the PDGF-B chain and the protein product of the simian sarcoma virus (SSV) oncogene v-sis bear a remarkable structural similarity, suggesting that the cellular gene has been incorporated into the viral genome {Stiles, 1983 for review}. A number of other viruses are now known to have acquired cellular genes encoding proteins responsible for

growth control, which are then used as viral oncogenes (e.g. VGF, Table 1; see also Receptors Section 1.1) {Bywater *et al.*, 1988 for review}. Subsequently both PDGF-B and PDGF-A expression have been observed in a wide range of virally and otherwise transformed cell types (Table 2) though not necessarily within the same cell type. For example, PDGF-AA homodimers seem to prevail in cells derived from certain tumors such as Osteosarcoma {Heldin *et al.*, 1986} and Glioma {Nistér *et al.*, 1988b}. This indicates that their expression is independently regulated and may also give a clue as to the possible normal role of the different homodimers in the respective cell types (see next section). Although it seems that the transformed cells are commonly derived from cells that normally express PDGF receptors, which would allow autocrine stimulation, it is still not clear whether aberrant PDGF production represents a primary event in neoplastic transformation {Ross *et al.*, 1986}. Once again, as for the normal process of wound healing, it will be interesting to determine which of the different dimeric forms are associated with neoplasia and abnormal proliferative diseases.

PDGF in development.

There is now a good deal of evidence for the involvement of PDGF during embryogenesis. An early study indicated that a PDGF-like activity could be detected in the culture medium of acutely isolated rat aortic smooth muscle cells (rASMCs) from postnatal day 13 (P13) to P18 rats but not those isolated from adults. Given that rASMCs are PDGF-responsive this suggests a role for PDGF in early postnatal formation and maintenance of the artery wall via autocrine or paracrine signalling {Seifert *et al.*, 1984}. Subsequent mRNA analysis indicated a difference in the regulation of PDGF-B and PDGF-A transcript levels. PDGF-A is expressed at roughly equivalent levels in both new-born and adult rASMCs, while PDGF B-chain transcripts were observed to accumulate in the new-born but not the adult cells in culture, confirming the independent expression of the two chains {Majesky *et al.*, 1988}.

PDGF A-chain expression in preimplantation mouse embryos has been detected along with TGF-α and TGF-β1 expression, by polymerase chain reaction (PCR) amplification and immunocytochemistry {Rappolee *et al.*, 1988a}. This was subsequently confirmed by RNase protection analysis which also demonstrated embryonic expression of both receptor subunits, although PDGF-αR mRNA appears earlier and is generally much more abundant than

that of PDGF-βR. This second study failed to detect any PDGF B-chain expression, providing further confirmation that the two PDGF genes and those for the receptor subunits are independently regulated in normal cells {Mercola et al., 1990}. Both PDGF-A and TGF-α were also detected as maternal transcripts in the unfertilised oocyte suggesting an early requirement for these two factors. A possible clue to the role of PDGF during development comes from the observation that its embryonic expression pattern correlates with that of ECM degrading metalloproteinases such as collagenase and stromelysin and their inhibitor TIMP. Since the metalloproteinases and TIMP are thought to be involved in remodelling the ECM to allow cell growth, migration and embryo implantation, it seems possible that PDGF may be acting alongside these enzymes to drive some or all of these processes during development {Brenner et al., 1989}. Increased PDGF-A expression has also been detected in human uterine smooth muscle cells during pregnancy implicating PDGF in the normal physiologic process of uterine expansion during pregnancy {Mendoza et al., 1990}.

PDGF is thought to play a crucial role in the development of the CNS. Early evidence came from the observation that some glioma cell lines express PDGF-A homodimers {Betsholtz et al., 1986} and intracranial injection of SSV causes a high incidence of glioblastomas {Deinhardt, 1980}. Studies of factors regulating rat O-2A glial progenitor cells, which give rise to myelinating oligodendrocytes and are thought to be migratory cells in vivo during development, revealed that purified PDGF is able to stimulate a mitogenic response, direct motility and prevent their premature differentiation in vitro {Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988}. Purified cultures of type-1 astrocytes isolated from the optic nerve have been shown to express PDGF A-chain mRNA and produce a mitogenic activity which is inhibitable by PDGF specific antibodies {Richardson et al., 1988} and PDGF activity has been isolated from the developing rat optic nerve {Raff et al., 1988}. In situ hybridisation of the developing rat optic nerve revealed RNA for PDGF A-chain but not B-chain, while analysis of the mitogenic response indicated that PDGF-A homodimers are more potent mitogens for O-2A progenitor cells than PDGF-B homodimers. By contrast, PDGF-B homodimers are the most potent form for fibroblasts {Nistér et al., 1988a; Pringle et al., 1989}, which have been shown to express both types of PDGF receptor subunit {Heldin et al., 1988}. O-2A progenitor cells were subsequently shown to have predominantly α-type

receptors {Hart *et al.*, 1989}. Thus, although α-type receptors are able to bind both A and B-chains of PDGF, it seems that, for O-2A cells at least, they are better able to direct mitogenic activity in response to PDGF-A homodimers, probably due to the different affinities of A and B for the α–receptor {Heldin *et al.*, 1988}. More recently both PDGF A-chain {Yeh *et al.*, 1991} and PDGF B-chain {Sasahara *et al.*, 1991} have been shown to be expressed by neurons indicating that neurons may also regulate the fate of glial cells such as O-2A progenitors. Alternatively, it has been suggested that PDGF B-chain may function as a neuronal regulatory agent though this remains to be tested {Sasahara *et al.*, 1991} {Raff, 1989; Richardson *et al.*, 1990 for reviews of glial cell regulation in the optic nerve}.

Alternative splicing of PDGF A-chain mRNA.

At the outset of the work described in this thesis sequence analysis of cDNA clones had revealed that there are two forms of the PDGF A-chain arising from alternative splicing of the primary transcript, and differing by the presence (PDGF-A_L) or absence (PDGF-A_S) of a highly basic carboxy-terminal "tail" that is derived from the sixth exon (Figure 4) {Betsholtz *et al.*, 1986; Collins *et al.*, 1987; Tong *et al.*, 1987; Rorsman *et al.*, 1988; Bonthron *et al.*, 1988}. Both forms of PDGF-A have been conserved in humans, mouse {Young *et al.*, 1990} and *Xenopus* {Mercola *et al.*, 1988} suggesting that the ability to select alternative versions of the PDGF A chain is important for some aspect of PDGF biology. This conclusion was subsequently reinforced by the observation that the gene for vascular endothelial growth factor (VEGF/VPF) {Leung *et al.*, 1989; Keck *et al.*, 1989} can be spliced in a manner analogous to the PDGF-A gene {Tischer *et al.*, 1989}, to produce alternative versions of VEGF, one of which has a basic, carboxy-terminal extension similar to that of PDGF-A_L (Figure 5) {Betsholtz *et al.*, 1990}.

There have been a variety of attempts to define the functional difference conferred by the presence or absence of the exon 6 encoded basic tail of PDGF-A_L. Evidence that PDGF is able to enter the nucleus and bind tightly to chromatin {Rakowicz-Szulczynska *et al.*, 1986} coupled with the sequence similarity between the PDGF-A_L basic tail and the SV40 nuclear transport signal (NTS) {Tong *et al.*, 1987} led to investigations which showed that the tail could direct nucleolar localisation of reporter proteins {Maher *et al.*, 1989}. However, since this observation depended on using nonsecreted

reporter products or secretion-deficient PDGF-A$_L$ mutants it was considered unlikely that this would be the normal *in vivo* function {Tong *et al.*, 1987}. Indeed it has now been demonstrated that the A$_L$ tail is not required for mitogenic activity {LaRochelle *et al.*, 1991}. Studies comparing the receptor affinity of PDGF-A$_L$ and PDGF-A$_S$ homodimers produced in a yeast expression system, failed to show any significant difference {Östman *et al.*, 1989} suggesting that the presence of exon 6 does not affect binding to receptor. However, studies carried out using mammalian cell expression systems have led to a number of apparently conflicting results. An early study using a plasmid-based expression system suggested that PDGF-A$_L$ was more efficiently assembled and secreted from both Cos-1 and NIH-3T3 cells than PDGF-A$_S$, which was thought to require dimerisation with PDGF-B chain for efficient processing {Collins *et al.*, 1987}. This was retracted, however, when it was shown that the expression vector they had used, directed aberrant expression of the PDGF-A cDNAs due to cryptic splicing of the RNA transcripts in the transfected Cos cells {Wise *et al.*, 1989}. A subsequent study using retroviral expression vectors showed that both rat-1 cells and human fibroblasts infected with a virus that directed expression of PDGF-A$_S$ released 10-fold more A-chain protein than cells infected with virus directing expression of PDGF-A$_L$ despite equivalent levels of mRNA {Bywater *et al.*, 1988}. However, another study using a different plasmid-based expression vector, with the mouse metallothionein (MMT) promoter directing expression of each isoform, showed that PDGF-A$_S$ and PDGF-A$_L$ secretion from NIH-3T3 cells were apparently no different {Beckmann *et al.*, 1988}.

A series of recent papers and work described in this thesis provide a model for how the two forms differ and suggest an explanation for these apparently contradictory results. Both PDGF-A$_L$ and PDGF-A$_S$ are synthesised as pre-pro-peptides that are proteolytically processed during their progress along the secretory pathway; the signal peptide is removed when the protein enters the lumen of the endoplasmic reticulum, and a maturation cleavage near the amino terminus occurs en route from the Golgi to the cell surface (Figure 10). Homodimers of PDGF-A$_S$ are then released from the cell as freely-diffusible molecules while PDGF-A$_L$ homodimers remain cell-associated {Östman *et al.*, 1991; LaRochelle *et al.*, 1991; Raines and Ross, 1992}. In this regard PDGF-A$_L$ resembles PDGF-B, which also remains associated with the cell surface after secretion {Robbins *et al.*, 1985}. Several lines of evidence indicate

that this association is mediated by a basic sequence present in the carboxy terminal region of the B-chain {LaRochelle *et al.*, 1990} and a similar sequence present in the basic carboxy terminus of PDGF-A$_L$ (Figure 5). Cell-associated PDGF-A$_L$ and PDGF-B can be released into the medium by addition of peptides corresponding to their basic "retention" domains or by soluble heparan sulphate proteoglycans (HPGs) {Raines and Ross, 1992}, and a synthetic PDGF-A$_L$ retention domain binds to cell surfaces in a manner that can be competitively inhibited by heparin {Khachigian *et al.*, 1992}, suggesting that the retention domains may function by binding to HPGs in the extracellular matrix (ECM).

1.3 Aims of thesis.

The evidence accumulating at the outset of this work implicated PDGF-A in a variety of developmental events. Of particular interest were the isolation of a cDNA representing the longer PDGF-A isoform, PDGF-A$_L$, from a human glioma cell line and the observations indicating that PDGF-A was involved in the regulation of rat glial progenitor cell differentiation and migration. It therefore seemed likely that an understanding of the expression pattern and function of both PDGF-A$_L$ and PDGF-A$_S$, might provide important clues to how glial progenitor cell proliferation is regulated *in vivo*. I therefore attempted to define the spatial and temporal distribution of the alternative PDGF-A transcripts in various rat glial cell types and to compare the functional activity of the two different PDGF-A homodimers *in vitro*, in the hope that this might provide a clue to their function *in vivo*.

Table 2. Cell types that express PDGF; their location and the proposed biological roles of the PDGF they produce. Adapted from Ross *et al.*, (1986).

Cell Types expressing PDGF	Anatomical considerations	Normal Activity	Abnormal Activity
Circulating Cells			
Platelets	In circulation for 9-11 days	Wound healing vasoconstrictor	Atherosclerosis
Monocyte / Macrophages	Can reside in tissue for months	Wound healing vasoconstrictor	Atherosclerosis; fibrotic disorders of Lung, Liver and kidney; inflammation and Arthritis.
Resident Cells			
Megakaryocytes	Bone marrow residents	For storage in platelets	Abundant in some forms of myelofibrosis.
Endothelium	Permeable barrier between circulating blood cells and underlying tissue	Wound healing vasoconstrictor	Atherosclerosis
Rat Pup Smooth muscle cells	Rat aorta	Development & Growth	Atherosclerosis
Adult Smooth muscle cells	Rat carotid, rat aorta media	Wound healing vasoconstrictor	Atherosclerosis
First trimester human placental tracts	Cytotrophoblasts	Development & Growth	Atherosclerosis
Type 1 Astrocytes & Neurons	Glial tracts	Myelination during development & wound repair. Neuroregulator	Developmental lesions & myelinating disorders ie. multiple sclerosis
Transformed cells			
PDGF-responsive cells	Mainly blastomas and sarcomas	None	Autocrine stimulation in atherosclerosis & neoplasia
PDGF-nonresponsive cells	Erythroleukemia, bladder carcinoma & hepatoma	None	Paracrine stimulation in myelofibrosis & desmoplasia
SSV (v-sis) transformed	Subcutaneous fibrosarcomas	None	Neoplasia

A.

RNA

B.

PDGF-A$_S$

DVR

PDGF-A$_L$

GRPRESGKKRKRKRLKPT

C.

PDGF-B

... RPPKGKHRKFKHTH ...
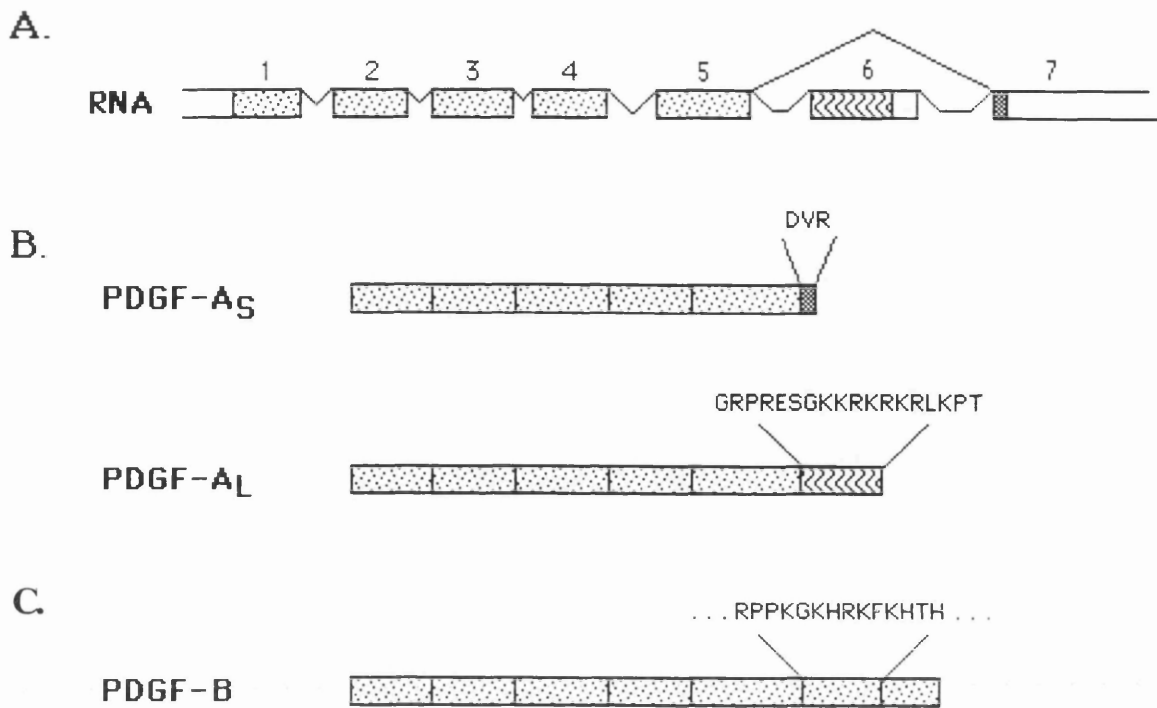
Figure 4.

Showing how the two forms of human PDGF-A protein arise by alternative splicing of a single mRNA transcript (**A**) differ by 18 amino acids in total but by only 15 amino acids in size (**B**) and how they compare to PDGF-B (**C**). See also Figure 5 for a direct comparison of all known PDGF-A$_L$ and PDGF-B carboxy terminal tails.

☐ = Non coding RNA sequences of RNA

▨ = Constant region

▧ = Exon 6 coding for the PDGF-A$_L$ basic carboxy-terminal tail.

▦ = Exon 7 coding for the PDGF-A$_S$ carboxy-terminus

(See Table 3 for single letter amino acid code)

```
...(V)R(R)P P(K)G K H R K F K H T H D(K)T A [L K E T]... human PDGF-B
...-G R P R E S G K K R K R K(R)L K P T     human PDGF-A_L
...-G R(R)R E S G K K R K R K(R)L K P T     mouse PDGF-A_L
...-G R T R E T G K K Q K R K(K)L K P T     Xenopus PDGF-A_L
.S(V)R G K G(K)G Q K R K R K(K)S R Y(K)S W S V....  human VPF/VEGF
```
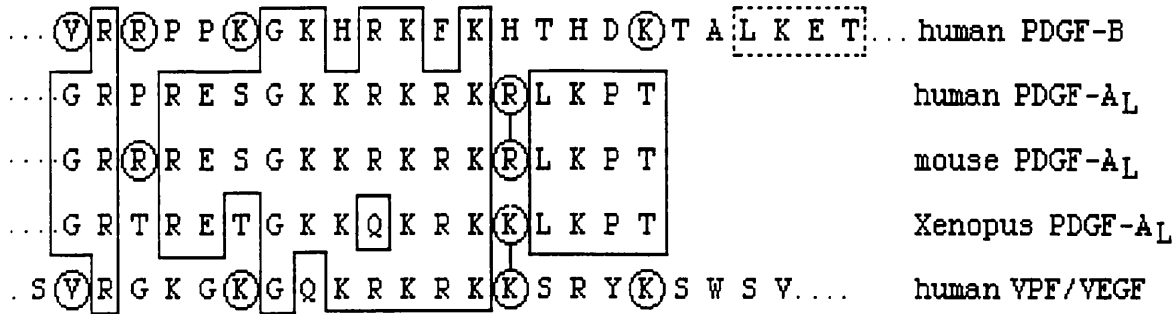
Figure 5.

Showing the amino acid sequences of the basic retention domains found in the human PDGF-B peptide, each of the known PDGF-A_L peptides, and the longer splice form of bovine VEGF. The regions which match in 3 or more cases are boxed while those that match in only two cases are indicated by circling. An additional region of homology present in the B-chain sequence but located nearer the carboxy-terminal tail is indicated in a [broken box].

Table 3. Single letter Amino Acid code indicating which are hydrophobic (H) and which are hydrophilic (P) including (Acidic and Basic).

ALANINE	A	(H)	THREONINE	T	(P)
VALINE	V	(H)	CYSTEINE	C	(H)
LEUCINE	L	(H)	TYROSINE	Y	(P)
ISO-LEUCINE	I	(H)	ASPARAGINE	N	(P)
PROLINE	P	(H)	GLUTAMINE	Q	(P)
PHENYLALANINE	F	(H)	ASPARTIC ACID	D	(ACIDIC)
TYPTOPHAN	W	(H)	GLUTAMIC ACID	E	(ACIDIC)
METHIONINE	M	(H)	LYSINE	K	(BASIC)
GLYCINE	G	(H)	ARGININE	R	(BASIC)
SERINE	S	(P)	HISTIDINE	H	(WEAKLY BASIC)

♉ Chapter Two ♉

"One fine morning in May a slim young horsewoman might have been seen riding a handsome sorrel mare along the flowery avenues of the Bois de Boulogne."

Chapter 2. Materials and Methods.

Unless otherwise stated all chemicals and reagents were purchased from BDH Chemicals Ltd. and were Analar grade wherever possible.

All restriction enzymes and molecular biology reagents were purchased from Pharmacia Ltd.

All radio-chemicals were purchased from Amersham International.

All specialised bacterial media components were obtained from Difco Laboratories Ltd.

Solutions were sterilised, where necessary, by autoclaving at 15 lb/sq. in. for 20 minutes and thereafter stored at room temperature unless otherwise stated.

2.1 Bacteriology.

Methods in this section were taken from Maniatis *et al.* (1982) unless otherwise referenced.

A) Bacterial strains.

For general cloning and sub-cloning of recombinant plasmids *Escherichia coli (E.coli)* strains TG1 (*supE hsdΔ5thi Δ(lac-proAB)* F'[*traD36 proAB* + *lac* I^q *lacZ* ΔM15] and JM101 (*supE thi Δ(lac-proAB)* F'[*traD36 proAB*+ *lac* I^q *lacZ* ΔM15] were used.

For phage lamda (λ) gt10 work strains NM514 (*hfl*+) and L87 supplied with the λ gt10 cloning kit and for λ gt11 strain Y1090 [*lac* U169 *pro* A+ D*lon ara* D 139 *str* A *sup* F *trp* C22: Tn10 (pMC9)] were used.

B) Growth media and agar plates.

Bacteria were grown in Luria Broth (LB) containing 10 g bacto-typtone, 5 g yeast extract and 10 g NaCl per litre, or on LB-agar plates (LB + 15 g bacto agar/litre).

Where appropriate Ampicillin (Amp) at a final concentration of 100 μg/ml was added after LB had cooled to below 50°C. Plates and solutions containing Amp were stored at 4°C in the dark. Amp was prepared as a 50

mg/ml stock by dissolving the sodium salt in deionised water (dH_2O), filter sterilised through a 0.22 μm filter (Millipore Corp.) and stored frozen at -20°C.

All strains of *E.coli* were grown at 37°C. Liquid cultures were continuously agitated in a rotating environmental shaker.

C) Preparation of competent bacteria.

i) Calcium chloride method.

50 ml of LB medium was inoculated with 1 ml of an overnight TG1 culture and incubated at 37°C with vigorous shaking until the density of the culture reached an absorbance of between 0.2-0.4 at 600 nm. The bacteria were pelleted (3000 G, 4°C, 10 minutes) and resuspended in 20 ml of ice cold 100 mM $CaCl_2$ (22 μm filter sterilised) and left on ice for 20 minutes. The cells were re-pelleted, as above, and resuspended in 1 ml of ice cold 100 mM $CaCl_2$ and kept at 4°C up to a maximum of 24 hours before use.

ii) Hexamine cobalt chloride method.

Where a higher efficiency of transformation was required, i.e. during cloning of newly ligated constructs, the following buffer was used in place of $CaCl_2$: [100 mM KCl, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 3 mM Hexamine Cobalt chloride, 5 mM 2 [N-morpholino]-etho-sulfonic acid adjusted to pH 6.3 with KOH (K.Mes)], 0.22 μm filter sterilised and stored in aliquots at -20°C {Hanahan, 1985}.

D) Transformation of bacteria with DNA.

DNA was added to competent cells at 50-100 ng DNA per 100 μl cell suspension and incubated on ice for 30 minutes. Then heat shocked for 90 seconds at 42°C. The bacteria were incubated in 1 ml LB at 37°C for 45 minutes with vigorous shaking. The cells were then pelleted at 3000 G for 5 minutes and resuspended in 100 μl LB before spreading onto LB-agar plates (supplemented with antibiotics where appropriate) and incubating at 37°C overnight. Individual colonies were placed into 10 ml of LB using sterile Gilson tips and incubated overnight at 37°C with constant agitation. The overnight cultures were then processed for plasmid mini preps.

When cloning into plasmids (or phage M13) where the cloning site forms part of a ß-Galactosidase gene it was possible to screen for inserts by

looking for loss of ß-Galactosidase activity. This was done by resuspending the transformed cells in 100 µl LB with 10 mM Isopropyl ß-D-thiogalacto-pyranoside in H$_2$O (IPTG) and 0.8% 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-GAL) in dimethylformamide and spreading as above. In this system native cloning vector gives blue colonies (or plaques) whereas vectors with inserts give uncoloured colonies (or plaques).

E) Long-term storage of recombinant bacteria.

Long-term stocks of bacterial strains and bacteria hosting recombinant plasmids were produced by adding 15% glycerol to overnight bacterial cultures and storing these at -20°C. Bacteria were recovered by inoculating 3 ml of LB with 10 µl of glycerol stock and growing the culture overnight at 37°C with constant agitation.

2.2 Molecular biology.

Methods in this section were taken from Maniatis et al (1982) unless otherwise referenced.

A) Phenol/chloroform extractions.

Samples to be extracted were made 0.5 M NaCl, vigorously mixed with an equal volume of UNC-phenol (containing 500 g phenol (melted at 65°C), 111 ml 2 M Tris pH 7.5, 114 ml dH$_2$O, 28 ml m-cresol, 1.1 ml ß-mercaptoethanol (2-ME), 555 mg 8-hydroxyquinoline) and centrifuged at 10,000 G for 10 minutes. The upper aqueous phase was removed and re-extracted with an equal volume of chloroform (containing 4% Isoamyl alcohol) centrifuged as before and the upper aqueous phase collected.

B) Ethanol precipitation of nucleic acids.

In general samples were made 0.5 M NaCl and 2 x the original volume of 95% ethanol was added. Samples were incubated on dry-ice for 20 minutes before centrifuging (10000 G, 4°C, 15 minutes). The pellet was washed once with 70% ethanol and to air dry before resuspending in an appropriate buffer. Samples containing less than 5-10 µg of DNA/RNA were precipitated with 25 µg of yeast transfer RNA(tRNA) to act as a carrier.

Table 4. Alternative Salts used for precipitation of nucleic acids {Wallace, 1987}.

Final Conc.	pH	EtOH	Recommended Uses :-
0.2 M NaCl	7.0	2 vols	General and any SDS in sample remains soluble but the Cl⁻ inhibits RNA translation.
0.3 M NaAc	5.5	2 vols	General uses.
2.5 M NH4Ac	5.5	3 vols	Precipitates only incorporated dNTPs but inhibits phosphorylation and tailing.
0.8 M LiCl		3 vols	Salt itself is not precipitated but Cl⁻ may still inhibit RNA translation and Li⁺ inhibits reverse transcriptase.
0.2 M KAc	5.5	2 vols	Used for RNA precipitation if in vitro translation was to be performed.

C) Restriction digest of DNA.

As a general rule up to 2 μg of DNA was digested in a final volume of 20 μl including enzyme, for a minimum of 1 hour at 37°C. Restriction enzymes were used in buffer containing 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT with an NaCl concentration of 0 mM, 50 mM, 100 mM or 150 mM depending on the suppliers' recommendations.

D) End labelling of DNA fragments.

Recessed 3' ends were radiolabeled by addition of 0.5 μl ^{32}P dNTP (10 μCi/μl) and 0.1 units of Klenow in 1 x 50 mM restriction buffer (see above) and incubating for 10 minutes at 37°C. The choice of the ^{32}P dNTP depended on the sequence of the overhanging 5' end and where necessary cold nucleotides (200 μM) were included to allow the reaction to proceed to the point at which a hot nucleotide could be incorporated.

E) Dephosphorylation of DNA termini.

To prevent religation of vectors cut at a single enzyme site the 5' phosphate groups were removed by dephosphorylation. 1-2 μg of digested DNA were incubated in 1 x CIP buffer (50 mM Tris pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine) with 0.2 units of Calf Intestinal Phosphatase (CIP) for 1 hour

at 37ºC. Samples were then heated to 65ºC for 15 minutes, in the presence of 0.5% SDS, to inactivate the CIP then phenol/chloroform extracted and EtOH precipitated with NaAc as previously described.

F) Ligation of DNA.

Ligations usually contained 50-100 ng of vector DNA and a 4 fold molar excess of the fragment to be inserted. In the case of blunt end ligations a 10 fold molar excess of insert was used. Ligations were incubated at 14ºC for at least 6 hours with 1 unit of T4 DNA Ligase in buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM Spermidine, 1 mM Adenosine Tri-phosphate (ATP) and 0.1 mg/ml Bovine Serum Albumen) in a total of 20 µl. Competent bacteria were transformed with the DNA and grown on agar plates containing 50 µg/ml Amp at 37ºC over night.

G) Screening plasmid DNA from transformed bacterial colonies.

i) Plasmid mini preparation.

Individual colonies from transformed bacteria were grown overnight at 37ºC as described. 1.5 ml was taken and the cells pelleted (13,000 G, 30 seconds) in eppendorf tubes. The cells were resuspended in 100 µl of 25 mM Tris pH 8.0, 10 mM EDTA, 50 mM Glucose and incubated at room temperature for 5 minutes. 200 µl 0.2 N NaOH, 1% (w/v) SDS was added and the tubes incubated on ice for 5 minutes, followed by addition of 150 µl of 3 M potassium acetate, 2 M acetic acid pH 4.5 (at 4ºC) and incubation on ice for a further 5 minutes. The solution was centrifuged for 5 minutes at 10,000 G, 4ºC, the supernatant taken and recentrifuged. The DNA was ethanol precipitated as described and the pellet resuspended in TE (10 mM Tris pH 8, 1 mM EDTA) and stored at -20 ºC.

ii) Transformed bacterial colony lifts and hybridisation.

This method was used in cases where it was necessary to screen large numbers of colonies either because several different clones were expected or if it was thought that few of the transformed cells contained the required clones. Having produced a number of colonies these were then picked onto two replica plates. On one plate a 60 mm nylon filter disc (Hybond-N from Amersham) with grid marks on it was laid on top of the LB agar and the colonies dotted onto each of the gridded squares respectively. After overnight

growth at 37°C the filter was lifted off the plate and laid down colony-side upmost on Whatman 3MM filter paper soaked in 10% SDS for 5 minutes, then onto 3MM paper soaked in 1.5 M NaCl, 0.5 N NaOH for 5 minutes and finally onto 3MM soaked in 1.5 M NaCl, 50 mM Tris-HCl pH 8 to neutralise for 5 minutes. The filter was then baked for 2 hours at 80°C and then washed in 50 mM NaOH for 5 minutes followed by 2 x washes in 1.5 M NaCl, 50 mM Tris-HCl pH 8.

The filter was hybridised in 10 ml of buffer containing 5 x Denhardt's (1% (w/v) ficoll 400, 1% (w/v) polyvinyl-pyrrolidone, 1% (w/v) BSA), 0.25 mg Salmon Sperm DNA, 6 x SSC (0.9 M NaCl, 0.09 M Sodium Citrate pH 7) and denatured ^{32}P labeled probe DNA at approximately 10 ng/ml overnight at 42°C. The filters were washed stringently (0.5 x SSC, 0.1% SDS, 65°C, 15 minutes x 3) before being wrapped in Saran wrap and exposed to pre-flashed X-OMAT AR diagnostic film (Kodak). Exposure times were often as little as 10 minutes at room temperature. Positives could then be picked from the appropriate colony on the replica plate.

H)   "Maxi"   plasmid   preparation.

200 ml bulk cultures were inoculated with 1 ml of an overnight bacterial culture and incubated overnight at 37°C with constant agitation. The cells were pelleted (6000 G, 15 minutes, 4°C) and resuspend in 4 ml of 25% sucrose in 50 mM Tris pH 8.0 containing lysozyme at 2 mg/ml (diluted from a fresh stock of 20 mg/ml in 50 mM Tris pH 8.0). Samples were incubated on ice for 10 minutes before addition of 0.2 ml of 0.5 M EDTA (pH 8.0) and incubation for a further 20 minutes on ice. 3.5 ml of 3 x triton lysis buffer (3% (v/v) Triton X100, 180 mM EDTA, 150 mM Tris pH 8.0) was added and mixed by inversion and incubated for 30 minutes on ice. The solution was then spun at 80,000 G, 4°C, for 60 minutes. The supernatant was removed and made 0.5 M NaCl before Phenol and Chloroform extraction as previously described. The final aqueous phase was made 10% (w/v) with solid PEG-6000 and incubated on ice for 60 minutes. The DNA was pelleted (10,000 G, 4°C, for 20 minutes) and resuspended in 500 μl of TE. RNA was removed by incubating in 0.1 mg/ml DNase-free RNase and the solution left at 37°C for 1 hour. An equal volume of 10 mM Tris pH 8.0, 1 M NaCl, 1 mM EDTA, 20% (w/v) PEG (Mwt. 6000) was added and the plasmid DNA allowed to precipitate on ice for 1 hour, before being pelleted (13,000 G, 15 minutes) and all traces of the supernatant removed. The pellet was resuspended in 400 μl of 10 mM Tris pH 7.5, 0.5 M NaCl

Phenol/Chloroform extracted and ethanol precipitated. The final DNA pellet was resuspended in TE and the concentration and protein levels determined by taking absorbance readings at 260 nm and 280 nm (A_{260}, A_{280}).

$$\text{DNA concentration (mg/ml)} = \frac{A260 \times \text{extinction co-efficient} \times \text{dilution factor}}{1,000}$$

At 20°C the extinction co-efficient for absorbance at 260 nm is 50 $\mu g^{-1}cm^{-1}$ for double stranded DNA and 40 $\mu g^{-1}cm^{-1}$ for single stranded RNA and 20 $\mu g^{-1}cm^{-1}$ for oligonucleotides.

I) Preparation of genomic DNA.

Genomic DNA was prepared from approximately 1.5 x 10^9 cells by a modification of the SDS lysis method {Maniatis *et al.* 1982}. The cells were lysed in 2 ml of resuspension buffer (RSB) (10 mM Tris, pH 7.4, 10 mM NaCl, 25 mM EDTA) with 0.5% Nonidet P40 (NP40) then spun at 2000 k for 2 minutes. The resulting pellet was resuspended in 1 ml of RSB with 2 mg of proteinase K and incubated at 37°C for 2 hours. The solution was made 0.5 M NaCl, phenol and chloroform extracted and ethanol precipitated as described previously. The dried pellet was resuspended in H_2O and treated with 0.2 mg/ml DNase free RNase at 37°C for 30 minutes before re-extraction with phenol/chloroform and precipitating again. The final yield was calculated as described previously.

J) Preparation of RNA.

Glassware was thoroughly washed in distilled water and oven baked at 250°C for at least 4 hours. Disposable sterile plasticware was also used. All solutions were treated with Diethylpyrocarbonate (DEPC) 0.1% (v/v). DEPC was added and mixed by shaking and the solutions autoclaved to destroy any remaining DEPC. Chemicals used were specifically set aside for RNA work only and disposable rubber gloves were worn at all stages of these protocols.

RNA extraction was carried out as described previously {Chomczynski and Sacchi, 1987}. For every 100 mg of cells to be extracted 1 ml of Solution D (4 M Guanidinium thiocyanate, 25 mM Sodium citrate pH 7, 0.5% (v/v) Sarcosyl, 0.1 M ß-mercaptoethanol [2-ME]) was added before homogenising on ice using a Dounce homogeniser. The solution was then transfered to a polypropylene tube and for every 1 ml of Solution D, 0.1 ml of 2 M Sodium acetate pH 4, 1 ml of

phenol (water saturated) and 0.2 ml of Chloroform-isoamyl alcohol mixture (49:1) was added. The final mixture was shaken vigorously and left on ice for 15 minutes before being spun at 10,000 G for 20 minutes at 4°C. The RNA in the aqueous phase was precipitated by adding one volume of Isopropanol and leaving at -20°C for at least 1 hour. The RNA was pelleted and resuspended in Solution D and reprecipitated. The washed and dried RNA was pellet was resuspended in an appropriate volume of 0.5% SDS by heating to 65°C for 10 minutes. The RNA was stored at -70°C until required. In most cases the integrity of the RNA was tested by northern blotting and probing for actin message prior to use.

K) Poly (A)$^+$ selection.

The total RNA concentration was adjusted to less than 1 mg/ml and Poly (A)$^+$ RNA was selected by oligo dT-cellulose chromatography. The total RNA recovered was run over a 2 ml oligo (dT) - cellulose column in 0.5 M NaCl, 10 mM Tris pH 7.5, 0.1% SDS. The column was washed extensively with the same buffer before eluting the bound Poly (A)$^+$ with 10 mM Tris pH 7.5, 0.1% SDS. Poly (A)$^+$ RNA was precipitated as previously described and the pellet dissolved in a suitable volume of 10 mM Tris pH 7.5, 1 mM EDTA. The absorbance was measured as described previously to calculate the concentration and the sample stored at -70°C until required.

L) Electrophoretic analysis of nucleic acids.

i) Agarose gels.

Nucleic acids were suspended in 1 x Loading buffer (0.025% bromophenol blue, 0.025% xylene cyanol, 2.5% ficoll-400) and electrophoresed through 1% agarose gels in a horizontal submarine gel system (Pharmacia). The agarose was melted in 1 x TAE Buffer (40 mM Tris Base, 20 mM Sodium Acetate, 10 mM EDTA, pH adjusted to 8.3 with acetic acid). Gels were stained by soaking them for 5 minutes in a solution of Ethidium Bromide (EtBr) at 5 μg/ml in H_2O and DNA visualised by illumination on a UV light box. Photographs were taken using Polaroid type 665 or 667 film.

ii) NaOH blotting procedure.

DNA was transfered to Nylon membranes (Genescreen-Plus, NEN Dupont) as previously described {Reed and Mann, 1985}. DNA fragments over 10 kb were hydrolysed by soaking the gel for 15 minutes in 0.25 M HCl twice. Then 0.5 M NaOH, 1.5 M NaCl was used as a transfer solvent which simultaneously neutralised and fixed the DNA to the membrane without recourse to baking or UV cross-linking.

iii) RNA gels.

RNA gels were electrophoresed at 17-21 mA overnight or at 100 mA for 2-3 hour runs, through 1% agarose gels in horizontal submarine gel systems. For a 100 ml gel 1 g of agarose was melted in 82 ml of 1 x MOPS running buffer (0.02 M MOPS (3-[N-Morpholino]-propane-sulfonic acid), 5 mM sodium acetate, 0.5 mM EDTA pH adjusted to 7.5) and this was cooled to $50^{\circ}C$ before addition of 18 ml of 2.2 M formaldehyde (final conc. 0.4 M). Aqueous RNA solutions were incubated at $65^{\circ}C$ for 3 minutes in 5 x volume of Sample buffer (50% formamide (de-ionised), 18% formaldehyde (2.2 M), 1 x MOPS) prior to loading in 1 x Loading buffer (5% glycerol, 0.1 mM EDTA, 0.04% bromophenol blue [runs at 500 bp] , 0.04% xylene cyanol [runs at 1 kb]). If dilute solutions of RNA were to be used the required amount, usually about 10-15 µg of Poly $(A)^{+}$, was precipitated and taken up directly in sample buffer. The running buffer (1 x MOPS) was recirculated to prevent alkainity build up.

iv) Northern blotting.

Gels were capillary blotted on to Nylon membranes (Genescreen-Plus, NEN Dupont). After transfer the membrane was baked for 2 hours at $80^{\circ}C$. The remains of the gel were stained with EtBr, as for DNA, and the positions of the 28s and 35s ribosomal bands noted.

v) Bisacrylyl-cystamine (BAC) gels.

This was based on a method used to separate DNA fragments of between 100-1000 bp prior to blotting {Hansen, 1981}. It gives better resolution than an agarose gel and, as I discovered, can be transferred to membrane more easily. A further adaptation was the inclusion of 50% (w/v) Urea in cases where DNA was to be kept denatured.

Gels were prepared by adding 20 ml of 10% acrylamide stock (47.7 g Acrylamide, 2.25 g Bisacrylyl-Cystamine in 500 ml of H_2O filtered through Whatman qualitative filter and stored at 4°C in a dark bottle) to 4 ml of 10 x TBE (890 mM Tris base, 890 mM Boric acid, 250 mM EDTA, adjusted to pH 8.3 with acetic acid) and either 1 ml of H_2O with 20 g of urea for denaturing conditions or 16 ml of H_2O giving a final volume of 40 ml . This mixture was heated to 37°C prior to polymerisation by the addition of 400 μl of freshly prepared 10% ammonium persulphate and 100 μl of TEMED. 1.5 mm thick vertical gels were electrophoresed at 200 V for approximately 1 hour. The DNA was visualised by staining with EtBr or in the case of radiolabeled samples exposed to pre-flashed X-OMAT AR diagnostic film (Kodak) at -70 °C.

vi) Electroblotting BAC gels.

This was adapted from a previously described method {Church and Gilbert, 1984}. Gels were pretreated in denaturing Buffer (0.2 M NaOH, 0.3 M NaCl) for 25 minutes, then the DNA was transferred, using western blotting apparatus, to a nylon membrane (Genescreen-Plus, NEN Dupont) at 250 mA for 3 hours or 50 mA overnight. After transfer the filter was washed in 0.4 M NaOH for 1 minute and neutralised with 0.2 M Tris pH 7.5, 2 x SSC for 10 minutes before drying and baking for 2 hours at 80°C.

vii) Sequencing size gels.

These gels were used for purification of oligonucleotides and separation of similar sized fragments as well as sequencing. The percentage of acrylamide used was adjusted according to the length of DNA to be resolved see (Table 5). These gels were run at 1400 volts for 2-7 hours on a BRL Sequencing Gel Electrophoresis System Model S2 with 0.4 mm spacers and 48 sample capacity Sharkstooth Combs. Stock solutions of 30% Acrylamide and 2.5% Bis-acrylamide were filtered through Whatman qualitative filter and stored at 4°C in dark bottles.

Table 5. Acrylamide sequencing gel mixes.

Non-denaturing	8%				
Denaturing (8.3 M Urea)		5%	8%	15%	20%
30% acrylamide	26.6 ml	16.6 ml	26.6 ml		
acrylamide (solid)				15 g	20 g
2.5% bis-acrylamide	6.4 ml	10 ml	16 ml	30 ml	40 ml
10 x TBE	10 ml	10 ml	10 ml	10 ml	10 ml
H_2O	57 ml	26 ml	10 ml	20 ml	5 ml
urea (solid)		50 g	50 g	50 g	50 g
ammonium persulphate	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.4 ml
TEMED	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
Dyes as markers :-					
xylene cyanol	275 bp	130 bp	90 bp	36 bp	28 bp
bromophenol blue	45 bp	35 bp	20 bp	8 bp	7 bp

M) Purification of DNA fragments.

i) From agarose gels.

This was either done with the Amersham GeneClean® system {Vogelstein and Gillespie, 1979} or by electro eluting onto a Glass fibre strip (Whatman GF/C) backed by dialysis tubing (Specta/POR™ m.w.t cut off 6,000-8,000) inserted into a slot cut in the gel in front of the DNA of interest with a minimum of running buffer. The DNA was released from the glass fibre by spinning at 13,000 G for 1 minute {Towner, 1991}.

ii) From acrylamide gels.

The region of the gel containing the DNA of interest was excised and left overnight in 0.5 ml of gel elution Buffer (500 mM NH_4Ac, 10 mM $Mg(Ac)_2$, 1 mM EDTA, 0.1% SDS, 5 µg/ml tRNA) at 37°C to elute the DNA. The DNA was

precipitated from the supernatant as described previously and the pellet resuspended in 0.3 M NaAc pH 4.8 and reprecipitated.

N) Preparation of DNA probes.

DNA was digested with appropriate restriction enzymes and the desired fragment purified as described previously. The DNA was labeled with ^{32}P to a specific activity of approximately 2×10^8 cpm/µg using the Random Oligonucleotide priming method {Feinberg and Vogelstein, 1984}. Oligo labelling Buffer (OLB^O) was prepared by mixing solutions A:B:C in a ratio of 10:25:15.

SOLUTION A : 1 ml of 1.25 M Tris-HCl, 0.125 M MgCl$_2$ containing 18 µl of ß-mercaptoethanol (2-ME), 500 µM dATP. 500 µM dGTP, 500 µM dCTP.

SOLUTION B : 2 M Hepes pH 6.6

SOLUTION C : Pd(N)$_6$ dissolved at 90 OD units/ml. This is the primer for the DNA polymerase and consists of 6 randomly coupled oligonucleotides.

The labelling reaction was carried out at room temperature by adding together the following reagents : 10 µl of OLB^O, 2 µl of DNase free BSA (10 mg/ml), 3-4 µl of ^{32}P dTTP (10 µCi/µl), 50-100 ng of DNA to be labelled (boiled for 3 minutes and cooled on ice prior to addition), X µl of H$_2$O to 50 µl total volume. 0.5 µl of Klenow (2 units) were added and the reaction left to proceed for at least 3 hours.

The unincorporated nucleotides were separated from the labeled DNA by centrifugation through a G-50 Sephadex spun column. Labeled DNA passed through while the unincorporated was retained by the G-50. The labeled dsDNA probe was denatured prior to use by boiling for 4 minutes.

O) Filter hybridisation.

Exact protocols used depended on the level of homology and the expected concentration of the target sequence on the filter being probed. DNA or RNA which had been transferred to a nylon filter membrane was prehybridised in 50% deionised formamide, 5 x Denhardt's (1% (w/v) ficoll 400, 1% (w/v) polyvinyl-pyrrolidone, 1% (w/v) BSA), 6 x SSC, 1 mM EDTA, 1% SDS, 100 µg/ml denatured sonicated salmon sperm DNA, 10% Dextran sulphate at 42°C for 2

hours before addition of the radio-labelled probe at a final concentration of approximately 10 ng/ml and hybridisation for at least 8 hours.

The blots were washed at an appropriate stringency; in most cases 3 washes in 1 x SSC, 0.1% SDS at 55°C for 15 minutes each time, was found to be enough. On occasions where high background was observed it was necessary to increase the stringency to 0.5 x or even 0.1 x SSC. Filters were then wrapped in Saran wrap and exposed to pre-flashed X-OMAT AR diagnostic film (Kodak) at -70 $^\circ$C.

As a control for probe specificity I standardly prepared strips of nylon filter with 1 μl spots of dilutions of the vector DNA containing the probe sequence (5 ng, 0.5 ng, 0.05 ng and 0.005 ng) as a positive and vector DNA alone (50 ng, 5 ng and 0.5 ng) as a negative control. The DNA was then denatured prior to probing by placing the filter onto 3MM paper soaked in 1.5 M NaCl, 0.5 N NaOH for 5 minutes then onto 3MM soaked in 1.5 M NaCl, 50 mM Tris-HCl pH 8 to neutralise for 5 minutes. The filter was then baked for 2 hours at 80°C and then washed in 50 mM NaOH for 5 minutes followed by 2 x washes in 1.5 M NaCl, 50 mM Tris-HCl pH 8.

P) DNA sequencing.

Sequencing reactions were performed according to the protocols of the Sequenase™ kit (United States Biochemical). Double stranded plasmids and purified DNA fragments were first denatured as follows: 2-3 μg of maxi prep plasmid DNA or 50 ng of fragment DNA were denatured in a total volume of 20 μl of 0.2 N NaOH at room temperature for 5 minutes {Zhang et al., 1988}. This was neutralised by addition of 2 μl of 2 M NH$_4$Ac (pH 4.6), quickly followed by 75 μl of 95% EtOH at -20°C all of which was well mixed and left at -70°C for 5 minutes. DNA was pelleted (10,000 G, 20 minutes) washed, dried as described previously and resuspended in 10 μl of 1 x Sequenase™ buffer with 25 ng of primer. The DNA could then be treated in the same manner as the single stranded control DNA supplied with the kit, giving similar quality results.

Q) Oligonucleotides.

Oligonucleotides were initially produced on an Applied Biosystems DNA Synthesiser and latterly obtained from either the ICRF oligonucleotide synthesis service or Dr. Colin Goding, Marie Curie Institute, Oxted.

Oligonucleotides were cleaved from the synthesis column matrix by elution with 1.5 ml of fresh ammonia at room temperature for 2 hours and subsequently deprotected by heating to 55°C for at least 8 hours. The ammonia was evaporated and the oligonucleotide resuspended in 200 µl of 2.5 M NH4Ac pH 7.0 and ethanol precipitated as described previously and resuspended in H2O at a final concentration of 1 µg/µl.

R) Polymerase chain reaction (PCR).

i) Preparing dNTP solution at pH 7.

Stock solutions of dNTPs at pH 7 were made by resuspending the dried nucleotides in 35 mM Tris-base to a final concentration of 10 mM for each of dATP, dCTP, dGTP and dTTP.

ii) Polymerase chain reaction (PCR).

Taq polymerase was obtained from Cetus Corp. The basic reaction mix (100 µl final volume) comprised 1 x Cetus Amplification buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 3 mM MgCl2, 0.001% (w/v) Gelatin), 10% DMSO, 200 µM of each dNTP (pH 7), 0.1 µg/µl of each oligo primer and between 0.1 and 5 ng of plasmid DNA or 100 ng of cDNA. This was covered with 60 µl of parafin oil to prevent evaporation. The mixture was heated to 95°C for 5 minutes to denature the Target DNA and cooled to 72°C for 30 seconds prior to addition of 1 unit of *Taq* polymerase under sterile conditions. Using a Cambio "intelligent heating block" a standard reaction was incubated at 55°C for 30 seconds (annealing), 72°C for 2 minutes (extension), and 92°C for 30 seconds (denaturing). This cycle was generally repeated 30 times followed by a final extension period of 10 minutes at 72°C. Variations to this procedure: such as using a lower annealing temperature when the primers were not 100% homologous; varying the MgCl2 concentration to alter the primer specificity; or even using a two temperature PCR with no anneal temp to increase specificity, are indicated in the appropriate figure legends. 10-20 µl of the reaction was then run on a either a 3% TAE / agarose or a Bisacrylyl-cystamine (BAC) gel and the product(s) visualised by EtBr staining. In some cases the products were further analysed by electroblotting the BAC gel and probing.

iii)   Contamination   avoidance   (Chapter   4   only)

Based on my experiences with contamination (See Chapter 3.7 b) I included a number of additional steps in my subsequent PCR attempts. All samples to be analysed by PCR were made in thoroughly washed or where possible sterile disposable vessels. Dedicated pipettes which had never been used in plasmid work were set aside for PCR work only. Solutions to be used for PCR were kept in small aliquots and only opened in a blow out tissue culture hood preferably in a separate lab from that used for plasmid work. For each amplification experiment all the reactions were made up, as far as possible, in a single "master mix" before aliquoting into separate tubes for addition of the elements specific for each reaction. The sample cDNAs, any positive control DNA and the *Taq* polymerase were all added to the final reaction mix using disposable positive displacement pipettes. For each PCR negative controls with no added RNA or DNA were amplified in parallel with the samples under investigation. Finally any clones of the sequence being amplified were prepared in a different lab and used carefully to avoid airborne contamination.

iv)   Primers.

For the location and sequence of the primers used to amplify human PDGF-A chain sequences see Figure 6 and Tables 6 & 7. For the sequence and location of the primers relative to the rat PDGF-A chain sequence see Chapter 4 Figure 27 and Tables 8 & 9.

λgt11 Forward sequencing primer 5'd(GGTGGCGACGACTCCTGGAGCCC)3' (FSP) and Reverse sequencing primer 5'd(TTGACACCAGACCAACTGGTAATG)3' (RSP) respectively 14 bases and 24 bases from the λgt11 *EcoRI* cloning site were employed to amplify this region in order to estimate the size of any insert. The resulting PCR product including both the primer sequences and the intervening λgt11 sequences was 85 bp longer than the actual insert size.

Primers K1 and K2 which amplify a 325 bp region of the alpha-1 anti-trypsin genomic sequence, a kind gift from Dr. K. Abbot, Department of Genetics, UCL. were used ., for control amplification of genomic DNA preps.

Figure 6.

Diagrams showing the annealing locations of the human oligonucleotide primers (No.s 1, 2, 6, 7, 9 and 10) used for Polymerase Chain Reaction, relative to Human PDGF-A$_L$ cDNA from which they are derived (A) and showing the actual sequences to which they anneal (B).

(▧▧▧▧) = Indicates the sequence of the alternatively spliced exon 6.

(▬) = Alternative Stop codons.

(⟶) = Indicates where the sequence shown is the actual sequence of the Oligo.

(⟵) = Indicates where the sequence shown is complementary to that of the Oligo.

(See also **Figure 27** for Rat Oligonucleotide Primers)

A.

850   950   1050   1150

69bp Exon

No.1   No.8   No.6   No.2

No.7

No.9   No.10

B.

```
          No.9
  840    |  850      860       |  870       880             890
CAGCGTCAAG GTGGCCAAGG TGGAATACGT CAGGAAGAAG CCAAAATTAA AAGAAGTCCA

     No.1                        No.7
  |      |  900      910  |     | 920      |  930       940       950
GGTGAGGTTA GAGGAGCATT TGGAGTGCGC CTGCGCGACC ACAAGCCTGA ATCCGGATTA

              970     |        No.8        |
  960       |   980        990    1000      1010
TCGGGAAGAG GACACGGGAA GGCCTAGGGA GTCAGGTAAA AAACGGAAAA GAAAAAGGTT
           ((((((((((((((((((((((((((((((((((((((((((((((((((((

                        No.10
  1020      1030      |         |1050     |1060|       1070
AAAACCCACC TAAAGCAGCC AACCAGATGT GAGGTGAGGA TGAGCCGCAG CCCTTTCCTG
           ))))))))))))))))))))))))))))))))))))))))

           No.6                            No.2
  1080      |   1090 |   1100       1110  |   1120      1130 |
GGACATGGAT GTACATGGCG TGTTACATTC CTGAACCTAC TATGTACGGT GCTTTATTGC
```

Table 6.

Showing the size and location relative to the Human PDGF-A$_L$ Chain cDNA sequence of the oligonucleotides used in the Polymerase Chain Reaction studies. For the oligonucleotides with cloning sites the length of uncomplementary "tail", the type of restriction enzyme site and the effective end site produced during PCR, expressed relative to the human sequence, are also indicated. The details for oligonucleotides No.3 and No.4 which are specific for human PDGF B Chain cDNA are also shown.

Table 7.

Showing the expected PCR product sizes for each combination of oligonucleotides in Table 5 when used to amplify each of three different possible target cDNAs including PDGF-A$_L$ Tag with its 44 bp Myc Tag insert in the alternatively spliced sixth exon. (See Figure 8 constructs Diagram).

Table 6.

	Size	Location on Human PDGF-A$_L$ Chain cDNA	Extra Tail	5' End enzyme site	Actual PCR end
Oligo No.1	36 mer	887 - 914	8 bases	*BamHI*	879
Oligo No.2	33 mer	1130 - 1109	11	*HindIII*	1141
Oligo No.6	35 mer	1090 - 1056	0	0	1090
Oligo No.7	20 mer	911 - 930	0	0	911
Oligo No.8	20 mer	971 - 990	0	0	971
Oligo No.9	30 mer	841 - 864	6	*HindIII*	836
Oligo No.10	28 mer	1060 - 1038	5	*BamHI*	1065
		Location on Human PDGF B Chain cDNA			
Oligo No.3	34 mer	786 - 810	9	*BamHI*	777
Oligo No.4	36 mer	967 - 941	11	*HindIII*	978

Table 7.

PDGF-A Chain	1 + 2	7 + 2	7 + 6	9 + 10	7 + 10	1 + 10
Short form (A$_S$)	194 bp	161 bp	110 bp	160 bp	85 bp	118 bp
Long form (A$_L$)	263 bp	230 bp	179 bp	229 bp	154 bp	187 bp
PDGF-A$_S$ Tag	307 bp	274 bp	223 bp	273 bp	198 bp	231 bp

PDGF-A Chain	8 + 10	8 + 6	8 + 2	1 + 6	B Chain	3 + 4
Short form (A$_S$)	0	0	0	143 bp	Only form	201 bp
Long form (A$_L$)	94 bp	119 bp	170 bp	212 bp		
PDGF-A$_S$ Tag	138 bp	163 bp	214 bp	256 bp		

S) cDNA synthesis.

The Amersham "cDNA Synthesis System Plus" kit (RPN.1256) was used to synthesis double stranded (ds) cDNA from either total RNA or Poly (A)$^+$ RNA using a modified version of the RNase H method for priming second strand synthesis {Okayama and Berg, 1982}. In most cases Oligo dT priming was used for first strand synthesis. For PCR it was only necessary to make first strand rather than ds DNA.

T) Lambda library production and screening.

Poly (A)$^+$ RNA from postnatal day 7 (P7) S/D rats was converted into ds cDNA (see above) and cloned into λ gt10 using the Amersham "cDNA cloning system λgt10" cloning kit (RPN.1257). Analysis of the products (ie titration of the phage; amplification; hybridisation screening; and clone isolation) were carried out according to the Amersham protocols (See also Chapter 4).

A λgt11 library made from 3 week old (P21) Lewis strain rat brain cDNA was obtained from Dr. W. Stoffel, Institut fur Biochemie, Der Medizinischen Fakultat, Universitat zu Koln. 2×10^7 plaque forming units (pfu) contained 1.2×10^6 independent clones.

A P30 mouse brain cDNA library (4×10^8 pfu/ml), a Rat Cerebellum cDNA library (6×10^9 pfu/ml) and a Rat Hippocampus cDNA library (2×10^8 pfu/ml) all in λ ZapII, were obtained from Dr. G. Lemke, The Salk Institute, San Diego, California.

U) S1 nuclease analysis.

i) Probe production.

A single stranded DNA probe complimentary to the RNA of interest was required for S1 analysis. This was generated as previously described {Myers *et al.*, 1985}, by primer extension on a single stranded (ss) M13 template (see Figure 7). A 540 bp region of PDGF-A$_L$ including the alternatively splice exon 6 (see Figure 10) was cloned into M13 vector MP10, using the standard cloning and screening techniques previously described. The single stranded cDNA form containing the same sequence as the original PDGF-A$_L$ RNA, allowed

production of a probe which was complementary to the RNA. (Appendix A for plasmid details).

The primer was annealed to ss M13 DNA template by mixing 200 ng of DNA with 100 ng of primer in a total of 10 μl 1 x Klenow buffer (50 mM NaCl, 2 mM DTT, 6 mM MgCl$_2$ and 6 mM Tris-HCl pH 7.4) and warming to 60°C for 30 minutes. Extension was carried out in sterile siliconised eppendorfs by adding 5 μl of the anneal mix to 70 μCi of ^{32}P dTTP with 150 μM each of dATP, dCTP and dGTP up to a final volume of 30 μl in 1 x Klenow buffer including 5 units of Klenow and the reaction allowed to proceed for 2 hours at 20°C. The Klenow was then inactivated by incubating at 70°C for 10 minutes. The extension product was digested to the desired length by adding 10 μg of *E.coli* tRNA, 1 μl of 50 mM spermidine and 10 units of *EcoRI* and placing at 37°C overnight. The digested probe was then ethanol precipitated using 2 M NH$_4$AC and taken up in Sequencing buffer (95% Formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol). The probe was then separated from the M13 DNA and unwanted extension products on either a 5% Sequencing gel or a Bisacrylyl-cystamine Urea gel. After electrophoresis the probe was visualised by exposing the gel to pre-flashed X-OMAT AR diagnostic film (Kodak). The area of the gel containing the probe was then excised and the probe eluted as previously described. The probe produced by this method had two short regions of M13 DNA on either end consisting of the primer with intervening sequence and the sequence from the end of the PDGF insert to the EcoRI site respectively 35 bp and 30 bp making a total probe length of 609 bp. These sequences do not affect the final band lengths because they do not hybridise and are therefore subject to S1 nuclease digestion.
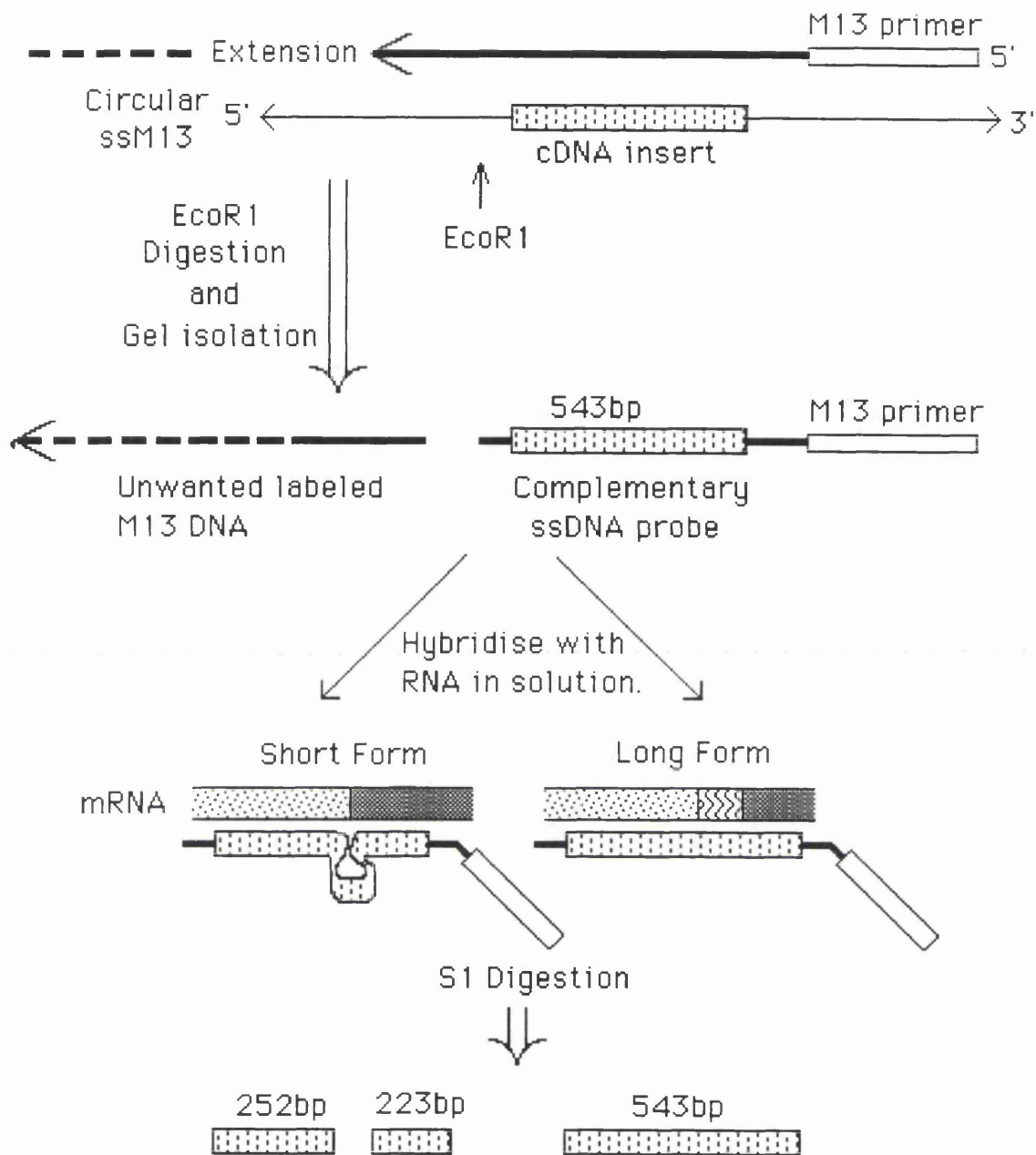
Figure 7.

Diagram showing the Primer Extension method for producing single stranded DNA probes for S1 nuclease analysis. After extension and restriction enzyme digestion the appropriate single stranded fragment is isolated from a denaturing gel, annealed to mRNA, digested with S1 nuclease and the products resolved on an acrylamide gel. Shading as in Figure 4.

ii) Single stranded M13 preparation.

Single stranded M13 DNA was prepared by growing bacteria infected with the required clone in liquid cultures and collecting 1 ml of supernatant after spinning out the cells. This was made 4% PEG-8000, 0.5 M NaCl and incubated for 10 minutes at room temperature before spinning down the precipitated M13 phage particles. All traces of the PEG solution were removed carefully and the pellet resuspended in 100 μl of TES buffer (20 mM Tris, 10 mM NaCl, 0.1 mM EDTA pH 7.4). This was phenol chloroform extracted and ethanol precipitated using 0.3 M NaAc pH 7.4. The final dried pellet was resuspended in 50 μl of TES buffer and used for primer extension or sequencing reactions.

iii) S1 nuclease protection.

The ^{32}P labeled single stranded (ss) probe and the RNA to be analysed were co-precipitated as follows: 5 μg of total RNA (or 500 ng of Poly A$^+$) was mixed with 5 μg of yeast total RNA and approximately 1-2 x 10^4 cpm of the purified ssDNA probe and ethanol precipitated using 0.3 M NaAc. The pellet was redisolved in 20 μl of Hybridisation buffer (80% deionised Formamide, 50 mM Pipes (Piperazine-N,N'-bis[2-ethane-sulfonic acid]; 1,4-piperazine diethane-sulfonic acid) pH 6.4, 500 mM NaCl, 1 mM EDTA). This drawn into a microcapillary tube which was sealed at both ends before incubating at 90°C for 15 minutes then immediately transfered to 52°C for 12 hours. After hybridisation the sample was transfered to 280 μl of ice cold S1 buffer (300 mM NaCl, 30 mM Na(AC)2 pH 4.6, 1 mM Zn2SO4 , 20 μg/ml Salmon sperm DNA) with 100 Units of S1 Nuclease and incubated at 37°C for 1 hour. After S1 digestion the sample was made 7 mM EDTA and 5 μg of *E.coli* tRNA was added prior to phenol/chloroform extraction. The aqueous phase was removed and the organic phase was re-extracted with 100 μl of TE buffer. The pooled aqueous phases were ethanol precipitated and resuspended in Sequencing buffer and run on a 5% sequencing gel. The protected bands were visualised by autoradiography as previously described.

2.3   Mammalian   cell   culture   methods.

All media, balanced salt solutions, trypsin (0.05% in Dulbecco's Modified Eagles Medium [DMEM]) and versene (0.02% in DMEM) solutions were obtained from the Imperial Cancer Research Fund, Clare Hall. Foetal calf serum (FCS) was obtained from Gibco-BRL. Disposable plastic culture-ware was obtained from Falcon Ltd. Cells were incubated in 5% CO_2 / 95% air atmosphere, with 100% humidity, at 37°C.

A)   Established   cell   lines.

3T3. A transformed rat fibroblast cell line obtained from ICRF.

C6. A rat glioma cell line derived from a chemically induced brain tumour {Benda *et al.*, 1968}.

157. A human glioma cell line isolated from autopsy material by Dr. M. Noble.

Cos-7. A monkey kidney cell line transformed with SV40 and expressing the large T antigen {Gluzman, 1981}.

All these cell were adherant and were grown in 75 cm^3 flasks in Dulbecco's Modified Eagle's Medium (DMEM) + 10% foetal calf serum (FCS). Cells were passaged by replacing the growth medium with 1:9 (v/v) Trypsin : Versene solution and incubating at 37°C until the cells had become detached from the surface of the flask. The cells were pelleted at 3,000 G for 3 minutes before resuspending in fresh medium and plating into a fresh flask.

B)   Storage   of   cell   lines.

Frozen stocks of cell lines were stored under liquid nitrogen in FCS + 10% dimethylsulphoxide at a density of 10^6/ml in round bottomed cryotubes.

C)   Primary   astrocyte   cultures.

Cultures of rat type 1 astrocytes from neonatal rat cerebral cortex were established by a modification of a previously described method {McCarthy and de Vellis, 1980}. Cerebral cortices from 2 day old rats (P2) were coarsely minced, digested with trypsin (0.025% w/v) for 30 minutes at 37°C, dissociated by trituration through a pasteur pipette, and cultured in DMEM + 10% FCS in Poly D Lysine treated 75 cm^3 tissue culture flasks (material from 3 brains to 1

flask), until the cells were confluent (3 - 5 days). Cells growing on top of the monolayer were removed by shaking the flask vigorously for 24 hour at 37°C (180 rotations/minute on a horizontal rotating platform with a 2 cm radius of rotation). The remaining cells were treated with cytosine arabinoside (araC, 10^{-5} M) for 24 hours to kill preferentially any rapidly dividing contaminating cells such as fibroblasts. The remaining astrocytes were passaged once (1 to 4) and grown to confluence before harvesting for RNA isolation.

D) PDGF-A expression vectors.

cDNAs encoding human PDGF-A$_S$ or PDGF-A$_L$ {Betsholtz *et al.*, 1986} were inserted as *SacII/EcoRI* fragments (See Chapter 3 Figure 10 for all restriction site locations) into a plasmid vector based on pHYK (obtained from H. Pelham), which contains the adenovirus serotype 2 major late promoter, splice donor and acceptor sites from the herpes virus type 1 thymidine kinase gene and the simian virus 40 origin of replication. A synthetic 44 mer double-stranded oligonucleotide encoding the antibody epitope (Tag) recognized by monoclonal 9E10 {Evan *et al.*, 1985}, followed by an *XhoI* site and a termination codon, was inserted into the *StuI* site unique to the PDGF-A$_L$ cDNA to generate PDGF-A$_S$ Tag (see Figure 8). This plasmid was converted to PDGF-A$_L$ Tag by cutting with XhoI, filling-in with Klenow and religating to shift the reading frame allowing translation of exon six encoding the basic tail exclusive to PDGF-A$_L$ (see Figure 8). (See appendix A for plasmid details). Transient expression of the proteins encoded by these vectors was achieved by transfecting Cos-7 cells as follows.

Figure 8.

Diagrams showing the 3' DNA sequence and respective carboxy terminal differences (single letter protein code) between each of the pHYK expression constructs used to produce both wild type and MYC tagged versions of human PDGF-A chain. (See Appendix)

(PDGF-A$_S$) Shows the final 2 codons common to all forms and the 3 codons which encode the short form terminus and includes an indication of the alternative splice junction.

(PDGF-A$_L$) Shows the same 2 common codons followed by the 18 codons encoding the mostly basic long form tail and includes an indication of the StuI restriction site used to create PDGF-A$_S$ TAG.

(PDGF-A$_S$ TAG) Shows the 44 bp sequence inserted into the StuI site, reforming the site in the correct orientation, coding for the MYC epitope and stopping translation of the long form tail making PDGF-A$_S$ TAG effectively short form. The XhoI site used to make PDGF-A$_L$ TAG is also shown.

(PDGF-A$_L$ TAG) Shows the result of digesting PDGF-A$_S$ TAG with XhoI, filling in the overhanging ends and religating to give PDGF-A$_L$ TAG in which the stop codon before the long form tail coding sequence is abolished allowing read through to produce of a tagged long form polypeptide.

PDGF-As

Splice Junction

```
DNA          GAC ACG G|AT GTG AGG TGA GGA TGA
Amino Acids  D   T   D    V   R   End
```

PDGF-A_L

StuI AGG/CCT blunt end cutter.

```
DNA          GAC ACG G|GA AGG CCT AGG GAG TCA GGT AAA AAA CGG AAA AGA AAA AGG TTA AAA CCC ACC TAA AGC
Amino Acids  D   T   G   R   P   R   E   S   G   K   K   R   K   R   K   R   L   K   P   T   End
```

PDGF-As TAG

---> MYC TAG   XhoI C/TCGAG SITE

```
DNA          GAC ACG G|GA AGG CCT GAG CAA AAG CTC ATT TCT GAA GAG GAC TTG CTC GAG TAG CC
Amino Acids  D   T   G   R   P   E   Q   K   L   I   S   E   E   D   L   L   E   End
```

PDGF-A_L TAG

tail

---> MYC TAG   Fill in to read through to Basic

```
                                                              /- -\              --->
DNA          GAC ACG G|GA AGG CCT GAG CAA AAG CTC ATT TCT GAA GAG GAC TTG CTC GAT CGA GTA GCC CCT AGG GAG
Amino Acids  D   T   G   R   P   E   Q   K   L   I   S   E   E   D   L   L   D   R   L   S   P   R   E
```

E) Cos cell transfection.

Transfections were carried out on 70% confluent Cos-7 cells in 60 mm dishes. Cells were washed 2 x with DMEM. 3 μg of the DNA to be transfected in 500 μl of Diethylaminoethyl (DEAE) Dextran at 1 mg/ml in TBS (50 mM Tris base, 150 mM NaCl pH 8.0) was mixed with 1.5 ml of DMEM and added to the cells and incubated at 37°C for 30 minutes. The supernatant was removed leaving the DNA on the cells. 5 ml of DMEM + 85 μg/ml of chloroquine (Disodium 7-chloro-4- [4diethylamino-1-methyl butyl-amino]-quinoline) dissolved at 10 mg/ml in DMEM was added to the cells and incubated at 37°C for 3 hours. This was replaced with 4 ml DMEM + 10% FCS and left for 24 hours at 37°C. Cos cells to be tested for mitogen production or processed for immunostaining were left in DMEM + 10% FCS for 24 hours, to recover from the transfection and then maintained in DMEM alone for a further 24 - 48 hours.

F) ^{35}S labelling of Cos cell proteins.

Prior to incubating in ^{35}S cysteine (>1000 Ci/mmol) the cells were gently washed 2 x in cysteine free DMEM. The cells were then incubated for 24-48 hours at 37°C in 2 ml of cysteine free DMEM +10% Normal DMEM containing 50 μCi/ml ^{35}S Cysteine. The supernatant, containing mainly secreted proteins, was removed for analysis. Cell debris was removed by centrifugation and the supernatant stored at 4°C with 2 mM PMSF (Phenylmethyl-sulfonyl fluoride stock at 100 mM in EtOH) and 0.02% Sodium Azide to prevent protease activity and bacterial growth. The adhering cells and associated matrix were lysed and extracted, where necessary, according to the following protocols.

G) Pulse chase ^{35}S labelling.

Pulse chase experiments involved modifications of the above labelling protocol. Firstly at least 4 dishes of Cos cells were transfected with each construct to be analysed. After washing the cells 2 x with cysteine free medium 2 ml of cysteine free DMEM (without 10% Normal DMEM) + 25 μCi/ml ^{35}S were added and left in contact with the cells for 20 minutes only. A zero time point was taken by collecting the medium from one of the dishes for each type of construct and keeping at 4°C until immunoprecipitation with the rest later. The cells of all the dishes were then washed 2 x with cysteine free DMEM

and the zero time point dish cells were either lysed and/or salt extracted (See next section). The later time point cells were once more covered in normal DMEM + 10% FCS and left for different lengths of time before collecting their supernatants, washing them as above and extracting them in the same way as the zero time point. The samples produced were store as described above.

H) Cell lysis and salt extractions.

Where analysis of the contents of cells was required; they were lysed with one of two buffers according to desired effect. Hypertonic lysis buffer (0.8 M NaCl, 10 mM Tris pH 7.5, 0.5% NP40, 1 mM EDTA) was used if simultaneous lysis and salt extraction were intended. Isotonic lysis buffer (150 mM NaCl, 10 mM Tris pH 7.5, 0.5% NP40, 1 mM EDTA) was used when subsequent salt extraction of the cells and/or matrix left on plate was to be performed separately. Hypertonic buffer (without the 0.5% NP40) was then used to perform the salt extractions of matrix and/or cell debris as required. All extraction and lysis was done at 4°C for at least 15 minutes. The supernatants produced were stored as described above.

2.4 Protein and immunological methods.

All the primary immunological work was done using antibody 9E10 isolated from a mouse immunised with a synthetic peptide immunogen with sequence derived from that of the human c-*myc* gene product {Evan *et al.*, 1985}. This antibody does not recognise native c-myc and is therefore ideal for immunoprecipitation and immunofluorescence of proteins engineered to contain the peptide epitope. The expression vectors described above (see Figure 8) allowed production of human PDGF-A homodimers tagged with this myc epitope which could then be studied with this antibody.

A) Immunoprecipitation.

Cell supernatants and lysates to be immuno-precipitated were adjusted to a uniform salt concentration by dialysis against 150 mM NaCl, 10 mM Tris pH 7.0 in presoaked dialysis tubing (Specta/POR™ m.w.t cut off 6,000-8,000). Samples were precleared of nonspecific proteins by adding 2 µl of Normal Hamster Serum and mixing by rotation for 1 hour at 4°C. 50 µl of Immunoprecipitin (Gibco BRL.) was added and mixed as before for 30 minutes before being pelleted (10,000 G, 5 minutes). The PDGF was then

immunoprecipitated from the supernatant by adding 9E10 antibody at 1 µl/ml and mixing as before. 9E10 antibodies with associated Myc-tagged PDGF were immunoprecipitated by conjugation with Immunoprecipitin as above. The pellet was washed 4 x in 400 µl Immunoprecipitation buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA. 0.05% NP40) and 1 x in 400 µl TE. The pellet was resuspended in 20 µl of protein sample buffer (2% SDS, 10% Glycerol, 0.05% bromophenol blue, 62.5 mM Tris pH 6.8), boiled for three minutes and the Immunoprecipitin pelleted (10,000 g, 5 minutes) leaving the proteins in solution prior to electrophoresis. Where proteins were to be reduced the sample buffer used contained 5% 2-ME. Samples not used straight after boiling were stored frozen at -20°C.

B) SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed as previously described {Laemmli, 1970} using either the Gibco-BRL large vertical gel system or the Biorad mini gel system. The proteins were electrophoresed through a stacking gel of 5% acrylamide, at 100 Volts, into a 15% acrylamide resolving gel and then at 200 Volts until the bromophenol blue dye had reached the bottom of the gel. Radiolabeled and/or prestained molecular weight markers were run alongside the test samples to allow identification of the protein band being studied.

10 ml of resolving gel (375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.05% bis-acrylamide, 15% acrylamide) was polymerised using 100 µl of 10% Ammonium Persulphate and 8 µl of TEMED This was overlaid with water saturated iso-butanol until set then 2.5 ml of stacking gel (125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.13% bis-acrylamide, 5% acrylamide) was polymerised by 25 µl of Ammonium Persulphate and 3 µl of TEMED with the well former in place. The gel was electrophoresed in 1 x running buffer (25 mM Tris-base pH 8.3, 192 mM glycine, 0.1% SDS).

C) Gel fluorography.

[35]S labeled proteins which had been electrophoresed were visualised by fluorography as described previously {Bonner and Laskey, 1974}. The gels were washed in 20 x gel volume of dimethylsulphoxide (DMSO) for 30 minutes followed by 1 hour in 4 x gel volume of 20% (w/v) 2,5,Diphenyloxazole (PPO) in DMSO. The PPO was then precipitated within the gel by washing in dH_2O for 15

minutes and finally the gel was dried onto 3MM paper (Whatman) and exposed to pre-flashed X-OMAT AR diagnostic film (Kodak) at -70 $^\circ$C.

D)   Immunofluorescence.

i)   Fixing   cells.

Transfected Cos cells on glass coverslips were washed 3 x in PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) and fixed in 4% formaldehyde (in PBS) for 10 minutes. The cells were washed a further 3 x in PBS and permeabilised by incubating in 0.5% NP40 (in PBS) for 10 minutes and finally washed 3 x in PBS. For surface staining the permeabilisation step was missed out leaving the outer membrane intact.

ii)   Antibody   staining.

Staining was achieved by indirect immunofluorescence. The coverslips were incubated in 50 µl of the first layer of 9E10 antibody diluted 1: 600 in MEM+H +10% FCS for 20 minutes at 20°C. The cells were then washed 3 x in MEM+H and incubated in 50 µl of Rhodamine (RITC) conjugated rabbit anti mouse antibody from Amersham (1:100 dilution in MEM+H +10% FCS) for 20 minutes at 20°C. The coverslips washed 3 x in MEM+H and mounted with Citifluor$^\circledR$ mountant. Staining was visualised using a fluorescent microscope.

E)   Heparin   binding   and   elution.

100 µl (swollen volume) batches of heparin-Sepharose CL-6B and Sepharose CL-6B were equilibrated in DMEM. Radiolabeled supernatants from transfected Cos cells were split into thirds; one third was applied to Sepharose, one third to heparin-Sepharose and one third was kept aside as a control. These were incubated for 2.5 hours at room temperature on a rotating catherine wheel, after which the solid substrate and supernatant were separated by gentle centrifugation. The Sepharose and heparin-Sepharose were washed 9 x with 1 ml of DMEM (90 column volumes) and the final wash kept for analysis. They were then washed with progressively higher salt concentrations (10 washes of 1 ml) collecting the first and last washes at each concentration for analysis. The samples were immunoprecipitated, as previously described, and subjected to SDS-PAGE.

F) ^{3}H-thymidine incorporation into 3T3 cells.

i) Measuring soluble mitogenic activity.

Soluble mitogenic activity was measured using a modification of a previously described method {Gospodarowicz *et al.*, 1978}. Confluent monolayers of NIH 3T3 cells growing in 96-well tissue culture plates were made quiescent by incubating overnight in DMEM containing 0.4% FCS. Cos cell supernatants were added at a range of dilutions in serum free DMEM in triplicate. These were incubated overnight at 37°C for cells to respond. Then 1 μCi of ^{3}H-Thymidine (Amersham, 25 Ci/mmol) was added to each well and the cells incubated for a further 4 hours at 37°C. The incorporated ^{3}H was precipitated by replacing the DMEM with 100 μl of 5% TCA at 4°C and the plates kept on ice for 10 minutes. After a further 2 washes with TCA the plates were dried at 37°C for 10 minutes. The precipitated ^{3}H-Thymidine was then solublilised in 100 μl of 0.5 N NaOH at 37°C for 30 minutes and added to 2 ml of scintilant (Aquasol: New England Nuclear). The samples were counted in an LKB liquid scintilation counter.

ii) Measuring matrix associated mitogenic activity.

Mitogenic activity bound to matrix was assayed by seeding quiescent 3T3 cells (approximately 5 x 10^4 / well of a 24 well tissue culture plate) onto matrix from which the cells had been removed and leaving them for 24 hours before TCA precipitating and scintilation counting triplicates as above.

G) Matrix preparation.

I prepared matrix using an adaptation {Lillien *et al.*, 1990} of a standard method {Vlodavsky *et al.*, 1987a; Bashkin *et al.*, 1989}. Cos cells transfected with various constructs directing expression of different forms of PDGF-A chain were allowed to secrete their respective proteins for 24 hours. Cells were then removed, leaving intact matrix, by incubating in NH$_3$ lysis Buffer (20 mM NH$_3$ in dH$_2$O) for 10 minutes at room temperature x 2. The matrix was then washed twice with 0.15 M NaCl, 2 mM Na$_{(2)}$ H$_{(2)}$ PO$_4$ pH 7 for 15 minutes at room temperature followed by 4 x with DMEM for 5 minutes. The matrix was then ready for the 3T3 cells to be seeded onto it.

☿ Chapter Three ☿

"One fine morning in May a slim young horsewoman might
have been seen riding a black sorrel mare along the
flowery avenues of the Bois de Boulogne."

Chapter 3. Analysis of the distribution of PDGF-A chain mRNA alternative splicing isoforms.

3.1 Introduction.

The first cDNA clones for both the long and short forms of PDGF-A chain were isolated from a human glioma cell line {Betsholtz et al., 1986}. However subsequent screening of cDNA libraries made from normal human umbilical vein endothelial (HUVE) cells isolated only the short form cDNA {Tong et al., 1987; Collins et al., 1987} giving rise to speculation that the long form was a tumor-specific or a glial-specific form. Cloning of the genomic sequence confirmed that these transcripts were produced by alternative splicing of a single primary transcript {Bonthron et al., 1988; Rorsman et al., 1988}. It therefore seemed reasonable to expect that there would be a functional difference between the two different forms of PDGF-A chain encoded by these differentially spliced mRNAs. An understanding of the spatial and temporal distribution of the two transcripts in different tissues and stages of development might provide some useful clues as to what this functional difference might be.

Northern blot analysis of the PDGF-A chain gene transcripts in a variety of both normal and transformed human cell lines indicated the production of 3 major transcripts at 1.9, 2.3 and 2.8 kb {Betsholtz et al., 1986}. A similar pattern of transcripts was also observed in RNA isolated from 3 day old rat brain {Richardson et al., 1988}. That this heterogeneity was likely to reflect the use of alternative Poly (A) sites was suggested by primer extension analysis which indicated a single transcription start site and sequence analysis of genomic clones which indicated the presence of near-consensus Poly (A) signals at appropriate sites in the 3' untranslated region of the gene {Bonthron et al., 1988}. These differences are unlikely to be associated with the differential splicing since this only dictates the presence or absence of the 69 bp exon 6 which would not be resolved via northern blot analysis.

In an attempt to investigate the pattern of expression of these two transcripts I employed two of the most sensitive detection procedures; S1 nuclease analysis and PCR, to examine RNA from rat primary astro-glial cells (Chapter 2 section 2.3 C) and whole rat brain both of which are known to express PDGF-A chain mRNA {Richardson et al., 1988}.

3.2 S1 nuclease analysis.

At carefully controlled temperatures and in high ionic strength buffers the enzyme S1 nuclease degrades single-stranded DNA and RNA but not double-stranded nucleic acids. S1 nuclease analysis of differential splicing requires a labeled probe of defined length containing sequence complementary to the RNA of interest, spanning the alternately spliced region (Chapter 2 Figure 7). Unlike Northern analysis of RNA which has been blotted and bound to a fixed membrane, both of which provide opportunity for target RNA loss or masking, S1 analysis depends on mixing of excess probe with untreated RNA in solution so that all the target sequence is hybridised. Two possible double stranded products result depending on whether the target RNA has the intervening exon or not. The probe either hybridises completely along its length preventing subsequent degradation by S1 nuclease or it hybridises to the remaining exon(s) leaving a single-stranded region, corresponding to the missing exon, which can be digested by the nuclease to produce a shorter product (Chapter 2 Figure 7). The labeled products once resolved on a denaturing polyacrylamide gel and visualised by autoradiography allow an accurate estimation of the relative abundance of the two RNA species according to the band intensities.

A 543 bp single-stranded probe complementary to the nucleotide sequence 716 to 1259 of human PDGF-A$_L$ mRNA (Figure 10) was hybridised in solution to Poly (A)$^+$ RNA from rat primary astrocytes and as a control to Poly (A)$^+$ RNA from human glioma cell line 157 which has also been shown to express high levels of PDGF-A chain mRNA {Richardson et $al.$, 1988}. After S1 Nuclease treatment the products were resolved on a 5% polyacrylamide sequencing gel (Figure 9). The expected products were either a single 543 bp band protected by the exon 6-containing long form or two bands of 252 and 223 bp respectively protected by the remaining exons 5 and 7 of the short form, or a mixture in proportion to the levels of each mRNA species present.

The observed results were quite different: the control reaction with the 157 RNA produced three bands at approximately 210, 170 and 90 bp two of which were much smaller than any of the predicted bands (Figure 9, Lane 3). However the combined length of the protected fragments was approximately 470 bp which was very close to the 475 bp sum of the two bands expected from an S1 reaction with the short form mRNA. This suggested that one of the two

expected bands, probably the longer exon 5 protected band, had been subjected to artefactual S1 attack. There is evidence to show that A:T rich regions of hybrids are very sensitive to S1 attack even when fully complementary {Miller and Sollner-Webb, 1981}. A close examination of the sequence contained within the probe revealed just such an A:T rich region in exon 5 around 90 bp from the alternative splice junction (Broken line box : Figure 10). It seemed likely that this unexpected banding pattern resulted from aberrant S1 nuclease digestion in this region. If this explanation is correct, the result suggests that the 157 glioma cell line produces exclusively PDGF-A$_S$ mRNA.

The S1 analysis of the rat primary astrocyte RNA gave no discernible bands (Figure 9, Lane 2) indicating that the S1 nuclease had effectively digested the probe to completion. This would be the expected result in the absence of any suitable target RNA to protect the probe, however rat astrocytes have been shown to express PDGF-A chain mRNA {Richardson et al., 1988}. It is known that S1 nuclease can attack small mismatches in otherwise perfectly complementary hybrids {Shenk et al., 1975}. In this case where the S1 probe was derived from human and the target RNA from rat, it is likely that there would have been a number of mismatches, this could explain the absence of detectable protected fragments (See Chapter 4 Figure 25 for sequence comparison).

The problems inherent in S1 analysis, illustrated by this experiment, make it clear that in order to use this strategy to examine the profile of PDGF-A chain mRNAs produced by rat cells the probe must be derived from the rat sequence. To this end I set about cloning the cDNA for rat PDGF-A chain as detailed in chapter 4 in the hope that I might then perform S1 nuclease analysis with a fully complementary probe. While in the process of cloning I tried another approach based on PCR as follows.

3.3 PCR analysis of mRNA splicing : the theory.

PCR is an *in vitro* method for producing large amounts of a specific DNA fragment of defined length and sequence from smaller amounts of a complex template {Saiki et al., 1985; Mullis and Faloona 1987; White et al., 1989 for review}. This enzymatic amplification requires two oligonucleotide primers that hybridise to opposite strands of the target sequence oriented with their 3'

ends nearest each other. In principle, repeated cycles of heat denaturation of the template, annealing of the primers and primer extension by a DNA polymerase result in amplification of the segment defined by the 5' ends of the primers. The extension product of each primer can serve as a template for the other primer during the next cycle, resulting in an exponential accumulation of the desired fragment.

Originally used to amplify genomic DNA, PCR provided a powerful tool for detecting and analysing sequences which would otherwise have been difficult to study. Using a protocol (Chapter 2.2 R) based on early papers describing PCR on cDNA {Todd *et al.*, 1987; Veres *et al.*, 1987} I extended its use to the analysis of gene expression and differential splicing by converting the cellular RNA to cDNA and amplifying across the alternative splice site (Chapter 2 Figure 6). This had the added perceived advantage of allowing discrimination between mRNA and unspliced RNA and/or contaminating genomic DNA which would produce much larger amplification products containing intron sequence. I carried out my initial experiments using the Klenow fragment of E.coli DNA polymerase I, which is inactivated by heating to 95 °C necessitating the addition of fresh enzyme after each denaturation step. However the experiments described in this chapter all used the thermostable *Taq* DNA polymerase avoiding the need for fresh enzyme each cycle {Saiki *et al.*, 1988}. Although the use of *Taq* allows temperature cycling automation (Chapter 2 Section 2.2) all the experiments detailed in this chapter used waterbaths for each temperature whereas all those in chapter 4 were performed with the aid of a Cambio "intelligent heating block".

3.4 PCR analysis of PDGF-A chain mRNA.

Poly (A)$^+$ RNAs from rat primary astrocytes and human 157 glioma cells were reverse transcribed to produce 1st strand cDNA and subjected to PCR amplification using oligonucleotide primers complementary to regions of human PDGF-A chain exons 5 and 6 as described above (also in Chapter 2 Figure 6). Evidence that Taq DNA polymerase could also function as a reverse transcriptase {Jones and Foulkes, 1989} led me to attempt amplification of 157 human glioma cell Poly (A)$^+$ RNA that had not been reverse transcribed to cDNA. PCR was also performed on human PDGF plasmid DNAs with different combinations of primers to check enzyme activity and produce size markers.

The amplification of the 157 cDNA produced what seemed to be two bands (Figure 11 Lane 5). However a band the same size as the lower of these two also appeared in the lanes beneath the products of two different control reactions (Figure 11 Lanes 2 and 3) indicating that these bands were unlikely to represent a specific reaction product. The remaining 157 band, though slightly smeared, ran at approximately the position expected for the short form PCR product. This corresponded to the S1 nuclease result that human glioma cell line 157 produces only short form RNA. The amplification of the rat astrocyte cDNA (Figure 11 Lane 6) produced only the non specific band observed in lanes 2, 3 and 5 which was brighter here than in the other lanes suggesting that it consisted of the unincorporated primers. This failure to prime amplification of the rat cDNA by human sequence primers raised the possibility that primers which were not 100% complementary would be unable to allow PCR amplification. The 157 RNA amplification reaction (Figure 11 Lane 7) did not produce any bands indicating no reverse transcription by *Taq*.

In an attempt to define conditions in which these primers would work on the rat cDNA I repeated the amplification using a less stringent annealing temperature (Figure 12 a). As previously human PDGF-B chain specific primers were used to amplify from the PDGF-B cDNA plasmid {Josephs *et al.*, 1984} both as a positive control for enzyme activity and to produce a size marker. In this instance B chain specific primers were also used to amplify 1st strand cDNAs synthesised from the rat and human Poly (A)$^+$ RNAs to establish whether different primers were better able to direct amplification from the cDNAs in question (Figure 12 Lanes 2 and 3). The resulting amplification products of both types of cDNA with A and B chain specific primers were smears rather than specific bands suggesting that the lower anneal temperature had indeed permitted priming, though mostly non specific. The gel was blotted and probed for PDGF-A chain to discover whether any specific bands could be identified from within the smear.

Probe specificity was indicated by lack of hybridisation to the PDGF-B chain PCR product (Figure 12 b Lane 1) and strong hybridisation to the PDGF-A chain product (Figure 12 b Lane 4). Despite poor fragment separation the autorad shows that the human glioma cDNA sample gave a specific band lower than the PDGF-A$_L$ marker band once more suggesting amplification of the PDGF-A$_S$ RNA (Figure 12 b Lane 5). The rat astrocyte cDNA, on the other hand,

gave a band of the size expected for PDGF-A$_L$ suggesting amplification of the PDGF-A$_L$ sequence (Figure 12 b Lane 6) as did the human glioma RNA sample which had not been converted to cDNA (Figure 12 b Lane 8).

This latter result, in direct contradiction to the result of the previous experiment (Figure 11), seemed to indicate that the *Taq* polymerase had reverse transcribed the RNA. However, if this were the case the product should have been the same size as that produced from the genuine cDNA of the same RNA (Figure 12 b Lane 5). In view of previous results showing no amplification of RNA that had not been reverse-transcribed into cDNA (e.g. Figure 11), it seems likely that the PCR product in Figure 12 Lane 8 was a result of PCR cross contamination by the PDGF-A$_L$ cDNA plasmid used for the positive control (Figure 12 Lane 4). This is now recognised as a common problem associated with PCR (Discussed later section 3.7b). This raised doubts about the rat astrocyte cDNA result which also seemed to show amplification of the long form. However the 157 cDNA results indicating short form amplification in both PCR experiments were consistent with the S1 nuclease result, suggesting that they represented amplification from cDNA rather than plasmid DNA.

3.5 Attempts to circumvent contamination of PCR reactions with amplifiable DNA.

To test if the above results were caused by amplification of contaminating DNA, I treated my Poly (A)$^+$ RNA samples with RNase-free DNase I before subjecting them to PCR amplification. 1 μg Human 157 glioma Poly (A)$^+$ RNA was treated with 5 mg of DNase I and 20 units of RNasin (RNase inhibitor) in a total volume of 60 μl of 1 x PCR buffer for 30 minutes at 37°C. The enzymes were heat inactivated at 90°C for 10 minutes and the RNA subjected to 40 cycles of standard PCR in parallel with untreated RNA (Figure 13 Lanes 1 and 2). The same experiment was carried out on RNA to which a known quantity of human PDGF-A$_L$ cDNA plasmid had been added (Figure 13 Lanes 3 and 4).

The 157 RNA amplification product, run on a higher percentage gel than previously, gave a well resolved band representing short form (Figure 13 Lane 1) as opposed to the 157 RNA long form amplification product indicated previously (Figure 12 Lane 8) (See Section 3.7 b for discussion). The DNase treated RNA gave the same band only much less intense (Figure 13 Lane 2).

The DNase had therefore removed some but not all the sequence being amplified confirming that some of the original target was DNA though not ruling out the possibility of RNA based amplification. The RNA with added plasmid produced the expected long form derived band (Figure 13 Lane 3) while the DNase treatment mirrored the above result by reducing but not eliminating the long form amplification product (Figure 13 Lane 4). This indicated that DNase I treatment of this kind was not sufficient to clear all the added DNA, suggesting that the band in Figure 13 Lane 2 may after-all have been derived entirely from contaminating DNA and not via reverse transcription by *Taq*. This result also showed that addition of a specific contaminant commandeers the amplification reaction, biasing the final outcome to represent only the abundant target. This is likely to be due to the much greater levels of the added long form relative to any contaminating short form (See Chapter 3.7 b).

To discover if a longer period of DNase treatment would be able to remove all contaminating DNA completely, I performed a time course experiment. Plasmid DNA was exposed to various periods of DNase treatment as above after which half of each sample was run on an agarose gel and EtBr stained to visualise any remaining DNA. The other half of each sample was subjected to 30 cycles of PCR amplification, the products run on a gel and visualised by EtBr staining. Of the unamplified samples only the untreated plasmid and that treated with heat inactivated DNase I remained visible when run directly on an agarose gel (Data not shown). The PCR amplification results, however, indicated that DNase I digestion seemed to have been complete after only 20 minutes and at 60 minutes but surprisingly failed to destroy all the target in both the 40 and 120 minute reactions (Figure 14). It is not clear what the explanation for this result might be, however, combined with the previous result (Figure 13) it lead to the conclusion that DNase I would not be a reliable antidote for contamination even using longer incubation times.

3.6 Contamination II : the final conflict.

Having established that my original RNA samples were contaminated with DNA, I replaced them with newly prepared samples in the hope that these would be free from DNA. PCR was performed on Poly (A)$^+$ RNA from both human 157 cells and rat P2 brain, with two different combinations of primers (Figure 15 Lanes 1, 3 and 6) in parallel with total 157 RNA (Figure 15 Lane 2). I

also amplified freshly made 157 genomic DNA with the same primers to define what, if any, contribution genomic DNA might make to contamination and with control primers K1 and K2 which span a 325 bp genomic fragment (Figure 15 lanes 4 and 5). Amplification of the rat astrocyte Poly $(A)^+$ RNA sample used in all the previous experiments was also carried out (Figure 15 Lane 7) for comparison.

All amplifications done with primers 1 and 2 gave the same three reaction products, although their relative abundance varied between reactions. The lower 2 bands in each lane corresponded in size to the expected long and short form of human PDGF-A chain cDNA products. However the third and highest band seemed to correspond to a PDGF-A form not previously identified. The uniformity and clarity of this pattern across human total RNA, Poly $(A)^+$ RNA and genomic DNA as well as rat RNAs indicated that it was likely to be due to plasmid contamination. A survey of possible contaminants suggested PDGF-A$_S$ Tag (Chapter 2 Table 7 and Figure 8) which has a 44 bp insert in the alternatively spiced exon 6 giving an expected PCR product of 307 bp with primers 1 and 2. This is close to the size of the third mystery band suggesting that this product was derived from PDGF-A$_S$ Tag. Although I was not using PDGF-A$_S$ Tag at that time it was being grown up by others in the laboratory in milligram quantities, as indeed were both PDGF-A$_L$ and PDGF-A$_S$ cDNAs. This experiment strongly suggested that indirect contamination, possibly via aerosols, may have taken place. The single band produced by the primer 6 and 7 amplification (Figure 15 Lane 3) suggested that much of the contamination was present in the solutions specific to the other PCR i.e. one of the two primers 1 and 2.

Using fresh aliquots of all solutions I attempted to amplify cDNA made from newly isolated human 157 glioma Poly $(A)^+$ RNA using both PDGF-A chain specific priming and oligo (dT) priming (Figure 16 Lanes 4 and 5 respectively). As a positive control I amplified the short form cDNA sequence while as a negative control I amplified both in the absence of any added nucleic acid and in the presence of Oligo (dT). I also amplified 157 Poly $(A)^+$ RNA (Figure 16 Lane 3) to check for any reverse transcriptase activity alongside good negative controls.

The result showed no sign of contamination in any of the negative controls and no sign of reverse transcription by *Taq* (Figure 16 Lane 3). The

amplification of the cDNA made using the PDGF-A specific primer 2 gave a band representing PDGF-A$_S$. The cDNA made by priming with oligo (dT) gave the same PDGF-A$_S$ band though more abundant probably because of more successful cDNA production. The lack of contamination in any of the negative controls engenders confidence in the result, that human 157 glioma cells express only PDGF-A$_S$. This therefore is further confirmation of the previous conclusion from S1 analysis and sequence analysis of cDNA clones.

Having isolated and removed the contaminated reagents I proceeded to amplify from rat cDNAs once again. These experiments, however, continued to show evidence of sporadic contamination. I therefore decided to concentrate on cloning the rat PDGF-A chain cDNA by a conventional library screening approach (See Chapter 4) in the hope that I would then be able to design rat-specific PCR primers which would not be affected by human PDGF-A chain contamination.

3.7   Summary

In this chapter I have described my attempts to analyse rat PDGF-A chain mRNA expression and differential splicing using the human cDNA sequence in two ways; as an S1 nuclease probe and via oligonucleotides designed from the human sequence in a PCR-based strategy.

A) The S1 lessons.

In retrospect this strategy appears to have been somewhat over-optimistic. The sequence chosen as a probe included a region prone to artefactual S1 nuclease digestion thus confusing the interpretation of the positive control result with fully complementary human RNA. The experimental analysis of the rat primary astrocyte RNA resulted in complete digestion of the probe, probably as a result of sequence mismatches (See Chapter 4 Figure 25). These results demonstrated the importance of using a fully complementary S1 probe carefully chosen to avoid sequences prone to artefactual digestion. Analysis of the human cDNA sequence reveals that the alternatively spliced exon 6 is very A:T rich. Since this is exactly the type of sequence which allows artefactual S1 attack (Miller & Sollner-Webb, 1981) any future S1 strategy would require a probe which did not include this region. Indeed a study published subsequently {Young *et al.*, 1990} used a 90 nt region

of mouse PDGF-A chain cDNA clone, including only 10 nucleotides from the 3'
end of exon 6, for S1 analysis of mouse RNA, thus avoiding both the problems I
encountered here (Figure 41).

B) The PCR lessons.

I considered the effect of sequence mismatches on the ability of primers
to direct amplification by PCR to be a potential problem at the start of this set
of experiments. For this reason I chose to make several different primers in
the hope that some of them, at least, would anneal sufficiently to allow
priming to occur. In the event the initial amplification reaction (Figure 11)
indicated primers 1 and 2 would amplify the human but not the rat sequence
under the conditions used. Altering the conditions appeared to indicate that
they were able to work at a lower stringency, however this experiment also
displayed the first signs of what was to become an insurmountable barrier to
continuing with this strategy: contamination.

A control, designed to show whether Taq polymerase would work as a
reverse transcriptase, produced an amplification product which seemed at
first to indicate that Taq could indeed convert RNA to cDNA. I did not consider
this surprising since it is known that E.coli DNA Polymerase I has reverse
transcriptase activity in vitro while reverse transcriptase can act as a DNA
based polymerase {Sambrook et al., 1989}· Furthermore only a very low level of
reverse transcriptase activity would be required because of the subsequent
amplification of any cDNA produced. However close analysis of this result
indicated that the band in question was more likely to be derived from
amplification of plasmid DNA. I then used DNase I to show that DNA
contamination of my RNA was partly if not entirely responsible for this
amplification product. Unfortunately, DNase I treatment, although able to
remove some of the DNA, proved unreliable at removing contamination
completely (Figure 14).

The DNase experiment (Figure 13) also indicated that preferential
amplification of target DNA may occur. The RNA sample alone when amplified
gave a band representing the short-form product; however, when long-form
plasmid DNA was added to the RNA only long-form product was observed after
amplification. Although it is possible that further rounds of amplification
would have produced the short-form product, it is clear that it would have

been unwise to infer that only one form was present in the original sample. A further indication of this effect was observed in the adjacent lanes where the DNase treated RNA sample shows one major and two approximately equivalent minor bands (later seen more clearly in Figure 15). In the untreated sample, amplification of the major contaminant, the short form, usurped the reaction so that one of the minor contaminants was not represented in the resulting product. It would appear that DNase treatment having reduced the overall levels of contamination allowed the minor species a chance to be amplified. Such an indication that potential target DNAs compete for amplification has a bearing on future analysis by PCRs which depend on co-amplification of two products with the same primers. It would be wise to consider strategies where alternative products could be detected with unique primers. In this case using a primer within the exon 6 would avoid this competition and therefore present a fairer estimate of the relative levels of each form.

The progression of contamination through these experiments illustrates what has since come to be termed "carry-over contamination" {Longo *et al.*, 1990; Shuldiner *et al.*, 1990}. Carry-over contamination from previous PCRs is due both to the abundance of PCR products, and to the ideal structure of the material for re-amplification such that trace amounts of these products easily yield false positive results. At the outset of these experiments the use of PCR was in its infancy and it was considered by many to be the panacea for all situations where it was difficult to study DNA; its use in studying RNA was even less well established. The potential for very small amounts of contaminating DNA to cause false positive results was certainly not fully appreciated. For my first experiment (Figure 11) I included as a positive control for enzyme activity the PDGF-A_L cDNA plasmid. By my second experiment my samples appeared to have been contaminated with this same DNA (Figure 12). The third experiment detailed here (Figure 13) indicated that my samples were contaminated with the short form cDNA sequence which I had used in the previous experiment (not shown here). The full range of contamination was completed by Figure 15 and included a sequence which I had not been directly associated with but which was present in abundance in the laboratory indicating that airborne contamination may also occur.

The drastic effects of contamination on amplification and the inability to clean it up once it had occured coupled with the apparent ease with which it

could occur led me to propose a number of improvements to the PCR protocol (Chapter 2. 2 R iii - Contamination avoidance). Many of these are now standard procedure for PCR workers {Kwok and Higuchi, 1989; Krone *et al.*, 1990; Sarkar and Sommer, 1990; Cimino *et al.*, 1990}. However the continuing opportunity for further contamination by any of a number of constructs being used, both by myself and others, in the laboratory and the unresolved uncertainty over the sequence homology of the primers convinced me to wait until I had cloned the rat cDNA sequence before I undertook further PCR analysis.
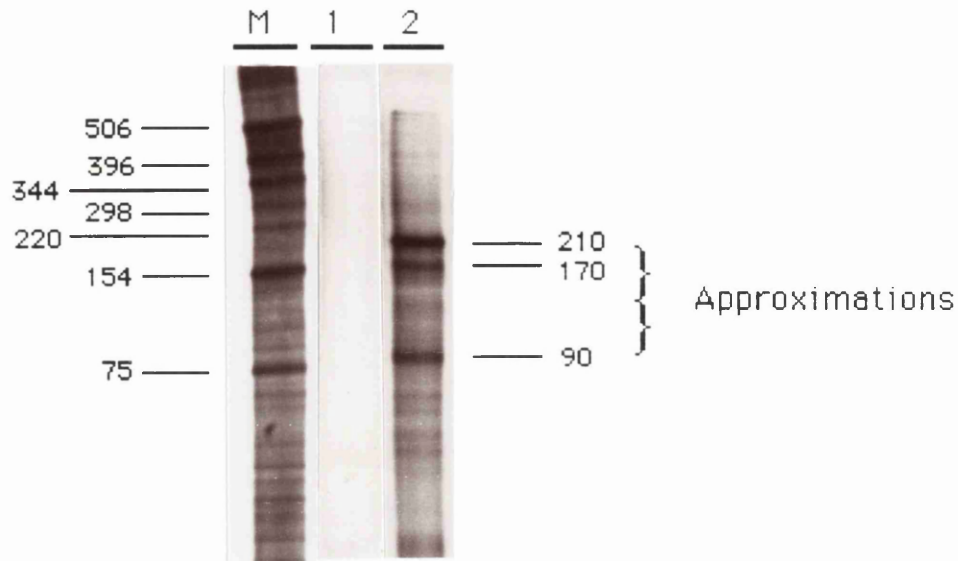
Figure 9.

Autoradiograph of a 5% polyacrylamide sequencing gel showing the results of an S1 Nuclease reaction (Chapter 2.2 U). The products of PBR322 digested with HinfI were run as molecular weight markers (**M**). The S1 probe was generated by primer extension on single stranded M13 containing the SalI/HindIII fragment of PDGF-A$_L$ form cDNA (Chapter 2.2 U i).

1) Protected fragments generated by annealing probe with 500 ng of Rat Astrocyte Poly (A)$^+$ RNA and treating with S1 nuclease.

2) Protected fragments generated by annealing probe with 500 ng of 157 Human Glioma cell line Poly (A)$^+$ RNA and treating with S1.

Figure 10.

Diagram showing the full Human PDGF-A$_L$ cDNA Sequence {Betsholtz *et al.*, 1986}. Indicating the location of the putative S1 Sensitive A:T rich region (Broken line box); the location of the alternatively spliced exon 6 (Solid line box) and the restriction enzyme sites used for sub-cloning into phage m13 (↑). Also shown are the translation start codon (ⅢⅢⅢ▷), the alternative termination codons (▬) and the cleavage sites for the Pre-Pro-polypeptide (✖) and the Pro-peptide (♣) relative to the cDNA sequence from which they are translated.

The *SacII, StuI and EcoRI* restriction sites used for cloning into expression vector pHYK (Chapter 2 Figure 8) are indicated (↓).

```
         10         20         30         40         50         60
TCCGCAAATA TGCAGAATTA CCGGCCGGGT CGCTCCTGAA GCCAGCGCGG GGAGGCAGCG

         70         80         90        100        110        120
CGGCGGCGGC CAGCACCGGG AACGCACCGA GGAAGAAGCC CAGCCCCGC CCTCCGCCCC

        130        140        150        160        170        180
TTCCGTCCCC ACCCCCATCC CGGCGGCCCA GGAGGCTCCC CGCGCTGGCG CGCACTCCCT

        190        200        210        220        230        240
GTTTCTCCTC CTCCTGGCTG GCGCTGCCTG CCTCTCCGCA CTCACTGCTC GCCGGCGCCC

        250        260        270        280        290        300
GTCCGCCAGC TCCGTGCTCC CCGCGCCACC CTCCTCCGGG CCGCGCTCCC TAAGGGATGG

                                                   SacII
        310        320        330        340        350        360
                                                       ↓
TACTGATTTT CGCCGCCACA GGAGACCGGC TGGAGCGCCG CCCCGCGGCC TCGCCTCTCC

        370        380        390        400        410        420
TCCGAGCAGC CAGCGCCTCG GGACGCGATG AGGACCTTGG CTTGCCTGCT GCTCCTCGGC
                              ‖‖‖‖‖‖‖→

        430        440        450        460        470        480
TGCGGATACC TCGCCCATGT TCTGGCCGAG GAAGCCGAGA TCCCCCGCGA GGTGATCGAG
                           *

        490        500        510        520        530        540
AGGCTGGCCC GCAGTCAGAT CCACAGCATC CGGGACCTCC AGCGACTCCT GGAGATAGAC

        550        560        570        580        590        600
TCCGTAGGGA GTGAGGATTC TTTGGACACC AGCCTGAGAG CTCACGGGGT CCATGCCACT

        610        620        630        640        650        660
AAGCATGTGC CCGAGAAGCG GCCCCTGCCC ATTCGGAGGA AGAGAAGCAT CGAGGAAGCT

                                            ♣
        670        680        690        700        710        720
GTCCCCGCTG TCTGCAAGAC CAGGACGGTC ATTTACGAGA TTCCTCGGAG TCAGG TCGAC
                                                     /\      Sall

        730        740        750        760        770        780
CCCACGTCCG CCAACTTCCT GATCTGGCCC CCGTGCGTGG AGGTGAAACG CTGCACCGGC

        790        800        810        820        830        840
TGCTGCAACA CGAGCAGTGT CAAGTGCCAG CCCTCCCGCG TCCACCACCG CAGCGTCAAG

                              ┌ ─ ─ ─ ─ ─ ┐
        850        860        870  880       890        900
GTGGCCAAGG TGGAATACGT CAGGAAGAAG CCAAAATTAA AAGAGGTCCA GGTGAGGTTA
                              └ ─ ─ ─ ─ ─ ┘

        910        920        930        940        950        960
GAGGAGCATT TGGAGTGCGC CTGCGCGACC ACAAGCCTGA ATCCGGATTA TCGGGAAGAG

     ┌ 970   ↓Stul 980        990       1000       1010       1020
GACACGGGAA GGCCTAGGGA GTCAGGTAAA AAACGGAAAA GAAAAAGGTT AAAACCCACC

       1030      ┐ 1040      1050       1060       1070       1080
TAAAGCAGCC AACCAGATGT GAGGTGAGGA TGAGCCGCAG CCCTTTCCTG GGACATGGAT
 ▬                     ▬

       1090      1100                 1120       1130       1140
GTACATGGCG TGTTACATTC CTGAACCTAC TATGTACGGT GCTTTATTGC CAGTGTGCGG

       1150      1160       1170       1180       1190       1200
TCTTTGTTCT CCTCCGTGAA AAACTGTGTC CGAGAACACT CGGGAGAACA AAGAGACAGT

       1210      1220       1230       1240       1250       126 0
GCACATTTGT TTAATGTGAC ATCAAAGCAA GTATTGTAGC ACTCGGTGAA GCAGTAAGAA
                                                             /\
       1270      1280       1290       1300         EcoRI    HindIII
                                                  ↓
GCTTCCTTGT CAAAAAGAGA GAGAGAGAAA AGAAAAAAAA AGGAATTC
```
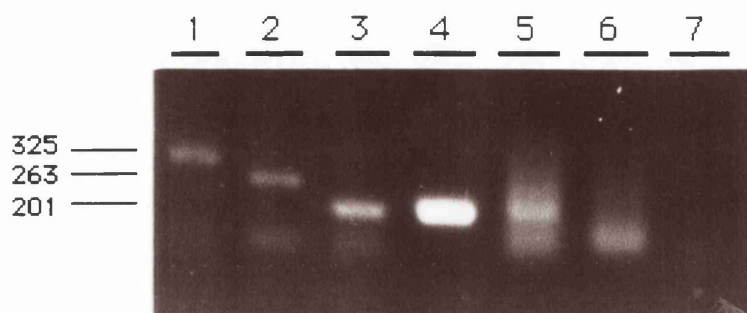
Figure 11.

1.4% Agarose gel showing the products of a 25 cycle PCR (Chapter 2.2 R) using an anneal temperature of 55°C performed on 20 ng of plasmid DNA (**2, 3** and **4**), on cDNAs generated from 1μg Poly (A)$^+$ selected RNA using the Amersham cDNA synthesis system (**5** and **6**) and on 1 μg Poly (A)+ RNA (**7**). Samples representing 10% of the total product were separated on the gel, stained with EtBr and visualised on a UV transilluminator.

1) 325 bp marker

2) Human PDGF-A$_L$ cDNA plasmid PCR (Oligos No.s 1 & 2)
   +ve control = 263 bp

3) Human PDGF-A$_L$ cDNA plasmid PCR (Oligos No.s 6 & 7)
   +ve control = 180 bp

4) Human PDGF B cDNA plasmid PCR (Oligos No.s 3 & 4)
   +ve control = 201 bp

5) 157 Human Glioma Cell Line cDNA PCR (Oligos No.s 1 & 2)

6) Rat Astrocyte cDNA PCR (Oligos No.s 1 & 2)

7) 157 Human Glioma Cell Line Poly A + RNA PCR
   (Oligos No.s 1 & 2) : NOT cDNA.
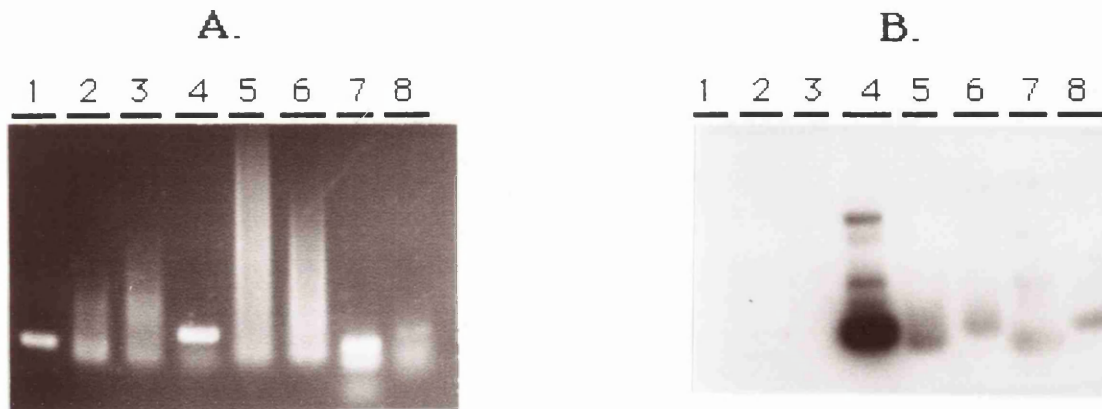
Figure 12.

1.4% Agarose gel showing the products of a 25 cycle PCR with either PDGF B Chain (**1, 2** and **3**) or A Chain (**4** to **8**) specific oligos. Using the highly permissive anneal temperature of 37°C, PCR was performed simultaneously on 20 ng plasmid DNA (**1, 4** and **7**), on cDNAs (**2, 3, 6** and **7**) generated from 1 μg Poly (A)$^+$ selected RNA using the Amersham cDNA synthesis system (Chapter 2.2 S) and on 1 μg Poly (A)$^+$ RNA (**8**). Samples representing 10% of the total product were run on the gel, stained with EtBr and visualised (**A**) on a UV transilluminator then alkali blotted (Chapter 2.2 L) and probed with radiolabeled Human PDGF-A chain cDNA sequence (**B**).

1) Human PDGF B chain cDNA plasmid PCR (Oligos No. 3 & 4)
   +ve control = 201 bp

2) 157 Human Glioma Cell Line cDNA PCR (Oligos No. 3 & 4)

3) Rat Astrocyte cDNA PCR (Oligos No. 3 & 4)

4) Human PDGF-A$_L$ cDNA plasmid PCR (Oligos No. 1 & 2)
   +ve Control = 263 bp

5) 157 Human Glioma Cell Line cDNA PCR (Oligos No. 1 & 2)

6) Rat Astrocyte cDNA PCR (Oligos No. 1 & 2)

7) Human PDGF-A$_L$ plasmid PCR (Oligos No. 6 & 7)
   +ve Control = 180 bp

8) 157 Human Glioma Cell Line Poly A+ RNA PCR (Oligos No. 1 & 2)
   : NOT cDNA
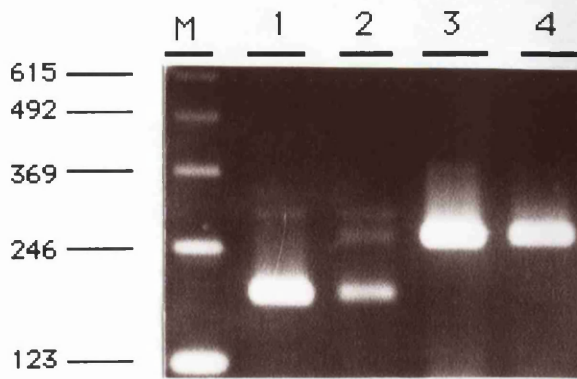
Figure   13.

3% Agarose gel showing the products of a 40 cycle standard PCR amplification (Chapter 2.2 R ii) on 1 μg of untreated human Glioma Cell line 157 Poly (A)$^+$ RNA (**1**), on an equivalent amount treated with DNase I (**2**) and the same for RNA combined with 200 ng of human PDGF-A$_L$ chain cDNA plasmid (**3** and **4**). Samples representing 10% of the total PCR product were run on the gel, stained with EtBr and visualised on a UV transilluminator. (**M**) = 123 bp ladder markers.

1) 157 Human Glioma Cell Line Poly (A)+ RNA PCR (Oligos No.1 & 2)

2) 157 Human Glioma Cell Line Poly (A)+ RNA PCR (Oligos No.1 & 2)
   DNase I treated.

3) 157 Human Glioma Cell Line Poly (A)+ RNA PCR (Oligos No.1 & 2)
   with 200 ng Human PDGF-A$_L$ cDNA plasmid.

4) 157 Human Glioma Cell Line Poly (A)+ RNA PCR (Oligos No.1 & 2)
   with 200 ng Human PDGF-A$_L$ cDNA plasmid :
   DNase I treated.
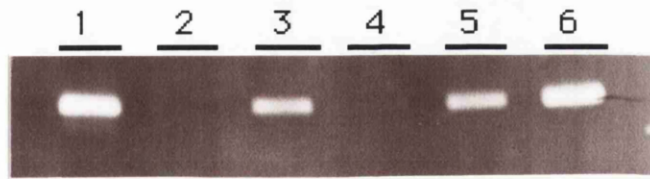
Figure   14.

1% Agarose gel showing the products of a 30 cycle standard PCR amplification (Chapter 2.2 R) of 440 ng human PDGF-A$_S$ cDNA plasmid treated for increasing lengths of time with DNase I which was inactivated at 90°C for 10 minutes prior to PCR. Samples representing 10% of the total PCR product were run on the gel, stained with EtBr and visualised on a UV transilluminator.

1) Untreated

2) Treated for 20 minutes

3) Treated for 40 minutes

4) Treated for 60 minutes

5) Treated for 120 minutes

6) As a control for DNase I inactivation 440 ng of plasmid was treated for 120 minutes with DNase I which had been inactivated as above before use.
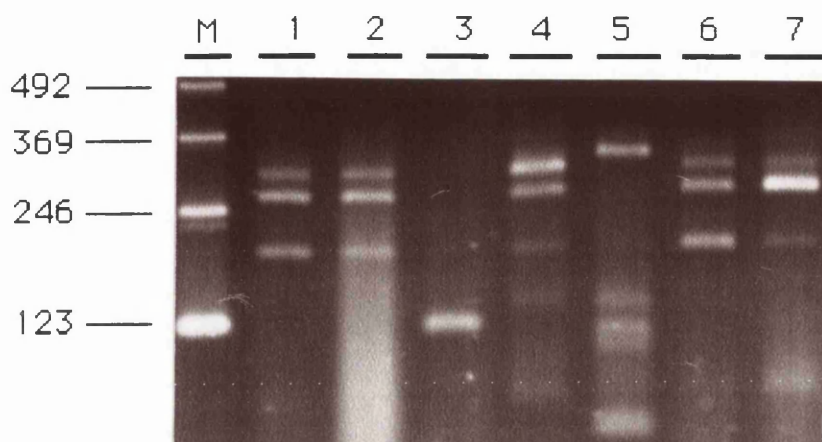
Figure 15.

3% Agarose gel showing the products of a 40 cycle standard PCR amplification (Chapter 2.2 R) on 600 ng 157 Human Glioma Cell Line Poly (A)$^+$ RNA (**1** and **3**), 10 µg 157 Human Glioma Cell Line total RNA (**2**), 7.5 µg of RNase treated 157 Human Glioma Cell Line genomic DNA (**4** and **5**), 1 µg Postnatal day 2 Rat Brain Poly (A)$^+$ RNA (**6**) and 1 µg Rat Astrocyte Poly (A)$^+$ RNA (**7**). Samples representing 10% of the total PCR product were run on the gel, stained with EtBr and visualised on a UV transilluminator. (**M**) = 123 bp ladder markers.

1) 157 Human Glioma Cell Line Poly A$^+$ RNA PCR (Oligos No. 1 &2)

2) 157 Human Glioma Cell Line Total RNA PCR (Oligos No. 1 & 2)

3) 157 Human Glioma Cell Line Poly A+ RNA PCR (Oligos No. 6 & 7)

4) 157 Human Glioma Cell Line Genomic DNA PCR(Oligos No. 1 & 2)

5) 157 Human Glioma Cell Line Genomic DNA PCR
    (Oligos No. K1 & K2) : +ve Control = 325 bp

6) P2 Rat Brain Poly A+ RNA PCR (Oligos No. 1 & 2)

7) Rat Astrocyte Poly A+ RNA PCR (Oligos No. 1 & 2)
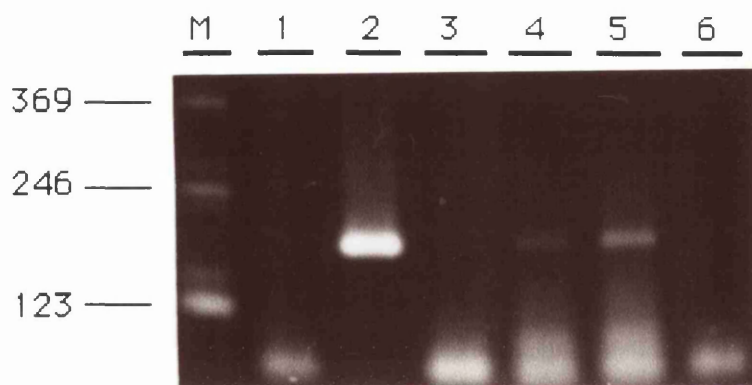
Figure 16.

3% Agarose gel showing the products of a 40 cycle standard PCR amplification (Chapter 2.2 R) on 20 ng Human PDGF-A$_S$ cDNA plasmid DNA (**2**), on 1 μg 157 Human Glioma Cell Line Poly (A)$^+$ RNA (**3**) and on 157 Human Glioma Cell Line cDNA generated by reverse transcription of 1 μg 157 Human Glioma Cell Line Poly (A)$^+$ RNA in the presence of the PCR oligos alone (**4**) and with the PCR oligos and oligo dT (**5**). Two PCR amplifications in the absence of any added RNA or DNA were carried out as negative controls (**1** and **6**). Samples representing 10% of the total PCR product were run on the gel, stained with EtBr and visualised on a UV transilluminator. (**M**) = 123 bp ladder markers.

1) PCR with no DNA added (Oligos No. 1 & 2) : -ve Control

2) Human PDGF-A$_S$ chain cDNA plasmid PCR (Oligos No. 1 & 2)
   +ve Control = 194 bp

3) 157 Human Glioma Cell Line Poly A+ RNA PCR (Oligos No. 1 & 2).

4) 157 Human Glioma Cell Line cDNA PCR (Oligos No. 1 & 2)
   reverse transcribed with only PCR Oligos present.

5) 157 Human Glioma Cell Line cDNA PCR (Oligos No. 1 & 2)
   reverse transcribed with Oligo dT and PCR Oligos present.

6) As 1) but with Oligo dT present : -ve Control

☿ Chapter Four ☿

"One fine morning in May a slim young horsewoman might
have been seen riding a glossy sorrel mare along the
flowery avenues of the Bois de Boulogne."

Chapter 4. Isolation and sequencing of rat PDGF-A chain cDNAs, design of rat-specific PCR primers and their use in analysing differential splicing patterns of PDGF-A mRNA in CNS cell types.

4.1 Introduction.

In the previous chapter I demonstrated the importance of having a fully complementary probe for S1 analysis and indicated the potential problems with non-homologous PCR primers. It was therefore necessary to isolate the 3' portion of the rat PDGF-A chain encoding the alternatively spliced exon 6 to allow analysis of the expression pattern of the two forms of rat A-chain transcript. To this end I chose to manufacture a cDNA library from postnatal day 7 (P7) rat brain, which is known to express moderate levels of PDGF-A transcript {Richardson *et al.*, 1988}, in the hope that this would increase my chances of obtaining suitable clones.

4.2 P7 Rat brain cDNA library production and screening.

Using the Amersham "cDNA Synthesis System Plus", 3 µg of P7 rat brain Poly (A)$^+$ RNA was reverse transcribed to ds cDNA and cloned into λgt10 using the Amersham "cDNA cloning System" (see also Chapter 2 Sections 2 S and 2 T). Two libraries were generated; Library No.4 and Library No.5 with respective cloning efficiencies of 1.5 x 10^6 and 7.3 x 10^5 plaque forming units (pfu)/µg of cDNA used, which compared favourably with the expected 10^6 pfu/µg efficiency required for a good library {Williams, 1981}. Based on a physical analysis of insert DNA from randomly picked single plaques (not shown) library No.5 was chosen for screening because it had a higher percentage with inserts (90%) which were also mostly longer than those in library No. 4.

To find a medium-to-low abundance sequence it is necessary to screen approximately 1.7 x 10^5 independent recombinants to have a 99% probability of obtaining the desired clone {Williams, 1981}. In order to do this with my library which had a total of 2.1 x 10^5 independent clones I would have had to use 80% for a single screening. I therefore chose to amplify it, using the Amersham protocols, to produce several millilitres of much higher titre library stock (6.5 x 10^{10} pfu/µg), allowing the opportunity for several screenings. Physical analysis of the amplified library (Figure 17) indicated

that although most inserts were within the range of 0.1 kb to 0.5 kb there were some inserts of greater than 2.3 kb and a good proportion (80-90%) of recombinants had inserts. I therefore screened a total of 1.9×10^5 plaques, including approximately 1.7×10^5 recombinants.

After a progression of three screening stages using the human PDGF-A chain cDNA sequence {Betsholtz et al., 1986} as a probe I isolated a total of six possible independent clones from single plaques and prepared their respective DNAs for further analysis according to the Amersham protocols.

4.3 Analysis of six potential rat PDGF-A chain cDNA clones.

λgt10 DNA from each of the six potential rat PDGF-A chain clones was digested with EcoRI to release the insert and run on an agarose gel to check the size range of the cloned sequences (Figure 18). Though only faintly visible each clone produced the same fragment size indicating that they might all be copies of a single clone in the original unamplified library. When the cloned inserts were blotted and probed with the human PDGF-A chain cDNA, all hybridised successfully (including DNA in lanes 2 and 4 which is not clear from this exposure) but not nearly as strongly as the homologous human PDGF-A chain included as a control (Figure 18 B Lane 7). The linearised pUC vector DNA, which had been run as a size marker, hybridised to the same extent as the insert DNA (Figure 18 Lane 8). This indicated that my probe DNA might have some complementarity to vector sequence despite having been separated from plasmid sequences by restriction digestion and gel electrophoresis (Chapter 2.2 M i). This raised my suspicions about the nature of the clones I had isolated.

A second blot of these digests when probed instead with labeled pUC vector DNA showed that the insert DNA hybridised to the vector sequence while the PCR-derived PDGF-A chain sequence did not (Figure 19). Furthermore, when the insert DNA was ligated in the absence of any added vector, they all displayed the ability to transform competent cells (Data not shown). This strongly suggested that, rather than isolating the rat PDGF-A chain cDNA, I had cloned a λgt10 containing some form of vector DNA sequence, though not pUC 8 since both gels show it to be smaller than the λgt10 inserts.

I re-screened the library with a probe made from newly-purified PDGF-A chain sequence using suitable controls (See Chapter 2.2 Section O) to ensure that only PDGF-A chain was being targeted and no vector sequence hybridisation was occuring. This time no potential clones were identified suggesting that the library carried very few, if any, sequences representing rat PDGF-A chain cDNA.

4.4 P21 Rat brain cDNA library screening and clone verification.

The improved screening procedure was used to screen a total of 1.8×10^5 clones from a λgt11 cDNA library (obtained from Dr. W. Stoffel: see Chapter 2.2 Section T) made with RNA from the brains of P21 Lewis strain rats. After a progression of six screening stages I isolated 12 possible independent clones from single plaques. This time, as a result of the hybridisation controls, I was confident that these clones represented the rat PDGF-A chain sequence. I used a number of different approaches in an attempt to identify which of these clones included the 3' portion of the rat cDNA sequence.

A) Sizing of lambda inserts by PCR amplification.

First I checked the size range of the cDNA inserts to establish whether the clones were truly independent and whether they were large enough to merit further investigation. DNA obtained from 10 of the 12 clones was amplified by PCR using primers complementary to either side of the λgt11 cloning site (RSP and FSP : see Chapter 2.2 Section R iv). The sizes ranged from approximately 500 bp to 1.4 kb (Figure 20) and included one which appeared to be a mixed clone giving two PCR products (Lane 1). Although several of them were of a similar size suggesting they might represent copies of a single clone it was clear that I also had several different clones. Furthermore, they were all of a sufficient size to contain the sequence information I required. I therefore decided to subclone a number of the longer inserts for sequence analysis (see Section 4.5).

B) Confirming clone identities by hybridisation to insert and PCR DNA.

As a further verification of the nature of the 12 clones, λgt11 DNA was isolated from fresh plaques, digested with *EcoRI* to release the insert, run on an agarose gel alongside PCR products for six of the clones (Figure 21 A),

blotted and probed with the human PDGF-A chain cDNA sequence (Figure 21 B). The result shows hybridisation to each of the clone inserts approximately in accordance with the amount visible by EtBr staining. The specificity of the probe was illustrated by its hybridisation to the human PDGF-A chain cDNA plasmid and fragment (F) but not to the vector (L) from which it was released (Figure 21 B Lane P).

Of the six PCR products run for comparison only five showed equivalent levels of hybridisation to their insert DNA and only 3 were larger than the equivalent insert. Thus the PCR products derived from clones 3.1, 5.3 and 5.4 apparently represent "false" amplification events indicating the value of this more standard type of analysis.

C) Looking for exon 6 by PCR.

I attempted to assess whether exon 6-derived sequences were present in the 3 largest clones (1.2, 3.4 and 5.3) by amplifying across the putative exon 6 boundaries with appropriate PCR primers and comparing the product sizes with those predicted for PDGF-A$_S$ and PDGF-A$_L$ (See Table 9). The appropriate PCR products from the previous experiment were ethanol precipitated (see Chapter 2.2 Section B) and re-amplified with human PDGF-A chain primer (oligo No. 7; see Chapter 2, Figure 6 and Tables 6 & 7) in pair-wise combination with each of the two λgt11 primers and a second human PDGF-A chain primer (oligo No. 10). PCR was also performed with both λgt11 primers as a positive control (Figure 22). Successful amplification with human oligo No.7 and one of the two λgt11 primers took place in each case indicating that all three clones contained sequence from the 3' end of the rat PDGF-A chain cDNA.

For each clone the oligo No. 7 amplification should only have worked with one of the two λgt11 primers to indicate the orientation of the clone with respect to the λgt11 cloning site. However close inspection of the apparently unsuccessful pairing in both 3.4 and 5.3 samples revealed a high molecular weight band. This band could even be seen in the successful oligo No. 7 + λgt11 (RSP) pairing for the 5.3 sample. This apparently anomalous result may be explained by comparison with the RSP + FSP positive control PCR which gave a band the same size as the unexpected band. It would appear that the unused RSP + FSP primers were co-precipitated with the product of the previous PCR

and subsequently took part in this PCR to give a product representing the full insert.

Unfortunately none of the amplifications with human oligo No. 10 showed any signs of a PDGF-A$_L$ PCR (154 bp) product and there were only very faint indications of a possible PDGF-A$_S$ product (85 bp). The successful oligo No. 7 / R or FSP amplification products in each case represented approximately 300 bp, which, by comparison with the human sequence, should easily have encompassed the sequence to which oligo No. 10 would anneal. This suggested that the rat sequence corresponding to oligo No. 10 was different from the human sequence, preventing efficient annealing and priming. Therefore although this experiment did not provide good information on the presence or absence of the rat exon 6 in these clones it did confirm that they included the region where differential splicing would occur.

The sizes of the products of the successful oligo No. 7 amplifications and the RSP + FSP amplifications indicate that the clones each contain a similar region of the rat PDGF-A chain cDNA. In fact clones 3.4 and 5.3 appear to contain closely similar fragments in different orientations relative to the λgt11 cloning site.

In a further attempt to characterise these 3 clones I performed a second experiment with human and λgt11 primers, this time exchanging oligo No. 10 for exon 6-specific oligo No. 8 (see Chapter 2 Figure 6 and Tables 6 & 7). All four primers were used simultaneously in the hope that each potential product would be represented. The amplification products (Figure 23) were, however, no different from those observed in the previous experiment indicating that no amplification from oligo No. 8 had occurred. This could be for one of two possible reasons: either the exon 6 sequence was not present or the rat sequence was different from the human sequence, preventing successful annealing and priming. Thus this result, although negative, did not necessarily mean that the alternatively spliced exon 6 was absent. The uncertainty over the ability of some human primers to amplify the rat sequence prevented me from pursuing this type of analysis with the remaining clones.

4.5 Analysis of rat PDGF-A chain sequence.

I sub-cloned the inserts from three of the longest λgt11 clones into phagemid vector pTZ18R {Rokeach *et al.*, 1988}; two (4.1 and 5.3) via EcoRI digestion of whole λgt11 DNA preparations and the third (3.3) from its PCR product. These were sequenced as described previously (see Chapter 2. 2 Section P) using a combination of standard λgt11 forward and reverse sequencing primers, human PDGF-A sequence-derived primers and a rat sequence primer R3 (See Figure 27 B). The complete sequence of clone 4.1 was obtained from overlapping sequence data and verified against the sequence of independent clone 5.3 (Figure 24). Clone 3.3 showed good sequence for the region covering the alternative splice junction but deteriorated to nonsense towards the ends of the insert sequence (see discussion 4.8 B). All three represented the PDGF-A$_S$ sequence.

Comparison of the clone 4.1 nucleotide sequence with the human PDGF-A$_S$ sequence (Figure 25) showed an 88% sequence similarity within the coding regions of the two clones. The 3' untranslated regions also showed high levels of sequence similarity with the exception of a number of inserts and/or deletions (overall 66%), whereas the 5' untranslated regions, though initially very similar and both highly GC rich (rat 75%; human 74%), showed significant divergence within 40 nucleotides of the start codon (overall 53%). This raised the possibility that such a difference may have been due to sequencing errors, however, comparison of the 5' untranslated region of *Xenopus* PDGF-A chain cDNA sequence {Mercola *et al.*, 1988} with that of human PDGF-A (not shown) indicated an analogous, though more extensive, divergence, as befitting their greater evolutionary distance (overall 25%).

Interestingly, a search of the EMBL/GenBank database revealed that an independent attempt to sequence rat PDGF-A$_S$ cDNA did not provide data on this region, indeed the sequence information stops precisely were the divergence occurs {Katayose *et al.*, 1991}. It seems possible that there may have been problems sequencing through this area or even that they opted to publish only the closely similar sequence information. In addition the sequence of the coding region differs by the absence of a guanine nucleotide at position 639 and the addition of a cytosine nucleotide at position 723 (Figure 25, human sequence locations). This has the effect of altering the reading frame such that the predicted mature protein would include a region of 28 amino acids that

have not been observed in any other form of PDGF-A. It is not clear whether this represents a new form of PDGF or is simply the result of cloning and/or sequencing artefacts.

A comparison of the predicted translation product from my rat cDNA indicates a 92% amino acid sequence similarity with that of human PDGF over the whole pre-pro-peptide and 95% within the mature peptide sequence (respectively 97% and 99% including conservative amino acid changes: see Table 3). It also shows a 96% similarity with mouse {Young *et al.*, 1990} and 76% with *Xenopus* {Mercola *et al.*, 1988}, reflecting their evolutionary relationships (Figure 26). N.B. The predicted *Xenopus* sequence includes 5 more amino acids than the mammalian ones.

4.6 Designing and testing rat-specific PCR primers.

By comparing the rat and human cDNA sequences I attempted to design rat-specific PCR primers that would not be able to amplify human PDGF-A chain cDNA that might be present as a low level contaminant (see Chapter 3). The high degree of sequence similarity meant that I had no option but to use rat sequence oligonucleotides which were largely complementary to the human sequence. I therefore chose two that were distinct at the 3' ends, in the hope that these would not prime DNA synthesis on a human target sequence (Figure 27 and Tables 8 & 9). I then tested the specificity of these primers in a number of ways, to establish conditions for analysing rat cDNAs.

The optimal buffer concentration of $MgCl_2$, which affects the specificity of primers, must be determined empirically for each set of primers (Perkin Elmer Cetus AmpliTaq specifications). I therefore compared amplification of both human and rat cDNA sequences using the rat-derived oligos No. 11 & 12 in a variety of $MgCl_2$ concentrations (Figure 28). Amplification of the rat sequence took place in all the $MgCl_2$ concentrations shown, but cross-species amplification of the human sequence only occured at the highest $MgCl_2$ concentration tested (5 mM) as determined by EtBr staining. This indicated that the specificity of the primers might be assured by using an $MgCl_2$ concentration of 3 mM in all subsequent PCR reactions.

Having only two rat-specific primers was potentially limiting. I therefore tested their specificity, when paired with human primers capable of

annealing to both rat and human sequence, by using them in pair-wise combinations with human oligos No. 7 & 2 which had only internal mismatches with the rat sequence (See Figure 27 and Tables 8 & 9). In a standard three-temperature cycle amplification reaction both rat primers, when paired with a human primer, directed amplification of human as well as rat sequence (data not shown). However, by using a two temperature PCR cycle with no $55^{\circ}C$ annealing step, specificity for the rat sequence was regained. Under these conditions the human primers were still able to direct PCR amplification from both human and rat cDNA when used together but in pair-wise combination with the rat-specific primers they only amplified the rat sequence (Figure 29). Therefore, by using stringent annealing conditions together with low $MgCl_2$ buffer concentrations, my rat-specific primers, even in combination with more permissive primers, would not amplify contaminating human cDNA sequence. This potentially afforded a means of avoiding false positives including hybrid human molecules with primer derived rat sequence at the end, which would represent a possible carry over contamination threat. The 5 mM $MgCl_2$ human cDNA amplification reaction (from Figure 28) was carefully disposed of to prevent future contamination by this hybrid molecule as were the products of all rat amplification reactions.

Having established that rat oligo No. 12 could amplify the rat sequence in concert with human oligo No. 7, I used them to amplify the nine unsequenced λgt11 clones to discover whether any included the alternatively spliced exon 6. This was done in preference to using both rat specific oligos which would have produced large quantities of potential contaminant (see Chapter 3.7 b). The product from this reaction did not include the rat oligo No. 11 annealing site (See Figure 27) so did not represent a potential contamination threat to future PCR amplifications using both rat-specific oligos. The result indicated that all the remaining clones contained the short form PDGF-A chain sequence (Figure 30). Negative controls (as described in Chapter 2.2 Section R iii), which were carried out alongside these amplifications and run on a separate gel, showed no signs of contamination (Not shown). This result indicated that most, if not all of the PDGF-A chain cDNAs in the library were short form, suggesting that this is the predominant transcript in the P21 rat brain.

4.7 Analysis of rat PDGF-A differential splicing with rat-specific primers.

Using the conditions established above to encourage primer specificity I attempted to identify the rat PDGF-A$_L$ sequence by amplifying cDNAs isolated from both normal and transformed rat cells known to express PDGF-A RNA {Richardson *et al.*, 1988} and those present in a number of cDNA libraries. However the two-temperature-cycle PCR produced no amplification products when presented with these more complex targets (Data not shown). I therefore repeated the experiment using a standard three temperature cycle PCR (with the resultant loss of species specificity). This allowed amplification of the PDGF-A from human 157 cell line cDNA (Figure 31 Lane 3) but not from either of the rat cDNA preparations (Lanes 1 and 2) or from two of the four rodent cDNA libraries tested (Lanes 4 and 7). However, PCR amplification products were obtained from one of the rat cDNA libraries and a mouse library (see Chapter 2.2 Section T). Both of these yielded only PDGF A$_S$ derived PCR products. The size of the human 157 cDNA confirms the previous observation (Chapter 3) that this cell line produces predominantly, if not exclusively, PDGF-A$_S$ transcripts.

A further attempt to amplify cDNAs made from freshly isolated P8 rat brain Poly (A)$^+$ RNA and C6 rat cell line Poly (A)$^+$ RNA using the standard three temperature cycle yielded amplification products from both (Figure 32). In this case the positive control was set up after I had finished setting up the experimental reactions, allowing me to be sure that no cross-contamination had occurred. Again, both gave bands representing only short form PDGF-A chain cDNA. I subsequently performed a large number of similar PCR amplifications on cDNAs made from both Poly (A)$^+$ and Poly (A)$^-$ RNAs from a variety of different rat sources including primary cortical astrocytes, O-2A lineage cells and different ages of rat brain. All these gave only the PDGF-A short-form product.

4.8 Discussion.

A) Library screening.

In an attempt to clone the rat PDGF-A$_L$ cDNA I constructed, amplified and screened a P7 rat brain cDNA library using the human PDGF-A$_L$ cDNA

sequence as a probe. Several positive clones were obtained but the inserts turned out to be plasmid vector sequence judging by their specific hybridisation to labeled plasmid DNA and their ability, once re-circularised, to transform *E.coli* to ampicillin resistance in the absence of added vector. The initial incorporation of plasmid sequence into λgt10 need only have occured at a very low level with the subsequent library amplification causing its apparent abundance in the final library. Cloning of plasmid sequence from libraries seems to be a relatively frequent event, as indicated by a recent study of the EMBL sequence database {Lopez *et al.*, 1992}. During a homology search using the complete M13mp18 sequence they showed that a "significant number" of the submitted sequences contained elements of vector and in some cases consisted of mostly vector sequence. Since they only used one vector to search the database the real number of vector derived sequences that are "contaminating" the database may be considerably underestimated. A further carefully controlled analysis of my P7 rat brain library using better purified probe DNA suggested that it contained few, if any, PDGF-A clones. This was subsequently confirmed by PCR amplification of the library (Figure 31 Lane 4) which also indicated the absence of any PDGF-A chain sequences.

B) Sub-cloning and sequencing.

PDGF-A clones were eventually obtained, using the improved screening procedure, from a P21 rat brain λgt11 library. A number of the inserts were sub-cloned and sequenced to determine the full coding sequence of the rat PDGF-A chain cDNA. The sequences of those subclones derived from insert DNA gel-purified from restriction digests of the λgt11 clones, were both consistent with each other and, in terms of open reading frame, with the known sequences from other species (Figures 25 & 26). However, the sequence of the subclone that was derived from a PCR amplification product, though identical with the others across the 3' region of the coding sequence, was apparently riddled with mutations towards the ends of the clone. This was observed consistently over a number of separate sequencing attempts. Together with the erroneous results of PCR amplifications using the λgt11 primers on other clones (see section 4.4 c and Figure 21) this evidence strongly suggested that cloning via PCR performed directly on cDNA would be prone to artefact. It is known that Taq polymerase has a much higher error rate than Klenow (1/90 for *Taq* as opposed to 1/270 for Klenow {Eckert and Kunkel, 1991}) although

this alone would not account for the level of difference observed. It is also possible that aberrant products arising from early mis-incorporation and mis-priming events might be amplified alongside the correct products giving rise to a mixture of amplified sequences instead of a single end-product. Any cloning strategy based on PCR would therefore require sequencing of a large number of clones derived from each amplification reaction to verify the nature of the sequence.

C) S1 analysis.

Sequence comparison of rat and human PDGF-A cDNAs confirmed that there would have been regular mismatches along the length of the human / rat hybrid formed during my S1 analysis (Chapter 3), the longest region of uninterrupted complementarity being only 35 bp in length (Figure 25; 1070 - 1105 bp). The discovery that none of the 12 clones represented the rat PDGF-A$_L$ form, however, precluded me from performing any fully complementary S1 analyses since it has been shown that a probe complementary to the RNA sequence on either side of a splice junction can be protected from S1 nuclease attack by both the spliced mRNA and the unspliced RNA {Sisodia et al., 1987}. An S1 probe corresponding to PDGF-A$_S$ could therefore be protected by PDGF-A$_L$ mRNA thus preventing discrimination between PDGF-A$_L$ and PDGF-A$_S$ mRNAs (see also Chapter 6 discussion).

D) PCR analysis of clones.

A further variation on a problem associated with PCR, previously discussed in chapter 3, was observed during amplification analysis of the λgt11 clones. Where a combination of primers was expected to amplify two different-sized products from the same target DNA, the shortest product was amplified preferentially (Figure 23). The earlier experiments had indicated that preferential amplification of a more abundant target sequence may take place. In this case there was only one target with different pairings of a combination of primers expected to give different-sized products. This emphasised that PCR analysis may not provide an accurate impression of the levels of the alternate forms of PDGF-A chain mRNA when used to survey cDNAs from a variety of sources.

E) PCR analysis of cDNAs.

Despite these reservations I attempted to design a set of rat-specific PCR primers with which to survey a range of rat cDNAs, in the hope that I could avoid the abundant human cDNA plasmid contamination observed in chapter 3 and perhaps obtain a clone of the rat PDGF-A_L. PCR analysis with human oligo No.10 (Figure 22) on rat target DNA had confirmed the observation (Chapter 3) that sequence mismatches in the primers (see Figure 27 and Table 8) may reduce their ability to direct primer extension during the PCR reaction. I therefore applied this principle in reverse by choosing rat sequence primers which were distinct from the human sequence at their 3' ends and defining conditions in which they were unable to prime amplification of the human sequence. In practice, however, these conditions proved unsuitable for amplifying from a complex target such as cDNA forcing me to use less stringent conditions, though, fortunately my contamination avoidance procedures prevented a resurgence of false positives. All the amplifications performed with these primers gave only the PDGF-A_S product.

Conclusion.

The results from the PCR analysis suggest that either rats do not express the PDGF-A_L sequence in the cells or tissues and at the developmental stages I examined, or that my primers preferentially amplified the short form when confronted with a mixture of PDGF-A_S and PDGF-A_L. However, in conjunction with the analysis of the cDNA clones the evidence points to the conclusion, that the predominant isoform of PDGF-A in the CNS is the short form, PDGF-A_S.
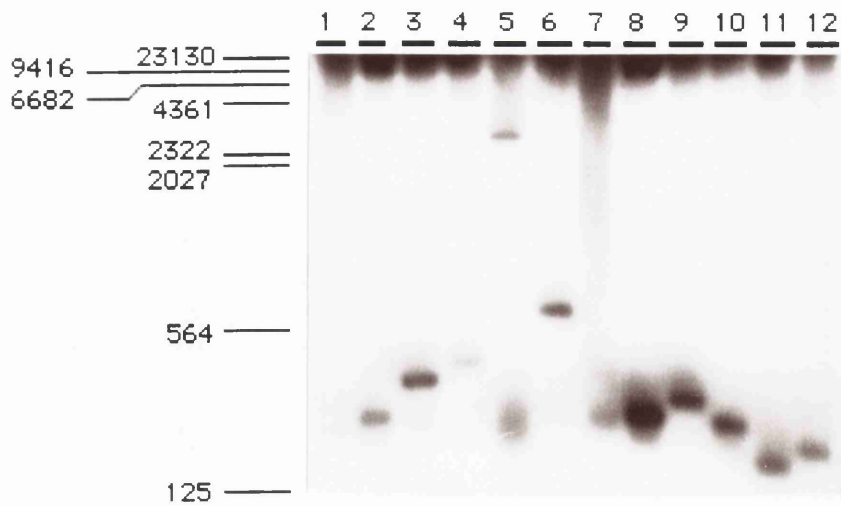
Figure 17.

Autoradiogram of a 1.5% agarose gel showing the radiolabeled inserts from 12 single plaques randomly chosen from the amplified λgt10 library No.5. DNA was prepared from each plaque, digested with EcoRI to release the insert fragment and end-labeled with ^{32}P (Chapter 2.2 D). The markers indicated were wild type λ phage DNA, HindIII digested and end-labeled.
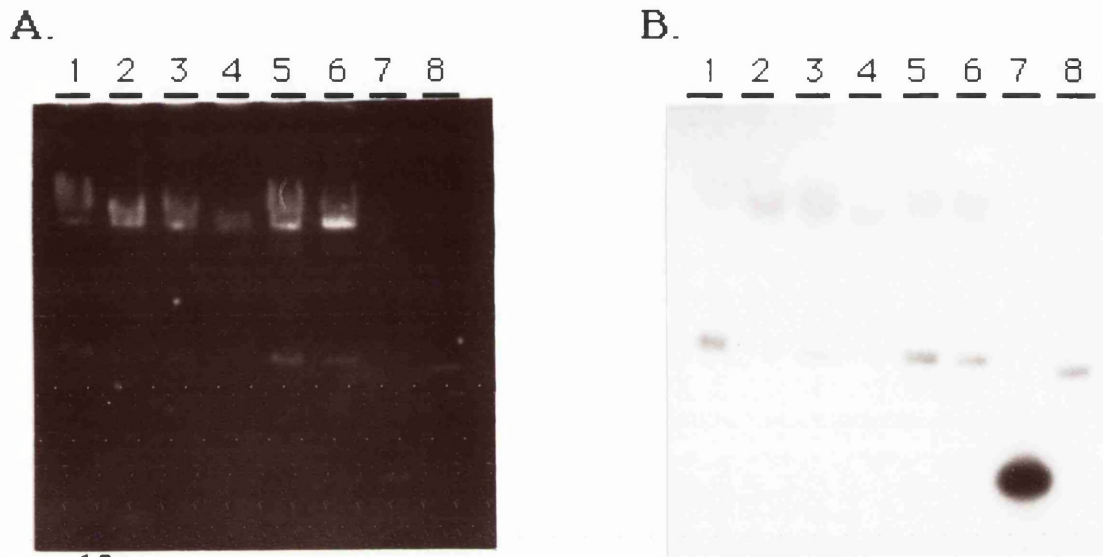
Figure 18.

1% Agarose gel showing the inserts from each of the six putative rat PDGF-A chain cDNA clones isolated from λgt10 library No.5. DNA was prepared from a single plaque of each clone, digested with EcoRI to release the insert fragment, run on the gel (**1-6**), stained with EtBr and visualised on a UV transilluminator (**A**) then alkali-blotted (Chapter 2.2 L ii) and probed with radiolabeled Human PDGF-A chain cDNA sequence (**B**). The 1.3 kb Human PDGF-A$_L$ chain cDNA clone (**7**) and 2.86 kb linearised pUC 8 vector DNA (**8**) were run as both size markers and probe specificity controls.

1) Putative PDGF-A clone 1 cut out of Lambda vector by EcoRI
2) Putative PDGF-A clone 2 cut out of Lambda vector by EcoRI
3) Putative PDGF-A clone 3 cut out of Lambda vector by EcoRI
4) Putative PDGF-A clone 4 cut out of Lambda vector by EcoRI
5) Putative PDGF-A clone 5 cut out of Lambda vector by EcoRI
6) Putative PDGF-A clone 6 cut out of Lambda vector by EcoRI
7) 1.3 kb Human PDGF-A chain cDNA clone : +ve Control
8) 2.86 kb Linearised pUC 8.
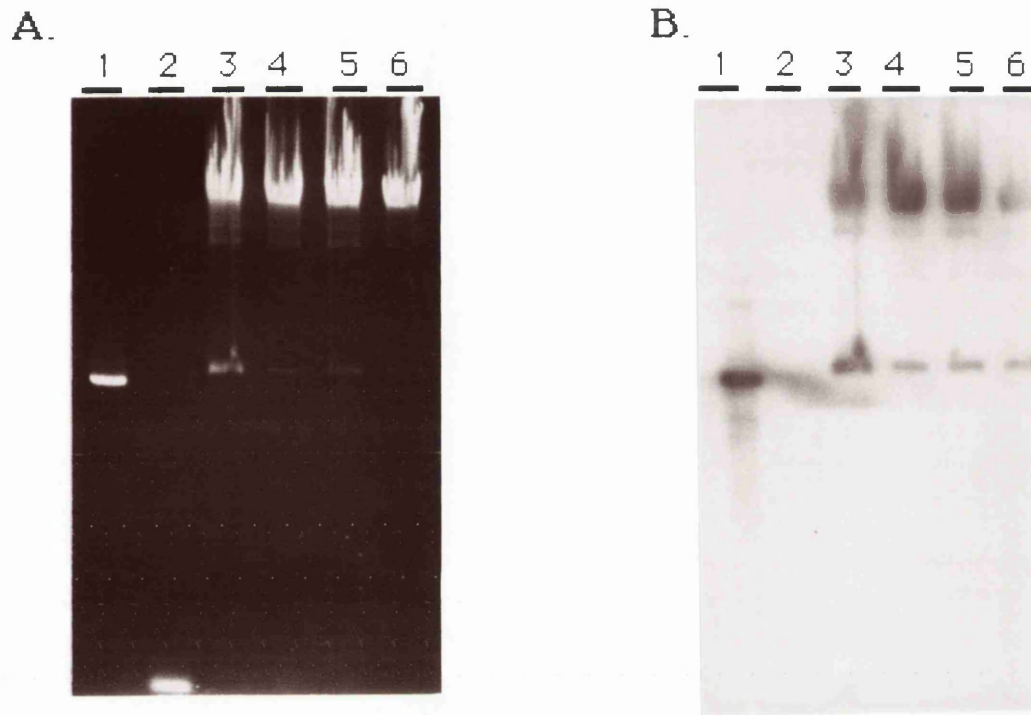
Figure 19.

0.8% Agarose gel showing the inserts from four of the six putative rat PDGF-A chain cDNA clones isolated from λgt10 library No.5. DNA was prepared from a single plaque of each clone, digested with EcoRI to release the insert fragment, run on the gel (**3-6**), stained with EtBr and visualised on a UV transilluminator (**A**) then alkali-blotted (Chapter 2.2 L ii) and probed with radiolabeled pUC 8 vector sequence (**B**). A 180 bp PCR fragment of Human PDGF-A_L chain cDNA clone (**2**) and 2.86 kb linearised pUC 8 vector DNA (**1**) were run as both size markers and probe specificity controls.

1) 2.86 kb Linearised pUC 8.

2) Human PDGF-A_L chain cDNA PCR Product (Oligos No.6 + 7)
     +ve Control = 180 bp

3) Putative PDGF-A clone 1 cut out of Lambda vector by EcoRI

4) Putative PDGF-A clone 3 cut out of Lambda vector by EcoRI

5) Putative PDGF-A clone 5 cut out of Lambda vector by EcoRI

6) Putative PDGF-A clone 6 cut out of Lambda vector by EcoRI
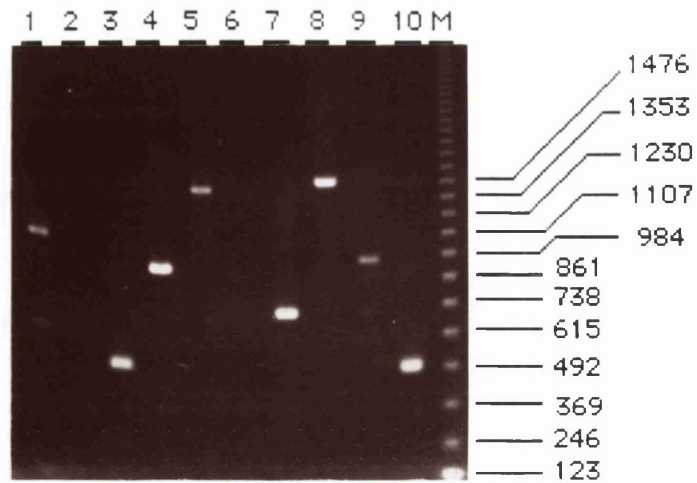
Figure 20.

1% Agarose gel showing the products of a 40 cycle standard PCR amplification using λgt11 Forward (FSP) and Reverse (RSP) sequencing primers (Chapter 2 R) on DNA isolated from single plaques of ten of the twelve putative rat PDGF-A chain cDNA clones isolated from λgt11 P21 rat brain cDNA library (**1-10**). Samples representing 10% of the total PCR product were run on the gel, stained with EtBr and visualised on a UV transilluminator. (**M**) = 123 bp ladder markers.

1) Clone 1.2 PCR

2) Clone 3.1 PCR

3) Clone 3.2 PCR

4) Clone 3.3 PCR

5) Clone 3.4 PCR

6) Clone 4.1 PCR

7) Clone 5.2 PCR

8) Clone 5.3 PCR

9) Clone 5.4 PCR

10) Clone 6.2 PCR

M) 123 bp ladder markers.

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Figure 21.

1% Agarose gel showing the inserts from each of the twelve putative rat PDGF-A chain cDNA clones isolated from λgt11 P21 rat brain cDNA library. DNA was prepared from a single plaque of each clone and digested with EcoRI to release the insert fragment and for six of the clones (*) the products of a standard 25 cycle PCR amplification using λgt11 Forward and Reverse sequencing primers were run on the gel alongside their restriction fragments, stained with EtBr and visualised on a UV transilluminator (UV Products Inc.) (A) then alkali blotted (Chapter 2.2 L ii) and probed with radiolabeled Human PDGF-A chain cDNA sequence (B).

(M) = 123 bp ladder markers.

(P) = Human PDGF-A$_L$ chain cDNA in pUC 8.

(L) = 2.86 kb linearised pUC 8.

(F) = 1.3 kb human PDGF-A chain cDNA *EcoRI* fragment.

(Ψ) = Uncut plasmid multimer.

(Ω) = Uncut open circle plasmid.

(Φ) = Uncut supercoiled plasmid.
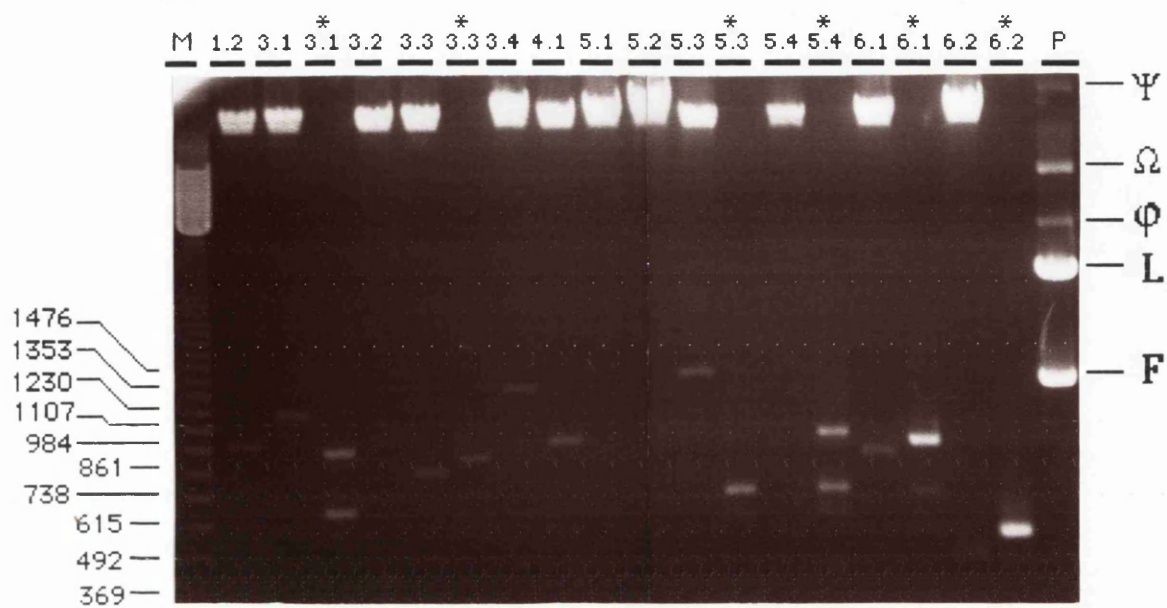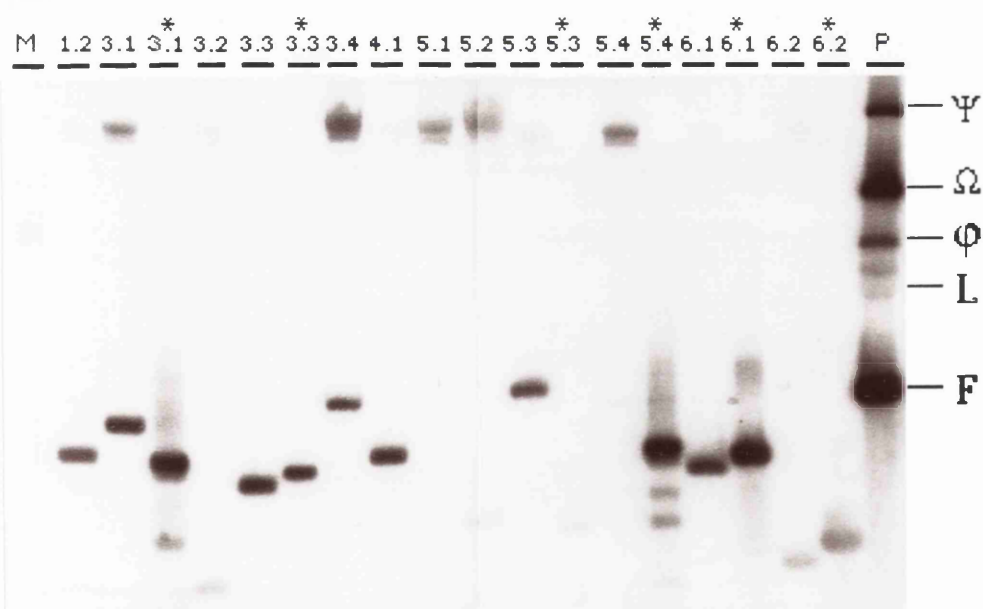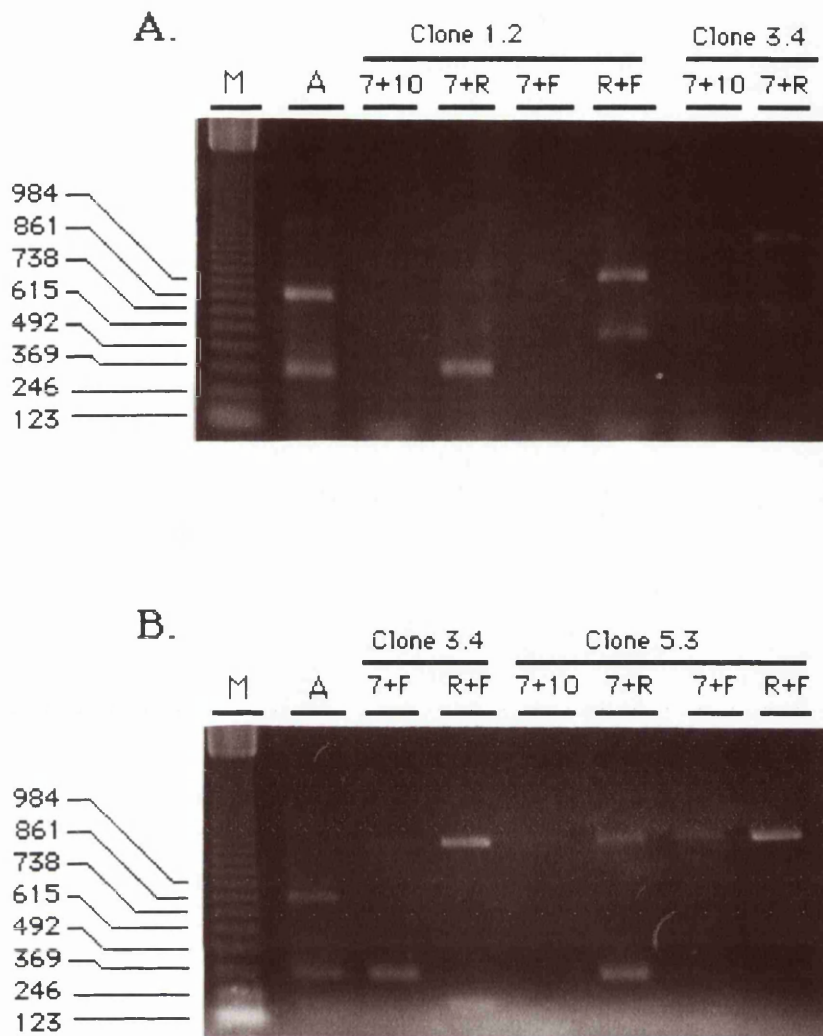
Figure 22.

1% Agarose gels showing the products of a 20 cycle PCR amplification with a permissive annealing temperature of 45°C. Combinations of λgt11 FSP (F) and RSP (R) sequencing primers and oligos No.7 and 10 (See Chapter 2 Figure 6) were used on DNA isolated from single plaques of three of the twelve putative rat PDGF-A chain cDNA clones isolated from λgt11 P21 rat brain cDNA library. Samples representing 10% of the total PCR product were run on the gel, stained with EtBr and visualised on a UV transilluminator. (**M**) = 123 bp ladder markers and (**A**)= PCR-derived Marker bands at 350 bp and 900 bp.
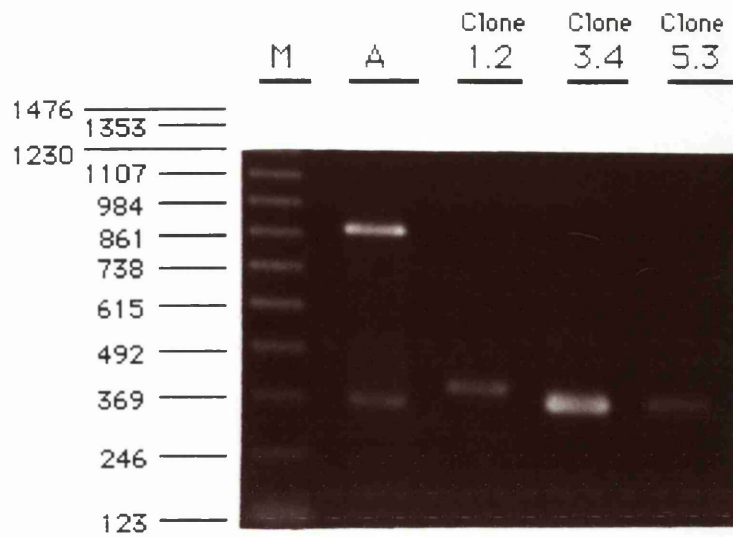
Figure 23.

1% Agarose gel showing the products of a 22 cycle PCR amplification with a permissive annealing temperature of 45°C. A combination of four oligonucleotide primers at the same time (λgt11 FSP and RSP and human oligos No.7 and 8) (Chapter 2 Figure 6) were used on DNA isolated from single plaques of three of the twelve putative rat PDGF-A chain cDNA clones isolated from λgt11 P21 rat brain cDNA library. Samples representing 10% of the total PCR product were run on the gel, stained with EtBr and visualised on a UV transilluminator. (M) = 123 bp ladder markers and (A) = PCR-derived Marker bands at 350 bp and 900 bp.

Figure 24.

Diagram showing how the complete sequence of rat PDGF-AS chain cDNA was determined by matching overlapping sequence information obtained from three independent clones using sequencing primers which anneal to the vector and those which anneal to the clone sequence itself. The numbering on the top refers to the Human PDGF-AS chain cDNA sequence and that on the bottom to the rat. The alternative splice site on the human sequence is indicated at 977 bp and the corresponding point is marked on the rat sequence at 710 bp.

(1) = Clone 5.3 sequenced with RSP and run for 2.5, 5 and 7 hours.

(2) = Clone 5.3 sequenced with FSP and run for 2.5, 5 and 7 hours.

(3) = Clone 5.3 sequenced with oligo No. 9 and run for 2.5 hours.

(4) = Clone 5.3 sequenced with oligo R3 and run for 2 hours.

(5) = Clone 5.3 sequenced with oligo R3 and run for 5 hours.

(6) = Clone 3.3 sequenced with RSP and run for 3.25 and 7 hours.

(7) = Clone 4.1 sequenced with RSP and run for 1, 3, 5 and 7 hours.

(8) = Clone 4.1 sequenced with FSP and run for 2.5, 3.25, 5.5 and 7 hours.

(9) = Clone 4.1 sequenced with oligo No. 10 for 2.5 hours.

(10) = Clone 4.1 sequenced with R3 and run for 2 and 5 hours.

(————) = Vector sequence

N.B Not all sequence gained in each case is represented here, only that used to confirm independent sequencing results e.g. 5 and 9 are shorter than actually available whereas the full length of 10 is shown because it was confirmed by sequencing from a different direction to give 7.
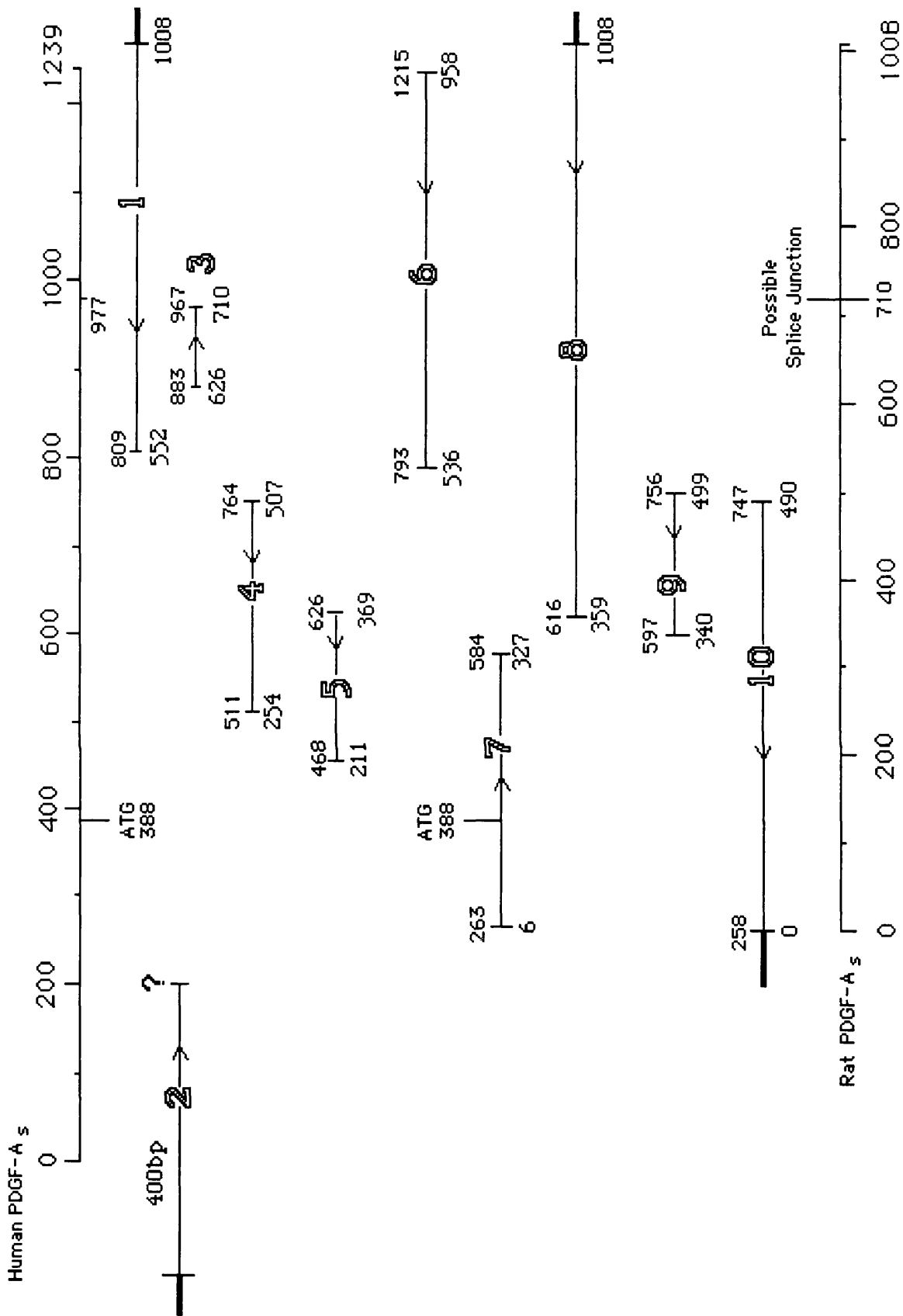
Figure 25.

Diagram showing alignment of the cDNA sequences for Human PDGF-AS chain cDNA (ℍ) (Betsholtz *et al.*, 1987) and rat PDGF-AS chain cDNA (ℝ). Numbering on the top refers to the Human sequence and on the bottom to rat sequence.

(•) = matching bases.

(-) = sequence absent.

(ⅢⅢⅢⅢ⟶) = Start codon.

(|) = Site of alternative splice junction.

(▬) = Stop Codon.

(↓) = Beginning and end of S1 nuclease probe used in Chapter 3.

```
                          30                                                70
TCCGCAAATA TGCAGAATTA CCGGCCGGGT CGCTCCTGAA GCCAGCGCGG GGAGGCAGCG CGGCGGCGGC  H
                          100                                               140
CAGCACCGGG AACGCACCGA GGAAGAAGCC CAGCCCCCGC CCTCCGCCCC TTCCGTCCCC ACCCCCATCC  H
                          170                                               210
CGGCGGCCCA GGAGGCTCCC CGCGCTGGCG CGCACTCCCT GTTTCTCCTC CTCCTGGCTG GCGCTGCCTG  H
                          240                                               280
CCTCTCCGCA CTCACTGCTC GCCGGGCGCC GTCCGCCAGC TCCGTGCTCC CCGCGCCACC CTCCTCCGGG  H
                                                                 •• •••• ••
                                                            GGA ATTCGGCACT GCAGTGGCGG
                          310                                               350
CCGCGCTCCC TAAGGGATGG TACTGATTTT CGCCGCCACA GGAGACCGGC TGGAGCGCCG CCCCGCGGCC  H
••••••••••  •• •••••• •    •• •••• •••• • ••  ••• •••• •  •••     •   •• •••••
CCGGGCCCGA TCTGGCCCGC GAGACCCCTG AGCGCTTCCG AGGAGCCCGG CTGGATCTCC CGCCCCGGCC  R
                          380                                               420
TCGCCTCTCC TCCGAGCAGC CAGCGCCTCG GGACGCGATG AGGACCTTGG CTTGCCTGCT GCTCCTCGGC  H
••••••• ••  •••••• ••• ••••••••••  •••••••••• •••••• •• •••••••••• ••••••••••
TCGCCTCCCT GCCGAGCTTC CAGCGCCTCG GGACGCGATG AGGACCTGGG CTTGCCTGCT GCTCCTCGGC  R
                                                ||||||||→
                          450                                               490
TGCGGATACC TCGCCCATGT TCTGGCCGAG GAAGCCGAGA TCCCCCGCGA GGTGATCGAG AGGCTGGCCC  H
•••••••••• •••••••••  •• •••••••• •••••••••• • •••••• •• •••••••••  •• ••••• •
TGCGGATACC TCGCCCATGC CCTGGCCGAG GAAGCCGAGA TACCCCGGGA GTTGATCGAG CGACTGGCTC  R
                          520                                               560
GCAGTCAGAT CCACAGCATC CGGGACCTCC AGCGACTCCT GGAGATAGAC TCCGTAGGGA GTGAGGATTC  H
  •••••••• •••••••••• •••••••••• •••••••• • •••••••••• ••••••• •• •••••••• •
GAAGTCAGAT CCACAGCATC CGGGACCTCC AGCGACTCTT GGAGATAGAC TCCGTAGGGG CTGAGGATGC  R
                          590                                               630
TTTGGACACC AGCCTGAGAG CTCACGGGGT CCATGCCACT AAGCATGTGC CCGAGAAGCG GCCCCTGCCC  H
  ••••• •• • ••••••••  •••••• •  •••• •• •• •••••••••• ••• ••••••• ••• ••••••
CTTGGAGACA AACCTGAGAG CCCATGGGTC CCACACTGTT AAGCATGTGC CGGAGAAGCG GCCTGTGCCC  R
                          660                                               700
ATTCGGAGGA AGAGAAGCAT CGAGGAAGCT GTCCCCGCTG TCTGCAAGAC CAGGACGGTC ATTTACGAGA  H
•• •• •••• •••••••••• ••••••••   ••• •• • • •••••••••• •••••••••• ••••••••••
ATCCGCAGGA AGAGAAGTAT TGAGGAAGCC ATTCCCGCAG TTTGCAAGAC CAGGACGGTC ATTTACGAGA  R
                  ↓                       730                               770
TTCCTCGGAG TCAGGTCGAC CCCACGTCCG CCAACTTCCT GATCTGGCCC CCGTGCGTGG AGGTGAAACG  H
••••••••••  ••• •••    •••••••• •• •••••••••• •••••••••• ••  •••••• •••••• ••
TACCTCGGAG CCAGGTGGAC CCCACATCGG CCAACTTCTT GATCTGGCCC CCATGTGTGG AGGTGAAGCG  R
                          800                                               840
CTGCACCGGC TGCTGCAACA CGAGCAGTGT CAAGTGCCAG CCCTCCCGCG TCCACCACCG CAGCGTCAAG  H
•••••• ••• •••• ••••• ••••• •• •• •••••••••• ••••• •• • •••••••••• ••• ••••••
CTGCACTGGC TGCTGTAACA CCAGCAGCGT CAAGTGCCAG CCCTCAAGGG TCCACCACCG CAGTGTCAAG  R
                          870                                               910
GTGGCCAAGG TGGAATACGT CAGGAAGAAG CCAAAATTAA AAGAAGTCCA GGTGAGGTTA GAGGAGCATT  H
•••••••••  •••• •• •• •••••••••• •••••••• • •••• •••••• •••••••••• •••••••• •
GTGGCCAAAG TGGAGTATGT CAGGAAGAAG CCAAAATTGA AAGAGGTCCA GGTGAGGTTA GAGGAGCACC  R
                          940                                               980
TGGAGTGCGC CTGCGCGACC ACAAGCCTGA ATCCGGATTA TCGGGAAGAG GACACGGATG TGAGGTGAGG  H
••••••••••  ••• ••••• •  ••• • •• • •••• • • •••••• • • •••••••••• ••••••••
TGGAGTGCGC ATGCGCGACC TCCAACCTGA ACCCAGACCA TCGGGAGGAG GAGACGGATG TGAGGTGAG-  R
                          1010                                              1050
ATGAGC-CGCA GCCCTTTCCT GGGACATGGA TGTACATGGC GTGTTACATT CCTGAACCTA CTATGTACGG  H
•• ••  •••• ••••• •••• •••••• ••• •••• ••••• ••••• •••• •••••• •• • •••••• •••
ATGAGCTGGCT GCCCTCTCCC GGGACACGGA TGTACGTGGC GTGTGACATT CCTGAACATA CTATGTATGG  R
                          1076                                              1116
TGCTTTATTG CCAGTGTGCG ----GTCTTT GTTCTCCTCC GTGAAAAACT GTGTCCGAGA ACACTCGGGA  H
•••• ••••• •••• •••••        ••• •••••••••• •••••••••• •••• •••••   
TGCTTCATTG CCAATGTGCG TGCAGTCTTT GTTCTCCTCC GTGAAAAACT GTGTCCGAGG ----------  R
                          1146                                              1186
GAACAAAGAG ACAGTGCACA TTTGTTTAAT GTGACATCAA AGCAAGTATT GTAGCACTCG GTGAAGCAGT  H
     ••••• ••••••• •   ••• •• ••• •••••••••• •••••••• • •••••••••• •   •• •••
--ACAAAGAG ACAGTGTCCG TTTGTTCAGT GTGACATCAA AGCAAGTACT GTAGCACTCG GAGAGACAGT  R
          ↓               1216                             1239
AAG-AAGCTT CCTTGTCAAA AAGAGAGAGA GAG------- --AAAGAAA AAAAAAGGAA TTC-------  H
•• •••••••  ••••••• • •••• •• •                   ••••••••• ••••••• ••
GAGAAAGCTT CCTTGTCACA GAGAGCGAAA ACAAAACCAC AAAAAAGAAA AAAAAAAAAA ACAAAAAATT  R
---------- --------- H
AAAAAAAAAC CGAATTCG  R
          1009
```

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Figure 26.

Diagram showing alignment of predicted amino acid sequences of PDGF-A$_S$ chain polypeptides from human (H), mouse (M), rat (R) and xenopus (X). The boxed areas define the regions which match in at least 2 of the 4 sequences. The filled bars above the sequence indicate the pre (▨▨▨) and pro (▨▨▨) peptide sections which are removed during processing to leave the mature polypeptide (▨▨▨). Conserved cysteines involved in disulphide bonding during dimerisation are indicated with a vertical bar (I). The alternative splice site position is also indicated relative to the amino acid sequence.

NB. Xenopus is 5 amino acids longer in total than the others.

(See Chapter 1 Table 3 for single letter amino acid code)

LEADER "PRE-PRO" PEPTIDE   PRO- PEPTIDE

MRTLACLLLL --GCGYLAHV LAEEAEIPRE VIERLARSQI HSIRDLQRLL   H
MRTYACLLLL --GCGYLAHV LAEEAEIPRE LIERLARSQI HSIRDLQRLL   M
MRTWACLLLL --GCGYLAHA LAEEAEIPRE LIERLARSQI HSIRDLQRLL   R
MRIWAWILLL SVCCSYLSPS LGEEAEIPQE LIERLAHSEI RSISDLQRLL   X

EIDSV-GSED -SLDTSLRA- -HGVHAIKHV PEKRPLPIRR KRSIEEAVPA   H
EIDSV-GAED A-LETSLRA- -HGSHAINHV PEKRPVPIRR KRSIEEAIPA   M
EIDSV-GAED A-LETNLRA- -HGSHTVKHV PEKRPVPIRR KRSIEEAIPA   R
DIDSVGGED ASA-ANIRSQ KHDFHHNRLV PEKRSVPSRR KRSVEEAVPA   X

VCKTRTVIYE IPRSQVDPTS ANFLIWPPCV EVKRCTGCCN TSSVKCQPSR   H
VCKTRTVIYE IPRSQVDPTW ANFLIWPPCV EVKRCTGCCN TSSVKCQPSR   M
VCKTRTVIYE IPRSQVDPTS ANFLIWPPCV EVKRCTGCCN TSSVKCQPSR   R
ICKTRTVIYE IPRSQIDPTS ANFLIWPPCV EVKRCTGCCN TSSVKCQPSR   X

                              Alternative Splice ₁ Junction

VHHRSVKVAK VEYVRKKPKL KEVQVRLEEH LECACATTSL NPDYREEDTDVR   H
VHHRSVKVAK VEYVRKKPKL KEVQVRLEEH LECACATSNL NPDHREEETDVR   M
VHHRSVKVAK VEYVRKKPKL KEVQVRLEEH LECACATSNL NPDHREEETDVR   R
IHHRSVKVAK VEYVRKKPKL KEVLVRLEEH LECTC-TANS NSDYREEETDVR   X

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Figure 27.

Diagrams showing the annealing locations of the human oligonucleotide PCR primers (No.s 1, 2, 6, 7, 9 and 10) and the rat oligonucleotide primers (No.s 11 and 12) relative to the two sequences from which they are derived (A) and showing the actual sequences to which they anneal on the rat cDNA including rat oligo R3 used for sequencing (B). In both A and B the top numbering refers to the rat PDGF-A$_S$ cDNA sequence and the bottom numbering in A refers to the Human cDNA sequence. (See also Figure 6).

N.B. The annealing location of human oligonucleotide No. 8 is not shown here because it lies within the exon 6 sequence not present in the PDGF-A$_S$ sequence shown here.

(▬) = Stop Codon.   (❙) = Alternative Splice Site.

(▢) = Oligos based on Human sequence and including indications of where within their length they differ from the given rat sequence.

(⬯) = Oligos based on rat sequence and therefore fully complementary.

(X)   = Indicates where the corresponding region of the Human sequence has been deleted or an insertion has occured in the rat sequence.

(G⤴) = Location of an additional G nucleotide in the human sequence.

(↑)   = Indicates where there is extra sequence present in the Human sequence not found to be in the rat sequence.

(⇀) = Indicates where the sequence shown is the actual sequence of the oligo.

(↽) = Indicates where the sequence shown is complementary to that of the oligo.

A.

RAT   650   750   850   910

No.11   No.12

No.1   No.2

No.6
No.7

No.9   No.10

850   HUMAN   950   1050   1150

B.

```
      550         560   R3   570         580         590         600
CGTCAAGTGC  CAGCCCTCAA  GGGTCCACCA  CCGCAGTGTC  AAGGTGGCCA  AAGTGGAGTA
                                                         G           A
                                                              No.9
```

```
      610         620         630         640  No.11  650         660
TGTCAGGAAG  AAGCCAAAAT  TGAAAGAGGT  CCAGGTGAGG  TTAGAGGAGC  ACCTGGAGTG
C                                                             TT
                                            No.1
```

```
      670         680         690         700         710         720
CGCATGCGCG  ACCTCCAACC  TGAACCCAGA  CCATCGGGAG  GAGGAGACGG  ATGTGAGGTG
         C
                                                    No.7          No.10
```

```
      730         740         750         760         770         780
AGATGAGCTG  GCTGCCCTCT  CCCGGGACAC  GGATGTACGT  GGCGTGTGAC  ATTCCTGAAC
         XC        A           T           T           A
G                                          No.6
```

```
      790         800         810         820         830         840
ATACTATGTA  TGGTGCTTCA  TTGCCAATGT  GCGTGCAGTC  TTTGTTCTCC  TCCGTGAAAA
              C           T          XXXX
                                No.2
```

```
      850         860         870         880  No.12  890         900
ACTGTGTCCG  AGGACAAAGA  GACAGTGTCC  GTTTGTTCAG  TGTGACATCA  AAGCAAGTAC
                                         T  A                        T
AGAACACTCGG
```

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Table 8.

Showing the size and location relative to the rat PDGF-A$_S$ chain cDNA sequence of the oligonucleotides used for the Polymerase Chain Reaction studies. (For position relative to Human cDNA sequence see Chapter 2 Tables 6 & 7). The type of restriction site, the length any uncomplementary tail and the effective end point of the PCR product, expressed relative to the rat sequence are also indicated. The number and location of any cross species mismatches are indicated as a guide to the likely specificity of each oligonucleotide.

Table 9.

Showing the PCR product sizes for amplifications of the known types of human and rat PDGF-A chain, with various combinations of oligonucleotide primers. Also shown are the predicted PDGF-A$_L$ product sizes for each combination of primers based on the PDGF-A$_S$ product size + 69 bp for exon 6.

N.B. Due to deletions and insertions the expected PCR product from amplifications with oligo No. 12 and any of oligos No. 1, 7, 9 and 11 would differ by 7 bp depending on whether it was from Human or rat sequence.

Table 8.

Oligo	Size	Location on RAT PDGF-A$_S$ Chain cDNA	Tail	5' End enzyme site	Actual PCR end	Mismatches with the RAT Sequence
No.1	36 mer	629 - 657	8	*BamHI*	621	2 centrally
No.2	33 mer	804 - 783	11	*HindIII*	815	2 centrally
No.6	35 mer	764 - 730	0	0	764	6 including the 3' end
No.7	20 mer	654 - 673	0	0	654	1 centrally
No.9	30 mer	584 - 607	6	*HindIII*	578	3 centrally
No.10	28 mer	734 - 712	5	*BamHI*	739	4 including a frame shift
Rat Specific						Mismatches with the Human Sequence
No. 11	20 mer	634 - 653	0	0	634	3 including the 3' end
No.12	23 mer	900 - 878	0	0	900	2 both at the 3' end

Table 9.

	7 + 10	11 + 12	12 + 7	11 + 2	7 + 2
Rat PDGF-A$_S$	85 bp	266 bp	246 bp	181 bp	161 bp
Rat PDGF-A$_L$ (Predicted)	154 bp	335 bp	315 bp	250 bp	230 bp
Human PDGF-A$_S$	85 bp	273 bp	253 bp	181 bp	161 bp
Human PDGF-A$_L$	154 bp	342 bp	322 bp	250 bp	230 bp
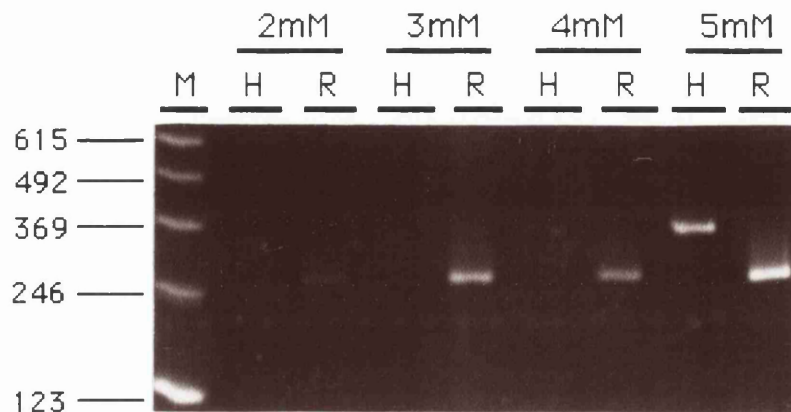
Figure 28.

Bisacrylyl-cystamine gel showing the products of a 20 cycle standard PCR amplification (Chapter 2.2 R ii) using rat specific oligos No. 11 & 12 on Human PDGF-A$_L$ chain cDNA plasmid (**H**) and on rat PDGF-A$_S$ chain cDNA plasmid (**R**) at four different concentrations of MgCl$_2$ indicated. Samples representing 10% of the total PCR product were run on the gel, stained with EtBr and visualised on a UV transilluminator. (**M**) = 123 bp ladder markers.

Figure 29.

Bisacrylyl-cystamine gel showing the products of a 20 cycle two temperature PCR amplification (Chapter 2.2 R ii) using combinations of rat specific oligos No. 11 & 12 and human sequence oligos No. 7 & 2 on human PDGF-A$_L$ chain cDNA plasmid (H) and on rat PDGF-A$_S$ chain cDNA plasmid (R). Samples representing 10% of the total PCR product were run on the gel, stained with EtBr and visualised on a UV transilluminator. (M) = 123 bp ladder markers.
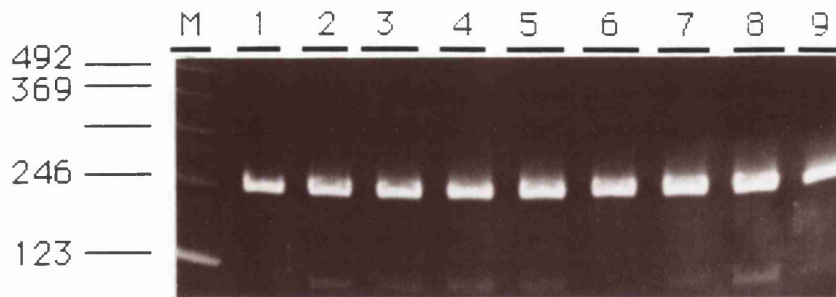
Figure 30.

Bisacrylyl-cystamine gel showing the products of a 25 cycle standard PCR amplification (Chapter 2.2 R ii) using oligonucleotides No.7 and No.12 (see Figure 27) on DNA isolated from single plaques of the nine unsequenced rat PDGF-A chain cDNA clones isolated from λgt11 P21 rat brain cDNA library (**1-9**). Samples representing 10% of the total PCR product were run on the gel, stained with EtBr and visualised on a UV transilluminator. (**M**) = 123 bp ladder markers.

1) Clone 1.2
2) Clone 3.1
3) Clone 3.2
4) Clone 3.4
5) Clone 5.1
6) Clone 5.2
7) Clone 5.4
8) Clone 6.1
9) Clone 6.2
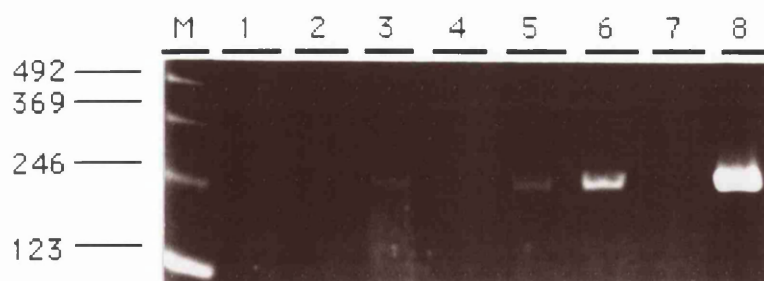
Figure 31.

Bisacrylyl-cystamine gel showing the products of a 30 cycle standard PCR amplification (Chapter 2.2 R ii) using of rat specific oligos No. 11 & 12 on cDNAs (1-3), rat cDNA libraries (4, 6 & 7), a Mouse brain cDNA library (5) and on rat PDGF-A$_S$ chain cDNA plasmid (8). Samples representing 10% of the total PCR product were run on the gel, stained with EtBr and visualised on a UV transilluminator. (**M**) = 123 bp ladder markers.

1) P8 Rat Brain cDNA
2) C6 Rat Cell Line cDNA
3) 157 Human Cell Line cDNA
4) P7 Rat Brain cDNA Library No. 5
5) Mouse Brain cDNA library
6) Rat Cerebellum cDNA library
7) Rat Hippocampus cDNA library
8) Rat PDGF-A$_S$ chain cDNA Plasmid :- +ve Control: 266 bp
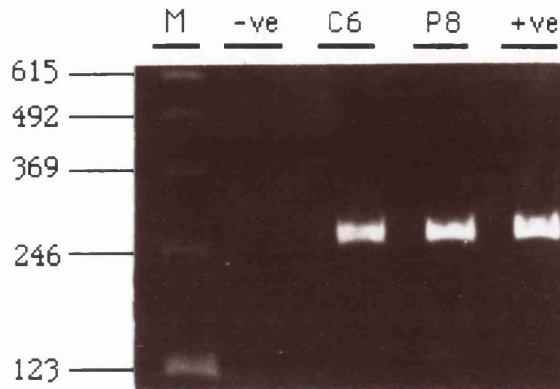
Figure 32.

Bisacrylyl-cystamine gel showing the products of a 30 cycle standard PCR amplification (Chapter 2.2 R ii) using of rat specific oligos No. 11 & 12 on P8 rat brain cDNA (**P8**), C6 rat cell line cDNA (**C6**) and on rat PDGF-As chain cDNA plasmid (**+ve**). PCR amplification in the absence of any added RNA or DNA was also carried out as a negative control (**-ve**). Samples representing 10% of the total PCR product were run on the gel, stained with EtBr and visualised on a UV transilluminator. (**M**) = 123 bp ladder markers.

Ⴞ Chapter Five Ⴞ

"One fine morning in May a slim young horsewoman might have been seen riding a glossy sorrel mare along the flower strewn avenues of the Bois de Boulogne."

Chapter 5. Analysis of PDGF-A chain isoform function.

5.1 Introduction.

In this chapter I describe experiments that attempt to define the functional difference(s) between the long and short alternative splice isoforms of the PDGF A chain (PDGF-A). In order to obtain plentiful supplies of the two PDGF-A isoforms, I used expression vectors encoding human PDGF-A$_S$ or PDGF-A$_L$ that allowed me to produce each homodimer by transient transfection of Cos cells (see Chapter 2.3 D & E and Appendix A). This system was chosen over a bacterial or yeast expression system because it is more likely to produce the proteins with appropriate post-translation modifications.

In this way I was able to produce both PDGF-A isoform homodimers for comparative mitogenic assays. However, the absence of any specific antibodies to PDGF-A chain at that time meant that my options for further analysis were limited. To facilitate immunological detection of the polypeptides encoded by the expression vectors, I used different constructs that directed production of PDGF peptides containing a c-myc epitope (Myc-Tag) recognised by monoclonal 9E10 {Evan et al., 1985} (See Chapter 2.3 D and 2.4 A and Appendix A). The Myc-Tag sequence included in the final polypeptide was located close to the alternative splice junction based on the assumption that variation in this region would not affect the mitogenic properties of the protein. Xenopus PDGF-A$_L$, for example, is thought to have an extra 11 amino acid sequence of unknown function at this position, which does not appear to block the mitogenic action of the peptide {Mercola et al., 1988}.

Cos cells transfected with the Myc-tagged PDGF-A expression vectors secreted the same amount of mitogenic activity as cells transfected under identical conditions with the untagged PDGF-A vectors, suggesting that addition of the Myc-Tag did not adversely affect the biological activity of the PDGF-A (Figure 33 a). Unless otherwise stated all the experiments described hereafter were performed with the Myc-tagged versions of PDGF-A$_L$ and PDGF-A$_S$.

5.2 PDGF-A$_S$ is efficiently secreted from Cos cells whereas PDGF-A$_L$ is not.

I tested the ability of the PDGF expression vectors to direct synthesis and

secretion of active PDGF-A$_L$ and PDGF-A$_S$ dimers from transfected Cos cells, by transfecting equal amounts of plasmid vectors encoding Myc-tagged or untagged PDGF-A$_L$ or PDGF-A$_S$, or the expression vector alone, into cultured Cos cells (see Chapter 2.3 E). After 2 days the culture media were collected and analysed for mitogenic activity (see Chapter 2.4 F). Supernatants harvested from untransfected Cos cells displayed similar levels of mitogenic activity to those from Cos cells transfected by vector alone (Data not shown), indicating that transfection *per se* did not induce endogenous secreted soluble mitogen production. Cells expressing PDGF-A$_S$ (tagged or untagged) secreted large amounts of mitogenic activity into the medium whereas cells expressing PDGF-A$_L$ (tagged or untagged) did not (Figure 33). Although variation in the relative levels of PDGF-A$_S$ and PDGF-A$_L$ mitogenic activity was observed between experiments (extremes shown Figure 33 a & b), the level of PDGF-A$_L$ activity recovered never exceeded 30% of that obtained from PDGF-A$_S$ transfected Cos cells (Figure 33 b) and was usually considerably less. Northern blot analysis of RNA from Cos cells producing almost no soluble PDGF-A$_L$, showed similar levels of PDGF-A RNA to those producing high levels of soluble PDGF-A$_S$, indicating that this effect was not related to differences in the efficiency of PDGF-A$_S$ and PDGF-A$_L$ transfection (Figure 33 a, inset). The variability in secreted mitogenic activity was mirrored by the amount of metabolically radiolabeled PDGF-A$_L$ that I could detect in Cos cell supernatants by immunoprecipitation with the anti-Myc antibody 9E10: sometimes ^{35}S-labelled PDGF-A$_L$ was almost undetectable in the Cos cell culture medium while in other experiments PDGF-A$_L$ could be readily detected though always below the level of PDGF-A$_S$ (e.g. Figures 34 & 36).

I performed pulse chase metabolic labeling of Cos cells expressing PDGF-A$_S$ or PDGF-A$_L$ (see Chapter 2.3 G) in an attempt to discover whether there was any difference in the timing of the appearance of each form in the supernatant. The result showed that PDGF-A$_L$ took longer to appear in the culture medium than PDGF-A$_S$ (Figure 34) indicating that PDGF-A$_L$ was either manufactured at a much slower rate or was in some way prevented from being released rapidly. The detection of equivalent mRNA levels for each isoform (Figure 33 a, inset) and the fact that both necessarily had identical translation signals favoured the latter explanation. These results might be explained by postulating that PDGF-A$_L$ is normally compartmentalised within or outside the cells and is released into the medium only after the available binding sites are

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saturated. Thus the amount of PDGF-A$_L$ that accumulates in the medium would depend critically on the absolute level of PDGF-A$_L$ expression, the number of binding sites in or on the cell and whether there was any mechanism for release once bound.

5.3   PDGF-A$_L$ can be visualised on and around cells by immunofluorescence.

I looked for evidence of differential compartmentalisation of PDGF-A isoforms by indirect immunofluorescence microscopy of transfected Cos cells expressing PDGF-A$_S$ or PDGF-A$_L$ (see Chapter 2.4 D). In fixed, detergent-permeabilised cells, similar levels of PDGF-A$_L$ and PDGF-A$_S$ could be seen in the endoplasmic reticulum (ER), Golgi apparatus and in presumptive transport vesicles in the cytoplasm (Figure 35 A & B) suggesting that the equivalent levels of mRNA were being translated into comparable levels of protein inside the cells. PDGF-A$_L$, but not PDGF-A$_S$, also gave rise to an indistinct halo of immunostaining that extended beyond the perimeter of the cell. This difference was even more pronounced when unpermeabilised cells were fixed and stained. Under these conditions the ER and Golgi staining was not apparent and no appreciable staining was observed on PDGF-A$_S$ transfected cells (Figure 35 C). The PDGF-A$_L$ transfected cells, however, were surrounded by areas of bright immuno-staining (Figure 35 D) suggesting that PDGF-A$_L$ may be sequestered outside the cells in association with the extra-cellular matrix (ECM) rather than inside or directly on the surface of the cells.

5.4   PDGF-A$_L$ can be released from the ECM with high salt.

The highly basic nature of the PDGF-A$_L$ carboxy-terminal tail has led to speculation that it may bind to oppositely charged ECM components, such as the acidic GAGs, *in vivo* {Ross *et al.*, 1986}. Indeed it has been shown that PDGF (type unspecified) has an affinity for the ECM component heparin *in vitro* and can be eluted at salt concentrations of O.5 M and higher {Shing *et al.*, 1984; Vlodavsky *et al.*, 1987b}. This suggested, together with the circumstantial evidence presented above, that the basic tail may function to direct binding of PDGF-A$_L$ to heparin in the ECM *in vivo*. It has been shown that members of the FGF family, which are known to associate with the ECM by binding to HPGs (see Chapter 1.6), can be released by extraction with NaCl {Baird and Ling, 1987}. I therefore attempted to test whether PDGF-A$_L$ was bound to the matrix in this

way, by salt extracting crude ECM, prepared by lysing Cos cells expressing either PDGF-A$_S$ or PDGF-A$_L$ and immunoprecipitating the eluted proteins (see Chapter 2.3 H and 2.4 A). The initial result showed clearly that, while Cos cells expressing PDGF-A$_S$ secreted higher levels of processed PDGF into the supernatant and showed signs of unprocessed PDGF in the lysate and matrix extracted samples, only PDGF-A$_L$ showed any sign of processed PDGF eluting from the matrix (Figure 36). It seemed likely that the unprocessed PDGF-A$_S$ present in matrix extraction sample represented the remains of that released during cell lysis whereas the processed PDGF-A$_L$ present in the extracted sample was more likely to represent protein that had been secreted from the cells and bound to the ECM prior to cell lysis.

Any doubts that the PDGF-A$_L$ might simply have been left behind after cell lysis rather than eluted specifically from the remaining ECM, were removed by repeating the procedure, this time washing the cells extensively after removal of the supernatant and washing the ECM after lysis. In each case the final washes were precipitated to check that all free PDGF had been removed prior to further extraction. I also washed and extracted the culture dishes to discover if any PDGF-A$_L$ remained associated with the cell-free ECM. The result confirmed that PDGF-A$_L$ was sequestered in the insoluble pellet of lysed cells and to a lesser extent in the cell-free ECM (Figure 37 b). PDGF-A$_S$, on the other hand, was not released from the cell pellet or the ECM, although copious amounts were present in the culture medium (Figure 37 a). In this more rigorous experiment no unprocessed PDGF-A$_S$ was detected in the cell pellet or the ECM fractions.

In this second experiment two different-sized forms of PDGF-A$_L$ were eluted from the cell pellet (Figure 37 b, Lane 6); the higher MW polypeptide appeared to be approximately the same size as that precipitated from the supernatant while the lower band was similar in size to that released from the cell-free ECM (Figure 37 b, Lane 4). Raines and Ross (1992) have suggested that the PDGF-A$_L$ tail may be proteolytically cleaved at a number of points along its length by endopeptidases known to act at multiple mono-, di-, and polybasic sites like those present in the PDGF-A$_L$ tail (Figure 5 & Table 3) {Fisher and Scheller, 1988} making it possible that this lower band represents a partial proteolytic cleavage product(s) which remains bound by residual PDGF-A$_L$ tail sequence(s).

5.5 Mitogenic activity is associated with the matrix produced by cells expressing PDGF-A$_L$ but not cells expressing PDGF-A$_S$.

Having shown by immunofluorescence staining that PDGF-A$_L$ localises on and around cells and by immunoprecipitation that it can be extracted from the lysed cell pellet and to a lesser extent from the cell-free ECM, I attempted to obtain direct biological evidence for association of PDGF-A$_L$ with ECM. I prepared ECM from Cos cells expressing either PDGF-A$_S$ or PDGF-A$_L$, as well as untransfected cells and looked for mitogenic activity associated with the ECM by seeding quiescent NIH-3T3 cells onto the respective ECM preparations (see Chapter 2.4 F and G). In each case I prepared four lots of ECM: one was washed with physiological salt, one extracted with 0.3 M NaCl buffer, another with 0.7 M NaCl buffer (see Chapter 2.3 H) and the fourth was supplemented with 10 % FCS. In this way I hoped to compare the mitogenic response elicited by the unsupplemented ECM with the maximal response elicited by serum. I also hoped to discover whether I could remove any bound mitogenic activity from the ECM by salt washing and if so at what salt concentration it was eluted.

The result showed that ECM prepared from cells transfected with PDGF-A$_L$ induced a maximal response in the 3T3 cells as judged by comparison with the cells grown on ECM in the presence of 10% FCS. ECM from PDGF-A$_S$ expressing Cos cells, on the other hand, elicited a similar a level of ^{3}H thymidine incorporation as the ECM from untransfected Cos cells (Figure 38) as did ECM prepared from Cos cells transfected with vector alone (not shown). The 0.3 M NaCl salt extraction of the PDGF-A$_L$ matrix did not significantly reduce the levels of mitogenic activity. The 0.7 M NaCl extraction did, however, remove much of the mitogenic activity bringing the response of the 3T3 cells down to within the range of that induced by the PDGF-A$_S$ transfected and the untransfected Cos cell ECMs. This provides further and more direct evidence for the specific association of PDGF-A$_L$, but not PDGF-A$_S$, to the ECM. Moreover it suggests that PDGF-A$_L$ sequestered in the ECM retains biological activity and is available to cells growing in contact with the ECM.

5.6 PDGF-A$_L$ binds specifically to the ECM component heparin in vitro.

Having specifically released PDGF-A$_L$, but not PDGF-A$_S$, from ECM using salt conditions known to dissociate PDGF (type unspecified) from heparin in

vitro, and with some preliminary evidence that the COOH terminal sequence of the soluble PDGF-A$_L$ produced by my Cos cell expression system remained intact, I sought to confirm that the differences observed in the affinity for ECM of the two PDGF-A isoforms were the result of a difference in affinity for heparin. Supernatants from Cos cells expressing PDGF-A$_S$ or PDGF-A$_L$ were divided into three: one third was kept as a control, one third was exposed to heparin covalently coupled to Sepharose and one third was exposed to Sepharose alone (see Chapter 2.4 E). The bound proteins were then eluted by sequential additions of increasing concentrations of salt, the first and last washes at each concentration dialysed to isotonicity, immunoprecipitated and the precipitate visualised by gel fluorography. In parallel I immunoprecipitated and electrophoresed the pooled heparin-Sepharose washes at each salt concentration.

PDGF-A$_S$ bound very poorly to either Sepharose (not shown) or heparin-Sepharose (Figure 39 A) the majority being removed during the physiological (0.15 M NaCl) washes (Figure 39 B) with no further elution being observed at concentrations up to and including 0.7 M NaCl. Furthermore approximately equivalent levels were precipitated from the supernatant which had been exposed to heparin-Sepharose and the control sample (respectively Lane 0.15 M α and lane U, Figure 39 A). PDGF-A$_L$ did not bind to Sepharose (Figure 40 B), but did bind to heparin-Sepharose (Figure 40 A) remaining stably bound in 0.3 M NaCl but eluting when the concentration was raised to 0.5 M NaCl. No further PDGF-A$_L$ was eluted at concentrations as high as 2 M NaCl (not shown). Comparison of the unbound and recovered PDGF-A$_L$ with the control sample shows that the amount bound to heparin-Sepharose was probably greater than that subsequently eluted in the high salt washes. This was true even if I included the PDGF-A$_L$ precipitated from the pooled 0.5 M NaCl heparin-sepharose wash (Figure 40 C). However, despite this discrepancy the results clearly showed that PDGF-A$_L$ can bind to heparin either directly or via a third party molecule present in the Cos cell supernatants, whereas PDGF-A$_S$ can not.

5.7   Discussion.

There is now ample evidence that the highly basic, exon 6-derived carboxy terminus of PDGF-A$_L$, and a similar sequence in PDGF-B, are responsible for immobilising these molecules on extracellular binding sites

{Östman *et al.,* 1991; LaRochelle *et al.,* 1991; Raines and Ross, 1992; Khachigian *et al.,* 1992}. In contrast PDGF-A$_S$, which does not possess an equivalent sequence, is secreted from cells as a freely-diffusible molecule. PDGF-A$_L$ and PDGF-B can be released from cells by addition of peptides corresponding to the PDGF-A$_L$ and PDGF-B retention domains, and also by soluble HPGs {Raines and Ross, 1992}. Moreover, a synthetic peptide corresponding to the PDGF-A$_L$ retention domain binds to cell surfaces in a manner that was inhibited by soluble heparin, while iodinated heparin binds to the peptide immobilised on nitrocellulose {Khachigian *et al.,* 1992}. These studies provided evidence that the PDGF retention domains bind to HPGs on the cell surface and in the extracellular matrix (ECM). The work described in this chapter provides additional evidence that strongly supports this view.

I have shown that PDGF-A$_L$, but not PDGF-A$_S$, can be detected by immunofluorescence microscopy on and around cells in a distribution suggesting association with the ECM and that metabolically labelled PDGF-A$_L$ can be eluted from cell and ECM preparations with a high salt wash. I was able to show that cell-free ECM, prepared from cells transfected with PDGF-A$_L$ expression construct, possesses significant levels of salt extractable mitogenic activity whereas ECM prepared from identical cells transfected in parallel with the PDGF-A$_S$ construct do not. Furthermore I have shown that the recombinant PDGF-A$_L$ released by Cos cells can bind to heparin-Sepharose *in vitro* and may be eluted by salt concentrations between 0.3 M and 0.5 M. It seems likely, therefore, that the reason PDGF-A$_L$ does not normally accumulate to high levels in cell culture medium is due, at least in part, to its ability to bind heparin-like proteoglycans on the cell surface and in the ECM.

There have been conflicting reports about the ability of PDGF-A$_L$ to be secreted from cells. The release of PDGF-A$_L$ from transfected NIH 3T3 cells {LaRochelle *et al.,* 1990} was found to be dependent on proteolytic removal of the carboxy-terminal retention domain and could be prevented by the drug monensin, which inhibits proteolytic processing of PDGF {LaRochelle *et al.,* 1991}. On the other hand, PDGF-A$_L$ could not be detected in the culture medium of rat-1 cells and human fibroblasts expressing retroviral vector encoded PDGF-A$_L$ {Bywater *et al.,* 1988}; constitutively expressing human endothelial cells and smooth muscle cells {Raines & Ross, 1992}; or transiently transfected Cos cells {Östman *et al.,* 1991}. It seems, therefore, that NIH 3T3 cells differ

from these other cells in their ability to cleave the PDGF-A$_L$ retention domain. In my experiments I found that PDGF-A$_L$ was released from transfected Cos cells; however, the level of PDGF-A$_L$ in the culture medium was always less (sometimes much less) than the amount of PDGF-A$_S$ that accumulated in the medium under similar experimental conditions. The fact that the soluble PDGF-A$_L$ was equivalent in size to that extracted from the cell surface and bigger than a second extracted form, which probably represented a partially COOH processed form, suggested that it possessed an intact retention domain. In addition, the observation, that soluble PDGF-A$_L$ was able to bind to heparin whereas PDGF-A$_S$ did not, provided further strong support for the view that the soluble PDGF-A$_L$ had not been processed to resemble the soluble PDGF-A$_S$. It would seem, therefore, that the reason PDGF-A$_L$ was released from Cos cells in these experiments was because the available binding sites in the ECM were saturated by the amount of PDGF-A$_L$ generated by my expression system rather than as a result of proteolytic processing of the retention domain.

The PDGF-B primary translation product is proteolytically processed at the amino-terminus to give a 30 kDa homodimeric form, low levels of which can be found in the supernatant {Bywater *et al.*, 1988; Östman *et al.*, 1991}, however, the vast majority undergoes further proteolytic cleavage of the carboxy-terminus to give a 24 kDa form that remains associated with the cell {Robbins *et al.*, 1985; Leal *et al.*, 1985; Bywater et al., 1988; Östman *et al.*, 1988}. This last observation caused some early confusion over the role of the basic COOH sequence in PDGF-B and the analogous sequence in the PDGF-A$_L$ tail because it is clear that the mature PDGF-B chain does not include the carboxy-terminal basic sequence now shown to be required for retention by the cell. There are, however, studies showing that it is possible to displace PDGF-B by competition with peptides corresponding to either the B or A$_L$ retention domain {Raines and Ross, 1992} and by elution with 0.5 M NaCl {LaRochelle *et al.*, 1991} suggesting that there is some association between intact PDGF-B and HPGs. In addition, there is evidence to suggest that the amino terminally processed 35 kDa PDGF-A$_L$ undergoes proteolytic processing, similar to that observed for PDGF-B, to produce a 28 kDa cell associated form {Östman et al., 1991}. In both cases it appears that this processing is dependent on retention {Robbins *et al.*, 1985; Östman et al., 1991}. It therefore seems possible that the association mediated by the basic COOH sequence present in PDGF-B and that in the PDGF-A$_L$ tail might facilitate a second, undefined, mechanism after which

the COOH pro-sequence is removed by proteolytic cleavage.

It has been suggested that this secondary retention mechanism may involve other sequences present in the mature form of PDGF-B {Raines and Ross, 1992}, though it is clear that these alone are insufficient to bring about retention; such sequences may only become important during or after removal of the primary retention signal. It is known, for example, that proteolytic processing by endopeptidases is commonly associated with other processing events such as amidation of the exposed COOH residue {Fisher and Scheller, 1988}. Detailed characterisation of endopeptidase KEX2, which cleaves at basic residues such as those present in the retention regions of both PDGF-B and PDGF-A_L (see Figure 5), revealed that it exists as a cell associated form {Fuller *et al.*, 1989}, providing a possible explanation for the apparent requirement for cell association prior to terminal processing. In addition, the amidation enzyme peptidyl-glycine α-amidating monooxygenase (PAM) has been shown to act on terminal glycine residues {Murthy et al., 1987} such as the conserved glycine present in both PDGF-B and A_L that may be exposed by proteolytic removal of the basic amino acid sequence (see Figure 5). However, it is not clear that amidation occurs in this instance or indeed whether it can lead to retention. The post translational modification isoprenylation, on the other hand, is known to play a critical role in promoting the cell membrane association of p21^{ras} {Hancock *et al.*, 1989}. In this case, although proteins which undergo isoprenylation have so far been thought to require a four amino acid carboxy terminal signal sequence "Cxxx" not present in either PDGF-B or PDGF-A_L, many also have a region of basic amino acids just prior to this motif. In the GTP-binding protein rac1 this additional motif includes the KKRKRK sequence conserved in the PDGF-A_L tail {Maltese, 1990}. Thus although the nature of this putative secondary retention mechanism is far from clear there are a number of interesting possibilities worthy of investigation.

Finally, although the evidence suggests that PDGF-B and PDGF-A_L may both be processed to COOH truncated forms that can remain associated with the cell, it is clear that proteolysis can also release PDGF-A_L {LaRochelle *et al.*, 1990 & 1991}. It seems possible that this difference may be due to the greater numbers of basic residues within the PDGF-A_L tail (see Figure 5) leading to a more general proteolysis whereas the PDGF-B sequence may only undergo a

more specific proteolytic cleavage, which in conjunction with further modification serves to prevent release. Certainly there seems to be no evidence for release of the 24 kDa mature form of PDGF-B and indeed it has been proposed that the soluble 30 kDa PDGF-B may be the result of overloading the processing capacity of the cells {Östman *et al.*, 1991} as I have suggested for the soluble PDGF-A$_L$ observed in my experiments. It is perhaps significant that of all the cell types used, only the NIH 3T3 cells, which do not normally express PDGF, release PDGF-A$_L$ in this way. All other types investigated to date retain the PDGF-A$_L$, these include human endothelial and smooth muscle cells, which express PDGF *in vivo* {Raines & Ross, 1992}. It seems likely, therefore, that PDGF-A$_L$ produced *in vivo* would normally be retained by the cell with any requirement for soluble PDGF-A being met by production of PDGF-A$_S$. Release from NIH 3T3 cells could then be explained either by aberrant endopeptidase activity or as the result of a release mechanism employed only in specific incidences rather than as a general system.

Figure 33.

Graphs showing mitogenic activities released into the supernatants of Cos cells transfected with the PDGF-A expression vectors indicated, estimated by stimulation of ^{3}H-thymidine incorporation in NIH 3T3 cells (Chapter 2.4 F i). Supernatants from Cos cells expressing either Myc-tagged and untagged versions of the two isoforms were compared along with the corresponding Northern blotted RNA probed with radiolabeled human PDGF-A$_L$ cDNA (A). Experimental variation in the relative recovery levels of PDGF-A$_S$ and PDGF-A$_L$ as demonstrated by their ability to stimulate ^{3}H-thymidine incorporation is indicated by the second panel (B).

A.

Legend:
- VECTOR (open square)
- PDGF A $_S$ + TAG (filled circle)
- PDGF A $_L$ + TAG (filled triangle)
- PDGF A $_S$ (open circle)
- PDGF A $_L$ (open triangle)

Experiment 1.

Y-axis: CPM x10^4
X-axis: Fold dilution

B.

Legend:
- VECTOR (open square)
- PDGF A $_S$ + TAG (filled circle)
- PDGF A $_L$ + TAG (filled triangle)

Experiment 2.

Y-axis: CPM x10^4
X-axis: Fold dilution
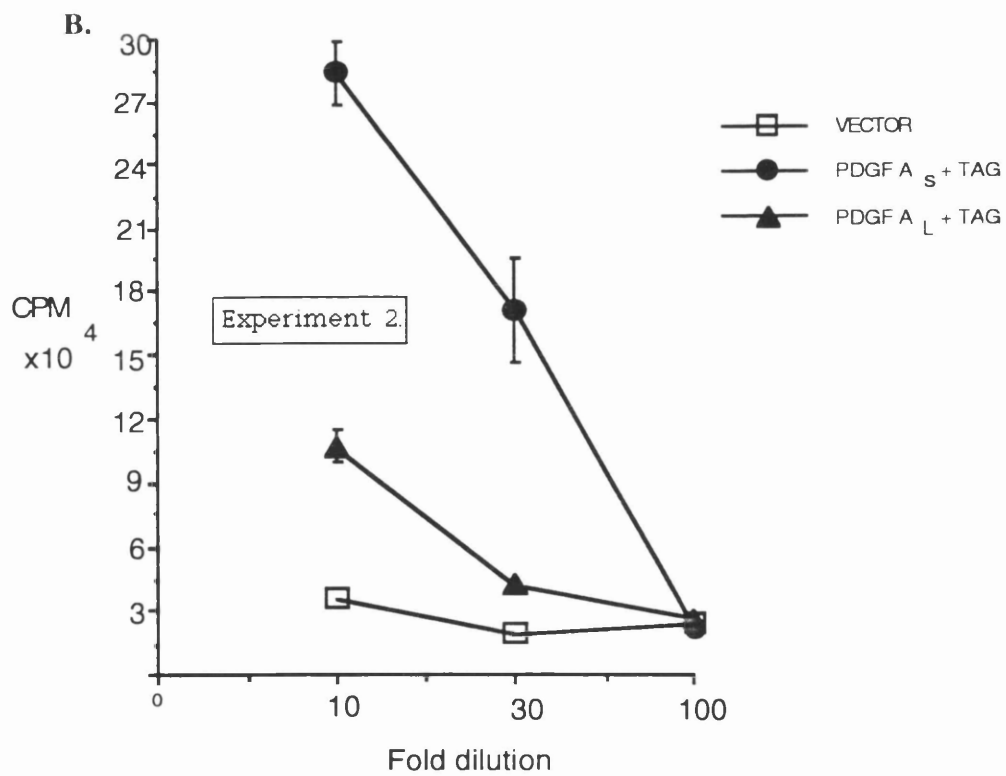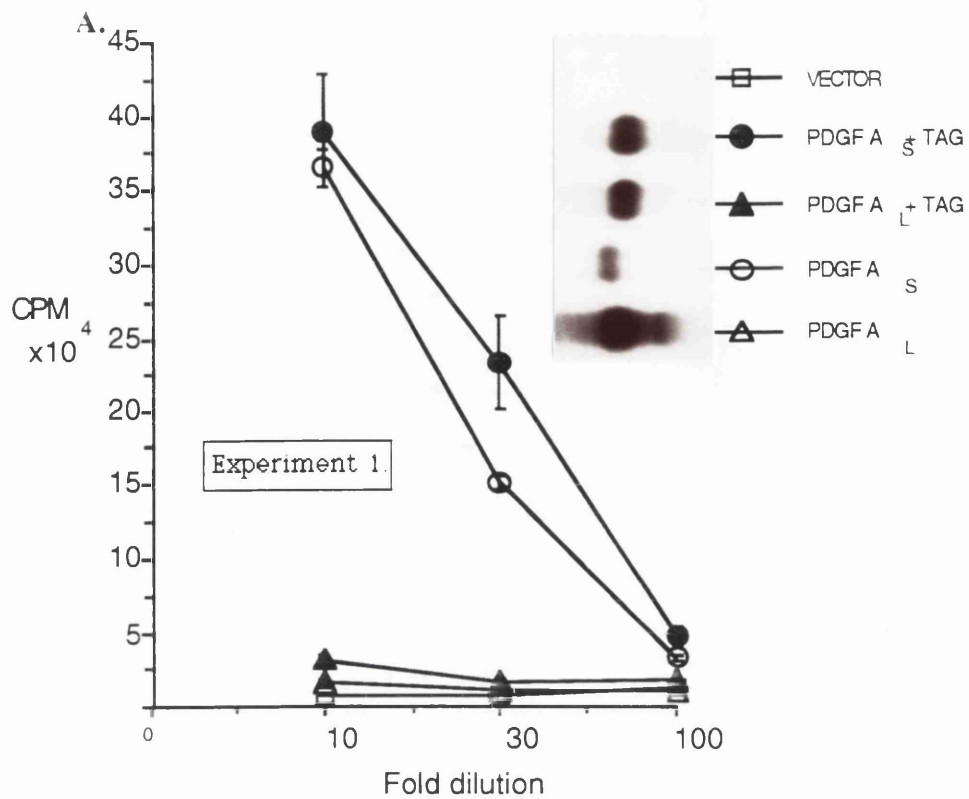
Figure 34.

Fluorograph of a 15% SDS-polyacrylamide gel showing the products of an anti-Myc (9E10) immunoprecipitation of the pulse chase ^{35}S labeled proteins in the supernatants of Cos cells transfected with PDGF-A$_S$ (**1-4**) and PDGF-A$_L$ (**5-8**). Pulse labeling was carried out as described (Chapter 2.3 G) with the chase periods in normal E4 medium being as follows:-

1) Immunoprecipitation of PDGF-A$_S$ after 30 minutes chase.

2) Immunoprecipitation of PDGF-A$_S$ after 90 minutes chase.

3) Immunoprecipitation of PDGF-A$_S$ after 3 hours chase.

4) Immunoprecipitation of PDGF-A$_S$ after 5 hours chase.

5) Immunoprecipitation of PDGF-A$_L$ after 30 minutes chase.

6) Immunoprecipitation of PDGF-A$_L$ after 90 minutes chase.

7) Immunoprecipitation of PDGF-A$_L$ after 3 hours chase.

8) Immunoprecipitation of PDGF-A$_L$ after 5 hours chase.
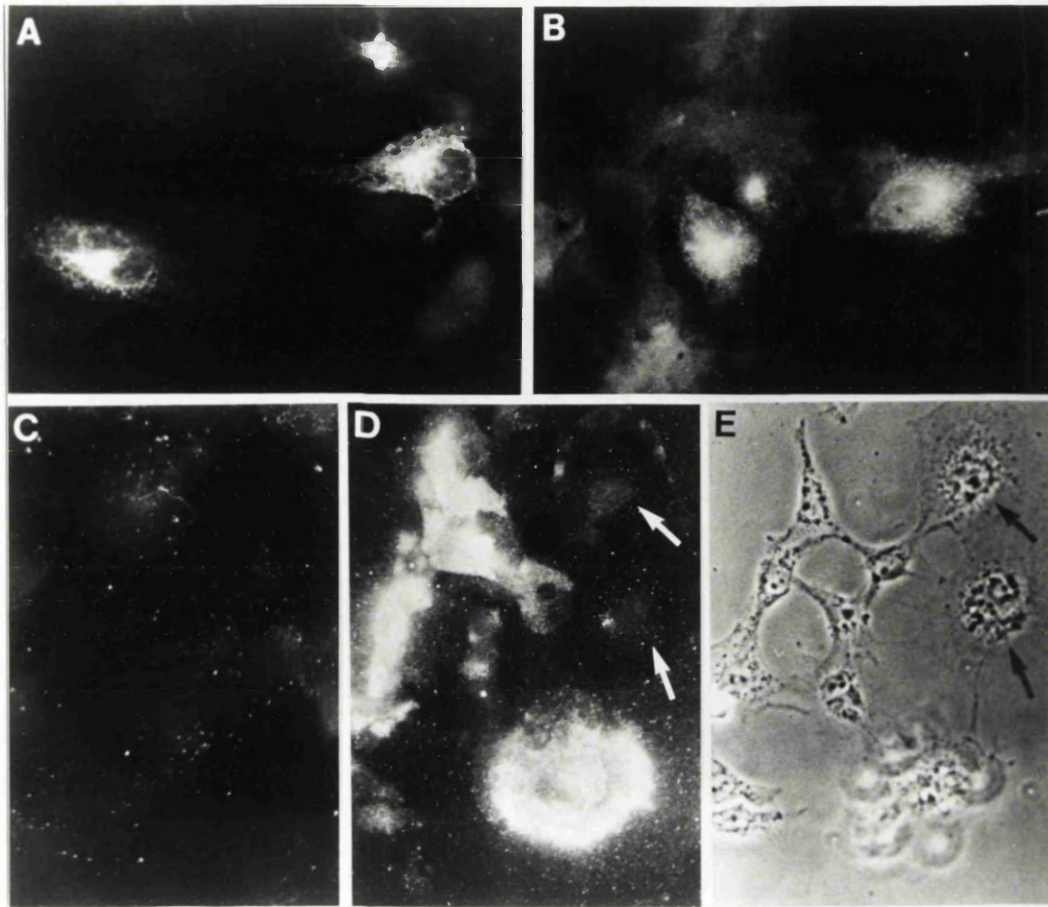
Figure   35.

Immunofluorescence   and   phase-contrast   micrographs   of
transfected Cos cells expressing Myc-tagged PDGF-A$_S$ (**A** & **C**) or
PDGF-A$_L$ (**B, D** & **E**). Cells were fixed and stained with anti-Myc
antibody 9E10 either with (**A** & **B**) or without (**C, D** & **E**) prior
permeabilisation with non-ionic detergent NP40 (Chapter 2.4 D).
Panels **D** and **E** are respectively immunofluorescence and phase-
contrast images of the same field with arrows sited in equivalent
positions in both panels as a visual aid to alignment.

Figure   36.

Fluorograph of a 15% SDS-polyacrylamide reducing gel showing the products of an anti-Myc (9E10) immunoprecipitation of the ^{35}S labeled proteins in the supernatants of Cos cells transfected with PDGF-A_S (**1**) and PDGF-A_L (**4**). The cells were lysed with isotonic lysis buffer (Chapter 2.3 H) and the lysate immunoprecipitated (**2** & **5**). The proteins remaining on the lysed cell debris (including ECM) were then extracted with 0.8M NaCl, the eluate dialysed to isotonicity and precipitated as above (**3** & **6**). All the samples were reduced by boiling in 2-mercaptoethanol sample buffer prior to loading on the gel.

1) Supernatant from Cos cell expressing PDGF-A_S

2) Lysate from Cos cell expressing PDGF-A_S

3) 0.8M NaCl wash of ECM from Cos cells expressing PDGF-A_S

4) Supernatant from Cos cell expressing PDGF-A_L

5) Lysate from Cos cell expressing PDGF-A_L

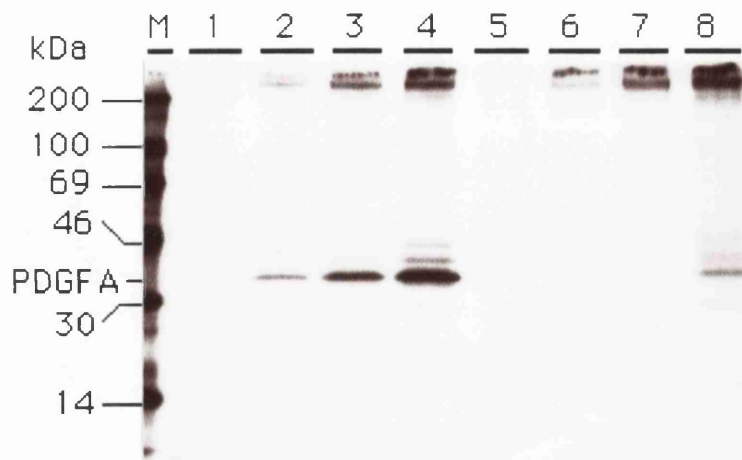6) 0.8M NaCl wash of ECM from Cos cells expressing PDGF-A_L

Figure 37.

Fluorographs of a 15% SDS-polyacrylamide gels showing the products of an anti-Myc (9E10) immunoprecipitation of the ^{35}S labeled proteins in the supernatants of Cos cells transfected with PDGF-A$_S$ (A Lane 1) and PDGF-A$_L$ (B Lane 1). The cells were washed 3x with 5 mls of E4 medium and a final wash of 2 mls kept for immunoprecipitation (2) before the cells were lysed with isotonic lysis buffer (Chapter 2.3 H) the lysate removed and the debris spun out. The tissue culture dish containing cell matrix and the lysed cell debris pellet were both washed as above and the final wash collected for immunoprecipitation (respectively 3 & 5). These were then extracted with 0.8M NaCl, 0.01M Tris pH7 which was immunoprecipitated after being dialysed to 0.15M NaCl (respectively 4 & 6). The dish and the pellet were washed as above in the 0.8M NaCl buffer and further extracted with 1.5M NaCl which in turn was immunoprecipitated after being dialysed to 0.15M NaCl (respectively 7 & 8).

1) Immunoprecipitation of Cos cell supernatant
2) Immunoprecipitation of last cell wash prior to cell lysis
3) Immunoprecipitation of last wash of tissue culture dish containing ECM
4) Immunoprecipitation of dialysed 0.8M NaCl extraction of culture dish
5) Immunoprecipitation of last wash of lysed cell pellet also containing ECM
6) Immunoprecipitation of dialysed 0.8M NaCl extraction lysed cell pellet
7) Immunoprecipitation of dialysed 1.5M NaCl extraction of culture dish
8) Immunoprecipitation of dialysed 1.5M NaCl extraction lysed cell pellet

A.

B.

Intact PDGF-A$_L$
Processed PDGF-A$_L$?
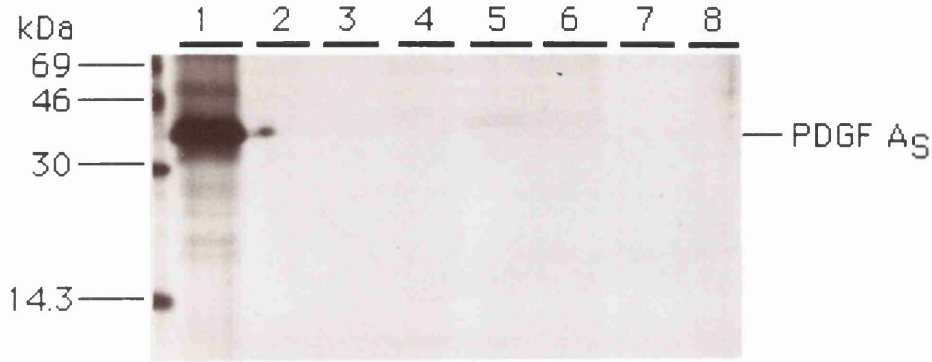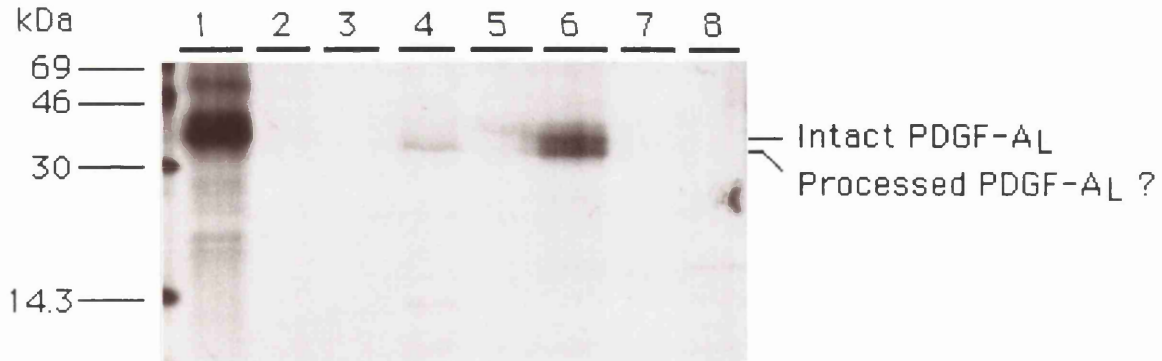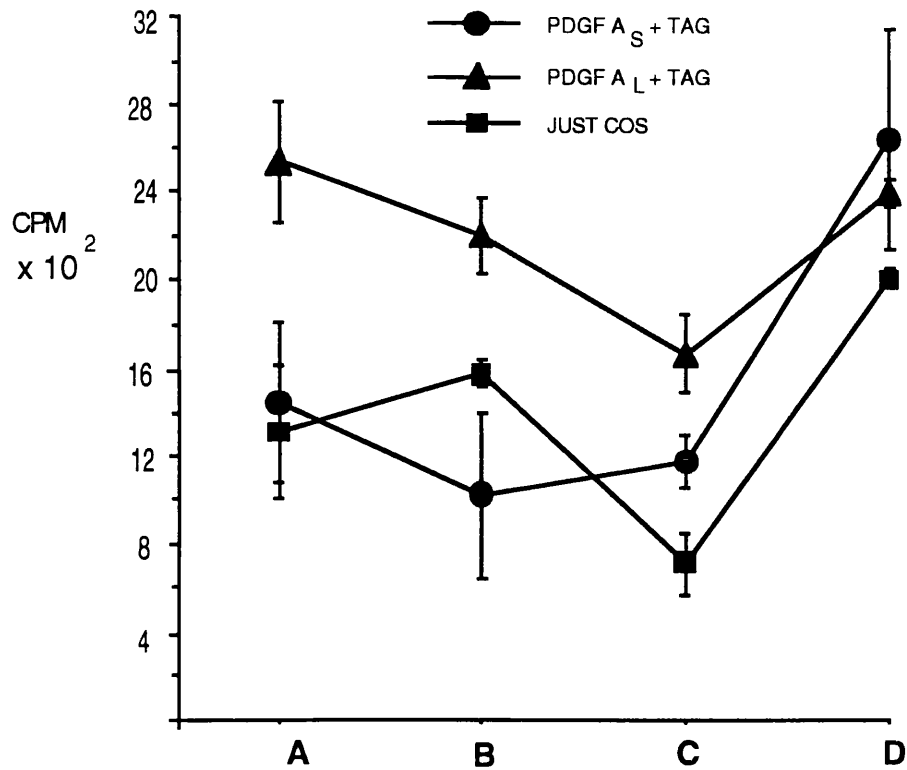
PDGF A$_S$

Figure 38.

Mitogenic activities associated with ECM prepared from Cos cells transfected with the PDGF-A expression vectors indicated, estimated by stimulation of ^{3}H-thymidine incorporation in NIH 3T3 cells seeded onto the ECM (Chapter 2.4 F ii). Error bars represent the observed error calculated from triplicate measurements of each experimental condition.

(A) Intact ECM.

(B) 0.3 M NaCl extracted ECM.

(C) 0.7 M NaCl extracted ECM .

(D) 10% FCS supplemented intact ECM.

A.

B.
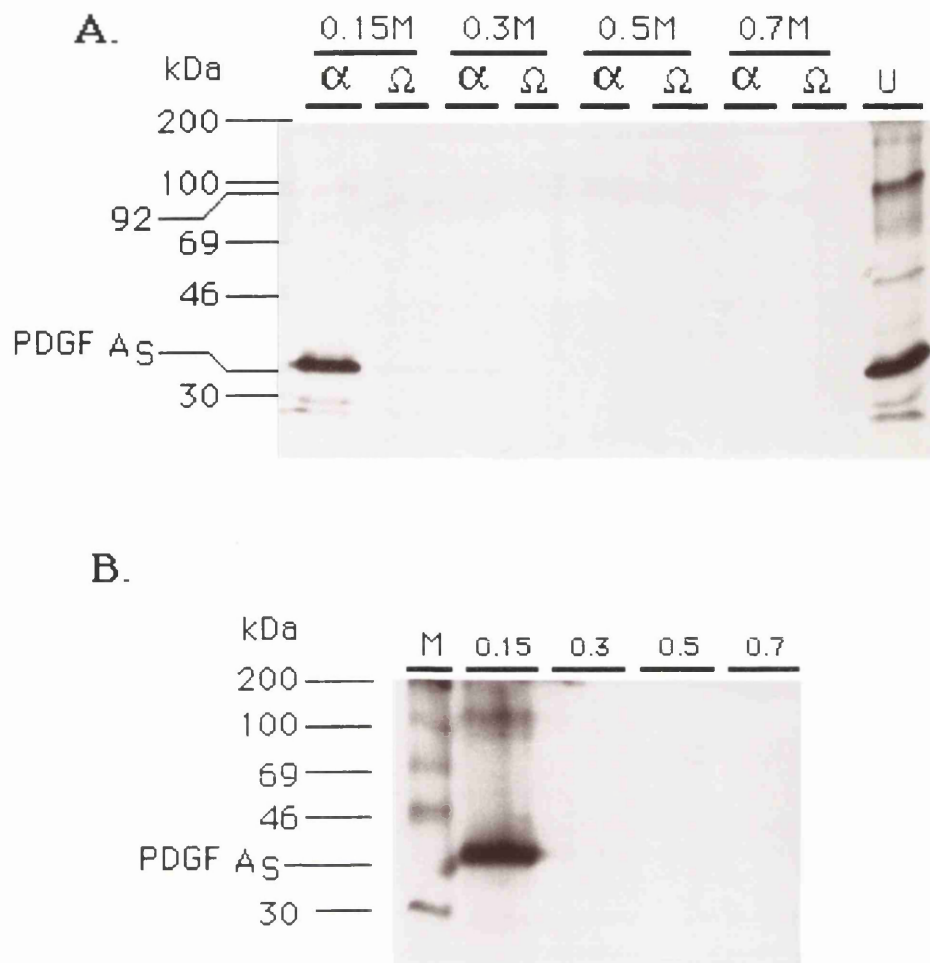
Figure 39.

Fluorographs of 15% SDS-polyacrylamide gels showing the products of Anti-Myc (9E10) immunoprecipitation of the ^{35}S labeled proteins in half the supernatant from Cos cells transfected with PDGF-A$_S$ (**U**). The second half of the supernatant was exposed to Heparin Sepharose at physiological salt (0.15M NaCl) concentrations and washed at the progressively higher salt concentrations indicated collecting the first (α) and last (Ω) washes at each concentration for immunoprecipitation (**A**) (Chapter 2.4 E). The washes at each salt concentration were also immunoprecipitated (**B**). Molecular weight markers shown (**M**).

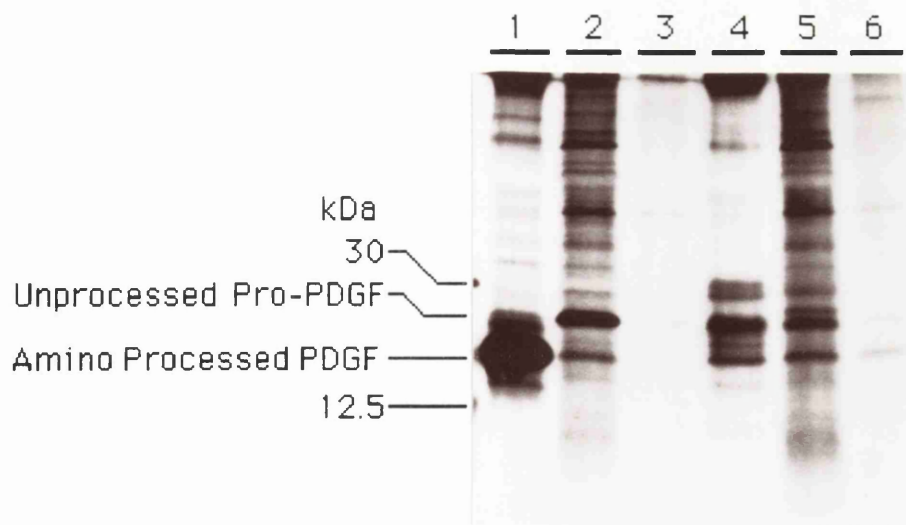**Figure 40.**

Fluorographs of 15% SDS-polyacrylamide gels showing the products of anti-Myc (9E10) immunoprecipitation of the ^{35}S labeled proteins in 1/6th of the supernatant from Cos cells transfected with PDGF-A$_L$ (**A** & **B** Lane **U**). 1/3rd of the supernatant was exposed to Heparin Sepharose (**A**) and 1/3rd exposed to Sepharose alone (**B**) at physiological salt (0.15M NaCl) concentrations. These were washed at the progressively higher salt concentrations indicated collecting the first (α) and last (Ω) washes at each concentration for immunoprecipitation (Chapter 2.4 E). The washes of Heparin Sepharose (**H**) and Sepharose (**S**) at each salt concentration were also immunoprecipitated (**C**). Molecular weight markers shown (**M**)
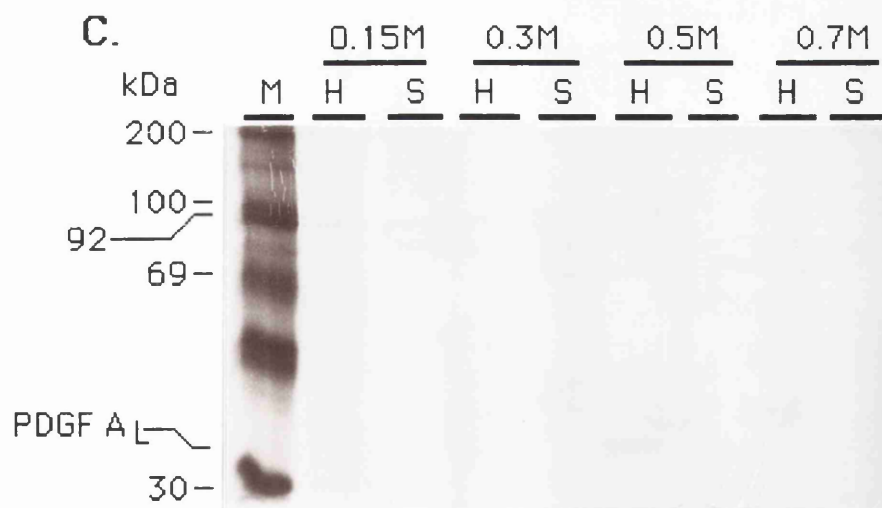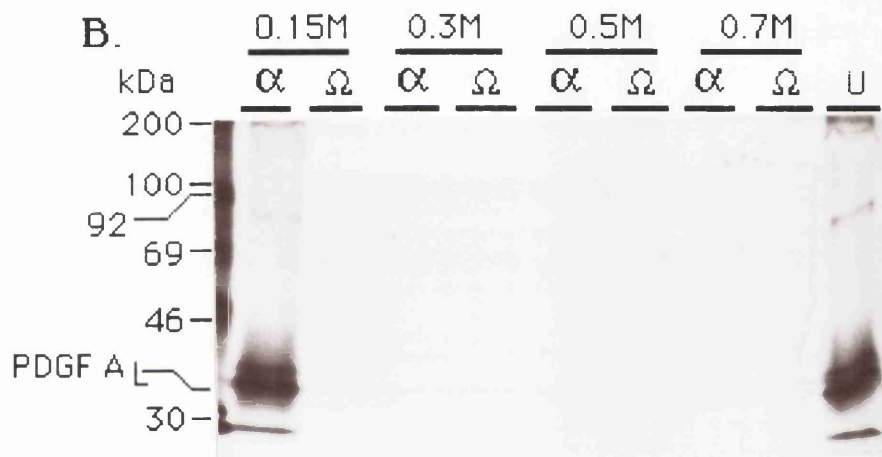
☿ Chapter Six ☿

"One fine morning in May, a slim young horsewoman might
have been seen riding a glossy sorrel mare along the
avenues of the Bois, amongst the flowers . . . ."

Joseph Grand

Chapter 6. Summaries, conclusions and future investigations.

6.1 Summary of investigations into the distribution of PDGF-A chain mRNA alternative splicing isoforms.

Chapters 3 and 4 described my attempts to determine whether the two PDGF-A isoforms, PDGF-A$_S$ and PDGF-A$_L$, are expressed differentially in a variety of both normal and transformed cell and tissue types known to produce PDGF-A mRNA. Although I encountered problems, particularly with regards to contamination and preferential amplification during PCR (discussed in Chapters 3 & 4), the results suggest that PDGF-A$_S$ is the predominant isoform expressed in the CNS (Table 10). Other studies, employing similar techniques to ask the same question of a wide range of cell types from several different species, have produced broadly similar results (Table 10).

A) Library screening and cloning.

In most instances clones obtained from library screening have all been derived from PDGF-A$_S$ mRNA {Collins *et al.*, 1987; Tong *et al.*, 1987; Mercola *et al.*, 1990; this thesis} indeed of the 32 independent PDGF-A cDNA clones described only 2 have been found to represent the PDGF-A$_L$ splicing isoform {Betsholtz *et al.*, 1986; Mercola *et al.*, 1988}. Thus, although disappointing, it is perhaps not surprising, that despite cDNA cloning and extensive analysis by PCR, I was unable to show conclusively that the rat PDGF-A gene encodes an alternatively spliced RNA allowing production of the two different isoforms. It seems likely, however, that rat cells will have this ability given that PDGF-A$_L$ cDNA clones have been isolated from species as diverse as human and *Xenopus* and genomic clones containing the exon 6 sequence have been isolated from mouse (Table 10 for references).

B) RNase and S1 nuclease protection studies.

Despite the fact that the cDNA cloning itself provided an estimate of the relative abundance of two RNAs, it would perhaps have been better to clone the rat PDGF-A gene instead of the cDNA. This might have afforded me the opportunity to perform S1 nuclease or RNase protection analysis on RNA from a variety of cell types, in the same way as the human PDGF-A$_L$ cDNA clone and the mouse genomic clone allowed such analysis of their respective RNA types {Collins *et al.*, 1987; Young *et al.*, 1990}. It is interesting to note that the first of

these studies used PDGF-A$_S$ and PDGF-A$_L$ human cDNA derived probes both of which indicated the presence of only PDGF-A$_S$ mRNA in all cell types studied. However, this result may not have been entirely accurate for reasons described previously in chapters 3.2 and 4.8 C, which indicate that short probes may be protected equally well by long or short form RNA {Sisodia et al., 1987} and that the A:T rich sequence in the exon 6 derived region of PDGF-A$_L$ probes may be prone to artefactual nuclease attack {Miller and Sollner-Webb, 1981}. The second study, this time on mouse RNA, used an S1 probe containing only 10 nucleotides from the 3' end of the exon 6 sequence thus avoiding the A:T rich region (Figure 41). In marked contrast to the previous study this protocol revealed that all the cell types expressed both forms, although in several samples, including brain, PDGF-A$_S$ was the predominant form {Young et al., 1990}. A further study on mouse RNA, using instead a mouse PDGF-A$_S$ cDNA probe, produced results which contained evidence of aberrant digestion, which they suggested might have been due to infrequently used polyadenylation sites though it also seems possible that they may be the result of artefactual RNase digestion {Mercola et al., 1990}. Thus although I may have been able to attempt S1 analysis using my rat PDGF-A$_S$ cDNA clone it seemed possible that this would have produced misleading results. Alternatively it may have been worth trying to use the successful mouse PDGF-A$_L$ S1 probe {Young et al., 1990} however comparison with the human exon 6 sequence indicates that it differs at 2 nucleotides out of the 10 exon 6 bases covered by the mouse probe (Figure 41). Thus although the mouse sequence could reasonably be expected to be more like that of the rat than the human it remained possible that sequence mismatches would cause difficulties in result interpretation. For these reasons then, I decided to direct my attentions to other forms of analysis.

C) PCR amplification analysis.

A number of publications have described the use of PCR to study the expression levels of the two PDGF-A mRNA isoforms, however, these were performed at a time when the hyperbole surrounding the use of this technique was at its peak with very few PCR studies including the controls which are now considered standard, as a result of work such as that detailed in chapters 3 and 4 {Kwok & Higuchi, 1989; Krone et al., 1990; Sarkar and Sommer, 1990; Cimino et al., 1990}. The first of these studies {Matoskova et al., 1989} indicated the presence of both isoforms in every cell and tissue type analysed, including cell types which had previously been thought to express only

PDGF-A$_S$ {Tong *et al.*, 1987} (see Table 10). In each case the PDGF-A$_S$ mRNA appeared to be at least 10 fold more abundant than the PDGF-A$_L$ mRNA. However, although there is some consideration of the risk of preferential amplification of the shorter mRNA species, since no controls of any sort are shown or discussed the possibility remains that these results may have arisen from a similar combination of contamination and preferential amplification to that I experienced in my early attempts at PCR. A second study, again detailing amplification of both forms in all tissues tested with no evidence of controls, was nevertheless somewhat more believable, because it showed variations in the relative abundance of the two isoforms {Young *et al.*, 1990}. In addition, since this study was not used in isolation, but served as confirmation of the S1 nuclease analysis results discussed above (Table 10), it seems likely that the combined results provide an accurate analysis of the RNAs present in the tissues studied. The question therefore arises as to why I, by contrast, was only able to show signs of PDGF-A$_S$. Part of the answer may lie in their use of whole tissues such as heart, testes, brain and various stages of whole embryo (Table 10) thus potentially missing subtle differences in expression between individual cell types within these tissues. However, since as I also examined mRNA from whole tissues such as P8 rat brain and adult rat cerebellum and still only detected PDGF-A$_S$, it seems that the difference may indicate, either an altered pattern of expression in the tissues and at the times I analysed, or a difference in my ability to detect PDGF-A$_L$ which they suggest is present in particularly low levels in the brain.

D) Northern blot analysis.

Two attempts have been made to assess the relative abundance of the two PDGF-A isoforms by northern blot analysis of mRNA from a number of human cell types (Table 10). Although both studies used probes based on the human PDGF-A cDNA sequence their approaches were quite different and, indeed, led to apparently contradictory results. The first used an oligonucleotide probe specific for sequence within PDGF-A$_L$ exon 6-containing transcripts, and an oligonucleotide complementary to 16 bp on either side of the exon 5/7 junction, which was specific for PDGF-A$_S$, to detect only the short form in each of the cell types tested {Tong *et al.*, 1987}. The second study used the entire exon 6 sequence, excised from the cDNA clone, as a PDGF-A$_L$ specific probe and subtracted the result from that produced by probing with the entire coding sequence to provide an estimate of the levels

of PDGF-A$_S$ mRNA present {Rorsman *et al.*, 1988}. The results of this study suggested that every cell type examined, including one (U-2 OS) which gave no signal in the previous study {Tong *et al.*, 1987}, expressed both mRNA types, although levels of PDGF-A$_L$ were generally much lower than PDGF-A$_S$. Interestingly, however, the human glioma cell line (U-343 MGa clone 2:6), from which PDGF-A$_L$ was originally cloned, showed by far the highest level of PDGF-A$_L$ mRNA indicating that this may be an unusually good source of this isoform.

It may have been worth my using human probes such as these or perhaps probes based on the more closely related mouse sequence to perform northern analysis on mRNA from different rat cell types since, although such experiments are limited in terms of sensitivity (previously discussed in chapter 3.2) I might nevertheless have been able to prove that rat can actually express PDGF-A$_L$.

E) Immunohistological analysis.

An immunohistological analysis of the distribution of the two PDGF-A protein isoforms was carried out by comparing the result produced with PDGF-A$_L$ isoform-specific antibodies on sections of foetal mouse tongue with that produced by commercially available PDGF-A antibodies {Young *et al.*, 1990}. The result showed PDGF-A$_L$ staining in skeletal muscle but not in the epithelium or connective tissue. General PDGF-A staining was more intense, and thus consistent with the body of evidence indicating PDGF-A$_S$ is the predominant form. However, these studies did not indicate a distinct tissue distribution for PDGF-A$_S$ and PDGF-A$_L$. Given that this was only a limited study of a relatively small number of cell types it is possible that future immunohistochemical studies of other tissues may yet reveal a distinct tissue distribution.

6.2 Conclusions on the differential expression of the PDGF-A isoforms.

The combined evidence of the work described in this thesis and that of others summarised above (Table 10), point to the conclusion that PDGF-A$_L$ is the less commonly used form of PDGF-A in the cell and tissue types analysed to date. There is, as yet, no evidence for there being a distinct pattern of expression for PDGF-A$_L$, since every cell or tissue type in which it has been

detected, also produces PDGF-A$_S$ and usually at levels several fold greater than PDGF-A$_L$. My experiments and, it would appear, most of the other investigations were carried out on the assumption that the two isoforms have different functions and are therefore likely to exhibit a distinct pattern of expression. On the basis of these studies it might appear that this assumption was wrong. From their coincident pattern of expression it is possible that they have a distinct but concurrent or inter-dependent function, indeed they may even form heterodimers though it is not clear what, if any, the function of such polypeptides might be. The work described in thesis does not address these possibilities; insofar as I and others have shown there to be a functional difference between the two PDGF-A homodimers it still remains a possibility that the two isoforms are independently and differentially expressed in some circumstances. It would therefore be of interest to use some of the above techniques, suitably refined, to carry out a more detailed analysis of a much wider range of cell types (e.g. Chapter 1, Table 2) from different stages in development.

6.3 Future investigations into the differential expression of PDGF-A isoforms.

A number of additional approaches may be worth investigating, including the use of *in situ* hybridisation with PDGF-A$_S$ and PDGF-A$_L$ specific oligonucleotides such as those used in the first PDGF-A northern blot analysis described above {Tong *et al.*, 1987}. However, unlike S1 nuclease or PCR analysis and in common with northern blotting this approach may lack the sensitivity to detect low levels of PDGF-A$_L$ such as have been shown to exist in most of the cell types examined so far. On the other hand, it would have the singular advantage of locating the expressing cells precisely rather than simply identifying whole tissues as expressing one or, more likely, both forms simultaneously.

Alternatively the *in vivo* function of the two PDGF-A isoforms may be inferred from studies of other similar factors, in particular the related VEGF, which exhibits alternative splicing of an analogous sequence to that present in the PDGF-A$_L$ tail {Betsholtz *et al.*, 1990}.

```
        10        20        30        40        50        60        69
         I         I         I         I         I         I         I
GAAGGCCTAGGGAGTCAGGTAAAAAACGGAAAAGAAAAAGGTTAAAACCCACCTAAAGCAGCCAACCAG  Human Exon 6
•••••• •••••• ••••••••••••• ••••••••• •••••••••••••••••••••• •• ••• •••••
GAAGGCGTAGGGAATCAGGTAAAAAGCGGAAAAGGAAAAGGTTAAAACCCACCTAAGGCGGCCGACCAG  Mouse Exon 6
```

S1 Nuclease probe

Figure 41.

Illustrating the differences between human and mouse exon 6
cDNA sequences {Betsholtz et al., 1986; Young et al., 1990}. Also
indicates region of mouse PDGF-A$_L$ exon 6 mRNA protected by the
S1 nuclease probe used by Young et al., (1990).

(•) = matching bases.   (━) = stop codon.

(⟵·····) = S1 oligonucleotide probe complementary to the mRNA
sequence shown.

Table 10. Showing the techniques used to study the expression of the two PDGF-A isoforms, the cell types studied and a summary of the results of each study in chronological order.

Techniques	Source of material	Results	References
cDNA library screening	Human glioma cell line U-343 MGa c 2:6	3 independent Short & 1 Long form clone	Betsholtz et al., 1986
cDNA library screening & RNase protection	Human endothelial "EC" cells EC cells & 2 human osteosarcoma lines (HOS & U1810)	4 independent Short form clones All Short form	Collins et al., 1987
cDNA library screening & Northern	Human umbilical vein endothelial "HUVE" cells HUVE cells & 3 human tumour lines (HOS, A172 &U-2 OS)	3 independent Short form clones All Short form	Tong et al., 1987
Northern	Human tumour cells (melanoma WM266-4, glioblastoma U-343, osteosarcoma U-2 OS, Squamous cell carcinoma A431)	All showed predominantly short form although the relative levels varied.	Rorsman et al., 1988
cDNA library screening	Xenopus oocyte Xenopus gastrula	1 Long form clone 1 Short Form clone	Mercola et al., 1988

Method	Source	Result	Reference
PCR	Wide range of normal & transformed human, pig, rat & hamster cells (e.g. HUVEs, U2 OS & U1810)	All showed both forms though Short form was at least 10 fold more abundant.	Matoskova *et al.*, 1989
S1 & PCR & Immuno-histochemistry	Adult mouse Liver, kidney, thymus, muscle, heart, testes Brain & ES cells and tissue from 6.5, 10.5 & 13.5 day embryos.	Both forms found in each with levels of short being much greater in those underlined.	Young *et al.*, 1990
	Mouse tongue	No distinct pattern of staining	
cDNA library screening & RNase protection	Mouse embryonal tetracarcinoma-derived cell line F9	7 independent Short form clones	Mercola *et al.*, 1990
	Undifferentiated & differentiated F9 cells and 6.5, 7.5 & 8.5 day mouse embryos.	Both forms found in each though Short form predominates.	
cDNA library screening & S1 & PCR	P7 rat brain	12 independent Short form clones	**Chapters 3 & 4 of this thesis.**
	Human glioma 157	Short form	
	Human glioma 157, mouse brain, adult rat cerebellum, P8 rat brain & rat glioma cell line C6.	All short form	

6.4 Possible biological reasons for cell and/or ECM retention.

Chapter 5 described my attempts to define the functional differences between homodimers of the two PDGF-A isoforms. The results of this work, along with that of a number of other studies discussed in chapter 5, provide evidence that PDGF-A$_S$ is soluble while PDGF-A$_L$ is retained on both the cell surface and within the ECM, at least initially, via association with HPGs. This raises the question why should PDGF-A be produced in either a diffusible or an immobilised form? It may be possible to answer this by analogy with other polypeptide growth factors including members of the TGF-β family {Andres et al., 1989} and the FGF family {Vlodavsky et al., 1991} that are known to associate with both the cell surface and the ECM.

A) Heparin binding may regulate receptor activation directly.

It has been shown that about 70% of FGF-2 binds to heparin-like molecules on the cell surface {Vlodavsky et al., 1987b} while the remaining 30% associates with the ECM {Vlodavsky et al., 1987a}. An explanation for this biased distribution probably lies in the recent evidence that association with cell surface heparin-like molecules is required for FGF-2 to bind to its high-affinity cell-surface receptor {Yayon et al., 1991}. On the other hand, while it is possible that the matrix bound FGF-2 also interacts with cell surface receptors (Figure 2), it seems likely that this association represents a storage system, or perhaps even a mechanism for titrating out excess FGF, given that it can be released in an active form by competition with soluble heparin or in complex with partial degradation products of heparin by the action of endoglycosidases such as heparanase and heparitinase {Bashkin et al., 1989; Presta et al., 1989}.

Although my experiments show evidence of a similar bias towards cell surface retention for PDGF-A$_L$ (Figure 37 b) there is no evidence that the activity of PDGF-A$_L$ can be augmented by heparin or related HPGs. Indeed the equivalent activity of PDGF-A$_S$ and PDGF-A$_L$ produced in yeast {Östman et al., 1989} and the fact that active PDGF-A$_L$ is released from cells by proteolytic cleavage of the heparin binding region {Beckmann et al., 1988; LaRochelle et al., 1991}, indicate that interaction between PDGF and heparin is not necessary for activity. However, since there are many different forms of HPG with different affinities for FGFs {Gordon et al., 1989; Ruoslahti, 1989; Herndon and

Lander, 1990; Ruoslahti and Yamaguchi, 1991 for review} it remains a possibility that some as-yet-unidentified form(s) of heparin or HPG may regulate PDGF receptor activation. Recently, interaction between PDGF-AB or BB dimers and the extracellular glycoprotein SPARC has been shown to inhibit binding of these isoforms to the PDGF-β receptor {Raines et al., 1992}. Thus, although this particular effect does not seem to require an interaction with heparin per se it provides evidence that PDGF activity can be regulated by extracellular glycoproteins.

B) Bound growth factors may be stored for future release.

Aside from the possibility of regulation by heparin, it seems likely that this association represents a storage mechanism given that a synthetic peptide corresponding to the PDGF-A$_L$ retention domain can be released from cells in vitro by using soluble heparin or heparan sulphate {Raines and Ross, 1992} suggesting PDGF-A$_L$ may be released in vivo in a manner analogous to that proposed for FGFs {Thompson et al., 1990; Whalen et al., 1989}. Moreover, during the process of clot formation and vascular repair, the proteolytic enzyme thrombin is known to induce release of soluble PDGF from endothelial cells without recourse to protein synthesis {Harlan et al., 1986}. This observation has been explained by suggesting that thrombin activates an inactive form of PDGF, because these cells, unlike platelets, do not contain intracellular stores of PDGF. It now seems possible that thrombin may release PDGF by proteolytic cleavage of the heparin binding retention domain. Although this remains to be tested it is interesting to note that a second and otherwise unrelated polypeptide, tissue factor pathway inhibitor (TFPI) shares some key features with PDGF-A$_L$, including a very similar COOH terminal sequence (Figure 42). TFPI is a multivalent kunitz type protease inhibitor that regulates fibrin clot formation by inactivating factor Xa directly and thereafter inhibiting the factor VIIa/tissue factor proteolytic cascade in an Xa-dependent manner {Broze et al., 1988}. Like PDGF it is known to be released from platelets by thrombin {Novotny et al., 1988} and perhaps most significantly it has been shown to exist in two forms that can be separated on the basis of heparin agarose affinity: a proteolytically cleaved carboxy-terminal truncated form eluting at 0.3 M NaCl and a full length form eluting at 0.6 M NaCl {Wesselschmidt et al., 1992}, reminiscent of the differential heparin-binding activity of PDGF-A$_S$ and PDGF-A$_L$. In addition it has been shown that soluble heparin increases the plasma TFPI levels when

administered intravenously {Sandset *et al.*, 1988}. Taken together, these similarities provide compelling evidence that the analogous COOH sequences present in TFPI and PDGF-A allow them to be stored in association with heparin molecules from which they can be released by proteolytic cleavage. Furthermore it is possible that this type of retention sequence may be present in other proteins involved in vascular repair and provide a general mechanism for storage and release by thrombin.

A) ···V R R P P K G K H R K F K H T H D K T A L K E T L G A

B) ····G R P R E S G K K R K R K R L K P T

C) ···V R G K G K G Q K R K R K K S R Y K S W S Y.....

D) ····S K G G L I K T K R K R K K Q R V K I A Y E E I F V K N M
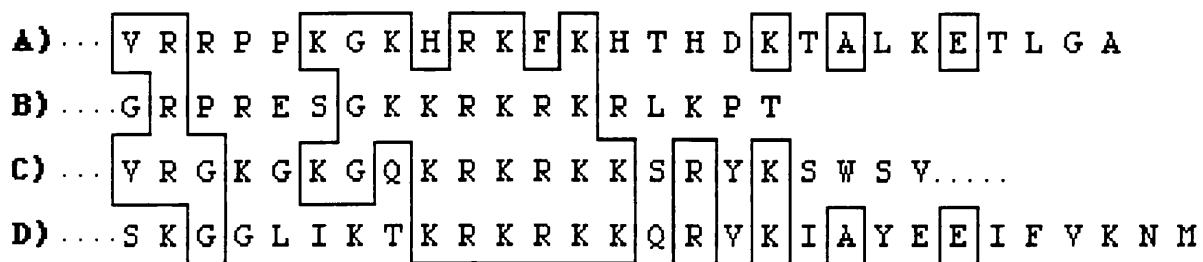
Figure 42.

A comparison of the basic retention sequences encoded by human PDGF-B exon 6 (**A**), human PDGF-A exon 6 (**B**), human VEGF exon 6 (**C**) and the sequence encoded by human TFPI exon 9 (**D**).

C) Retention may serve to localise growth factor activity.

Alternatively, immobilisation of growth factors on the ECM may facilitate precise localisation of proliferation and differentiation signals and possibly even allow formation of discrete pathways for migrating cells as has been proposed for kit ligand (KL or *Steel* factor) {Flanagan and Leder, 1990}. RNA encoding KL, the ligand for the c-*kit* receptor which is a member of the PDGF tyrosine-kinase subfamily (Figure 1), undergoes alternative splicing giving rise to two forms of KL one of which has a transmembrane domain that anchors it to the cell surface {Flanagan *et al.*, 1991}. Mutations affecting either KL or its receptor c-*kit* are known to affect the development of three different migratory cell lineages: primordial germ cells, melanocytes and hematopoietic stem cells {Russell, 1979; Silvers, 1979}. An aberrant form of KL, the mouse mutant Steel-Dickie (*Sl^d*), has a deletion of the sequence coding for the transmembrane domain, allowing production of a soluble form of KL that is

able to stimulate proliferation of mast cells *in vitro* but apparently unable to direct the normal development of its target cells *in vivo* {Flanagan *et al.*, 1991}. This indicates the importance of membrane association for normal KL activity *in vivo*. Recently *in situ* studies examining the relative distribution patterns of c-*kit* and KL mRNAs have revealed that they are expressed in separate but adjacent cell layers suggesting the range of KL activity may be regulated jointly by restricted expression and cell retention {Motro *et al.*, 1991; Keshet *et al.*, 1991}.

A similar pattern of 'appositional' expression has been observed for a number of other related ligand-receptor pairs including CSF-1, which also has a transmembrane domain {Rettenmier *et al.*, 1987} and its receptor c-*fms*, another member of the PDGF tyrosine-kinase subfamily, (Figure 1) {Regenstreif and Rossant, 1989}; PDGF-B and the PDGF β-receptor {Holmgren *et al.*, 1991}; and more recently PDGF-A and the PDGF α-receptor {Orr-Urtreger and Lonai, 1992; Mudhar *et al.*, In preparation}.

D) Cell adhesion and growth factors as receptors.

A number of other activities have been proposed for immobilised growth factors. For example, it has been suggested that transmembrane growth factors including EGF, TGF-α and the related *Drosophila Notch* and *C.elegans lin-12* gene products may mediate cell-cell adhesion {Massague, 1990 for review}. It has also been suggested that the highly conserved intracellular domains of these same transmembrane growth factors may function as signalling domains in their own right, although they have so far shown no homology with any of the known growth factor receptors {Massague, 1990}. Growth factors such as PDGF-A$_L$ may also act as cell surface receptors by association with HPGs such as Syndecan that have a membrane spanning protein core {Saunders *et al.*, 1989}. It is even possible that ECM bound factors may act as receptors by causing conformational alterations in the ECM leading in turn to signalling via transmembrane proteins such as integrins, according to the 'dynamic reciprocity' model for matrix regulation of gene expression {Bissell *et al.*, 1982; Bissell *et al.*, 1987; Ruoslahti, 1989}.

6.5 Conclusions.

In each of the above examples the growth factors can be made in both soluble and cell-surface-bound forms, except perhaps FGF which appears to

require association with HPG for activity and may therefore all be considered bound. There are a number of different mechanisms for this including production of a transmembrane precursor that can be selectively processed as for CSF-1 and EGF family members {Massague, 1990 for review}, or production by alternative RNA splicing of separate forms with differing affinities for HPGs, as has been shown for PDGF-A. The cell membrane localisation of KL also seems to be regulated by alternative splicing leading to production of two different forms; however it seems that they differ, not by the presence or absence of the transmembrane region, which they both have, but rather in their susceptibility to proteolytic release {Flanagan et al., 1991}.

TGF-βs, on the other hand, do not seem to be produced in different forms but can nevertheless be localised to the cell surface or the ECM by interaction with various differentially expressed betaglycans that have been shown to bind to cell membranes and to associate with the ECM and which it binds to via the core protein rather than the GAG side chains {Andres et al., 1989; Ruoslahti and Yamaguchi, 1991}. So far only one other growth factor, Leukemia inhibitory factor (LIF), has been shown to regulate its association with ECM by producing alternative forms of the growth factor. Although the mechanism of association is not yet understood it appears that alternative promoter usage produces two forms of LIF that differ at their amino-termini, one of which codes for a signal sequence that is removed after directing incorporation into ECM {Rathjen et al., 1990}. Vascular endothelial growth factor (VEGF) displays analogous alternative splicing of sequence similar to the PDGF-A chain exon 6 (Figures 5 & 42) {Tischer et al., 1989; Leung et al., 1989; Keck et al., 1989; Betsholtz et al., 1990}. However, although there is evidence for an affinity with heparin {Ferrara and Henzel, 1989} it has, so far, not been shown to associate with either cell surface or ECM bound HPGs. It seems possible that V-EGF, PDGF-A$_L$ and TFPI (see Section 6.3) may be members of a larger family of proteins which use this polypeptide motif to regulate their mode of action. Further work is required to understand when and where these polypeptides are expressed and the mechanism and circumstances leading to their release in vivo.

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Appendix :- Plasmid constructs.

M13 MP10 PDGF-A$_L$:- Used for S1 nuclease probe production (See Figure 7).

pHYK :- Original plasmid expression vector obtained from Hugh Pelham (See also Figure 43).

pHYK 2 :- Version of the expression vector adapted to allow subsequent cloning steps (See also Figure 43).

pHYK 3 :- Version of expression vector adapted further to allow subsequent cloning of untagged PDGF-A$_S$ and PDGF-A$_L$. This version was used as a negative control for the effects of transfection by vector alone (See also Figure 43).

PDGF-A$_S$:- pHYK vector encoding human PDGF-A short form cDNA sequence (See Figure 8).

PDGF-A$_L$:- pHYK vector encoding human PDGF-A long form cDNA sequence (See Figure 8 and Figure 43).

PDGF-A$_L$ 2 :- pHYK vector encoding human PDGF-A long form cDNA sequence adapted for further alterations (See Figure 43).

PDGF-A$_L$ 3 :- pHYK vector encoding human PDGF-A long form cDNA sequence adapted for further alterations (See Figure 43).

PDGF-A$_L$ 4 :- pHYK vector encoding human PDGF-A long form cDNA sequence adapted for further alterations (See Figure 43).

PDGF-A$_S$ Tag :- pHYK vector encoding PDGF-A short form sequence including Myc Tag epitope (See Figure 8).

PDGF-A$_L$ Tag :- pHYK vector encoding PDGF-A long form sequence including Myc Tag epitope (See Figure 8).

Figure 43.

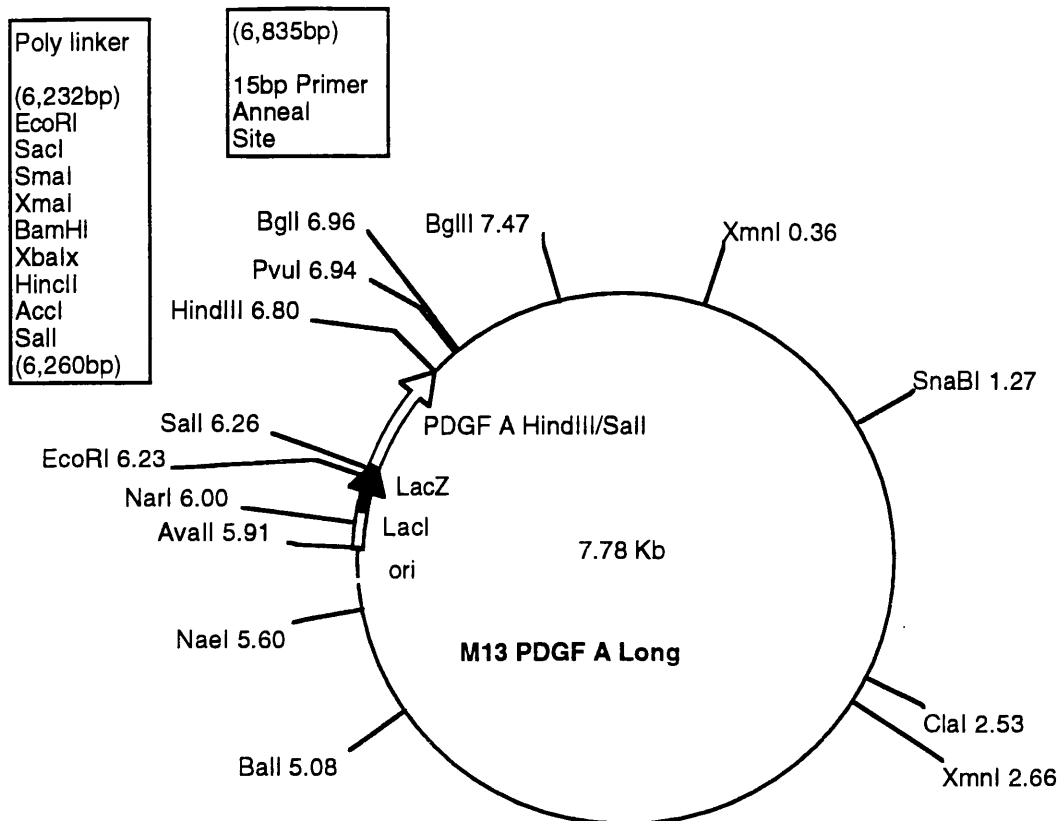Diagram indicating the pHYK vector manipulations prior to sub-cloning human PDGF-A cDNA as described in Figure 8. Changes in each case highlighted with an oval ⬭.

PHYK

Chicken Lysozyme
600bp cDNA

SacII   HindII

Ad 2 Late

EcoRI   SacI   XbaI   BamHI   SmaI
SeoI
MYC
TAG

StuI

Herpes
TK Poly A
Signal

SV40
ORI

Amp R

pBR322 / PUC hybrid vector

⇓

PHYK 2

Chicken Lysozyme
600bp cDNA

SalI   HindII

Ad 2 Late

EcoRI   SacI   XbaI   BamHI   SmaI
SacI
MYC
TAG

StuI

Herpes
TK Poly A
Signal

SV40
ORI

⇓

PHYK 3

Chicken Lysozyme
600bp cDNA

SalI   SacII

Ad 2 Late

EcoRI   SacI   XbaI   BamHI   SmaI
SacI
MYC
TAG

StuI

Herpes
TK Poly A
Signal

SV40
ORI

⟹ PDGF-A short

⇓

PDGF-A Long

Human PDGF A_L chain cDNA
SacII/EcoRI fragment

SalI   SacII

Ad 2 Late

HindIII   EcoRI   SacI   XbaI   BamHI   SmaI

StuI
Stop
Codon

Herpes
TK Poly A
Signal

StuI

SV40
ORI

⇓ Kill PDGF HindIII

PDGF-A Long 2

Human PDGF A_L chain cDNA
SacII/EcoRI fragment

SalI   SacII

Ad 2 Late

EcoRI   SacI   XbaI   BamHI   SmaI

StuI
Stop
Codon

Herpes
TK Poly A
Signal

StuI

SV40
ORI

⇓ Recreate PHYK HindIII

PDGF-A Long 3

Human PDGF A_L chain cDNA
SacII/EcoRI fragment

SalI   HindIII

Ad 2 Late

EcoRI   SacI   XbaI   BamHI   SmaI

StuI
Stop
Codon

Herpes
TK Poly A
Signal

StuI

SV40
ORI

⇓ Change StuI to SacII

PDGF-A Long 4

Human PDGF A_L chain cDNA
SacII/EcoRI fragment

SalI   HindIII

Ad 2 Late

EcoRI   SacI   XbaI   BamHI   SmaI

StuI
Stop
Codon

Herpes
TK Poly A
Signal

SacII

SV40
ORI

⇓

PDGF-A short TAG
&
PDGF-A Long TAG

See Figure 8

(6,835bp)

15bp Primer
Anneal
Site

BglI 6.96   BglII 7.47   Xmnl 0.36

Pvul 6.94

HindIII 6.80

PDGF A HindIII/SalI

SnaBl 1.27

SalI 6.26

EcoRI 6.23

Narl 6.00

Avall 5.91

LacZ

LacI

ori

7.78 Kb

M13 PDGF A Long

Nael 5.60
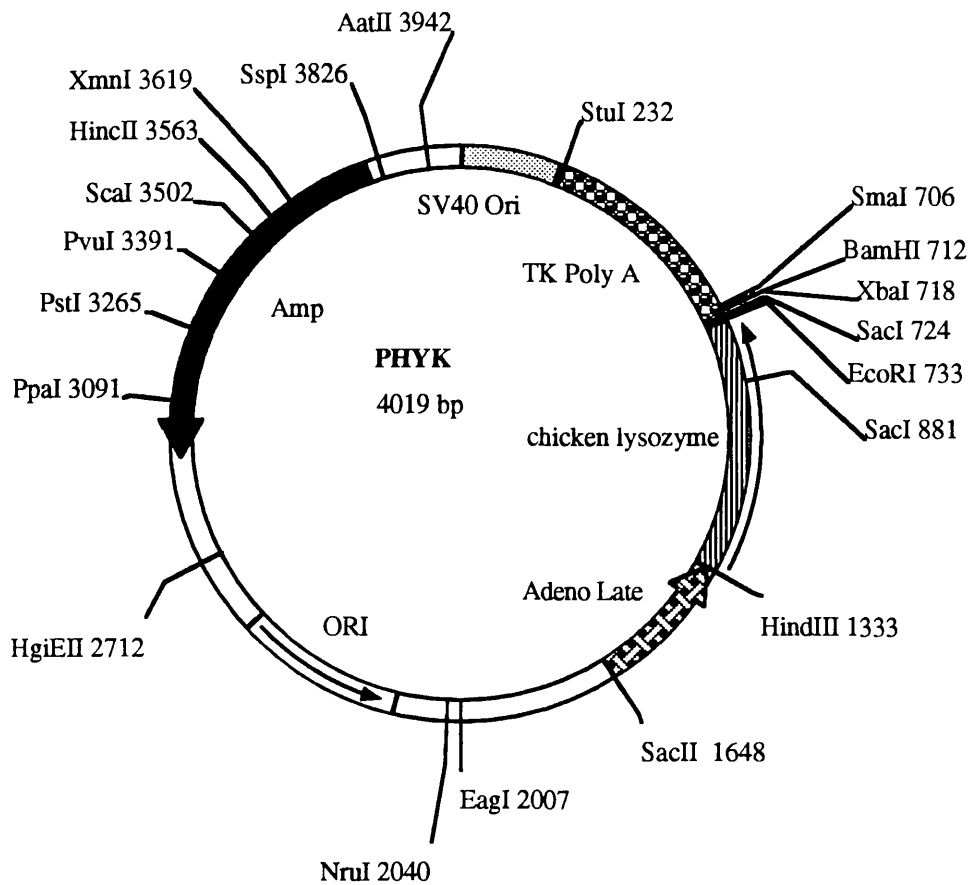
Clal 2.53

Xmnl 2.66

Ball 5.08

Plasmid name: M13 PDGF A Long

Plasmid size: 7.78 kb

Constructed by: inserting the SalI / HindIII fragment of human PDGF
A-chain long form cDNA.

Construction date: 9-2-88

Comments/References: Used to produce S1 probes by primer extension. The correct
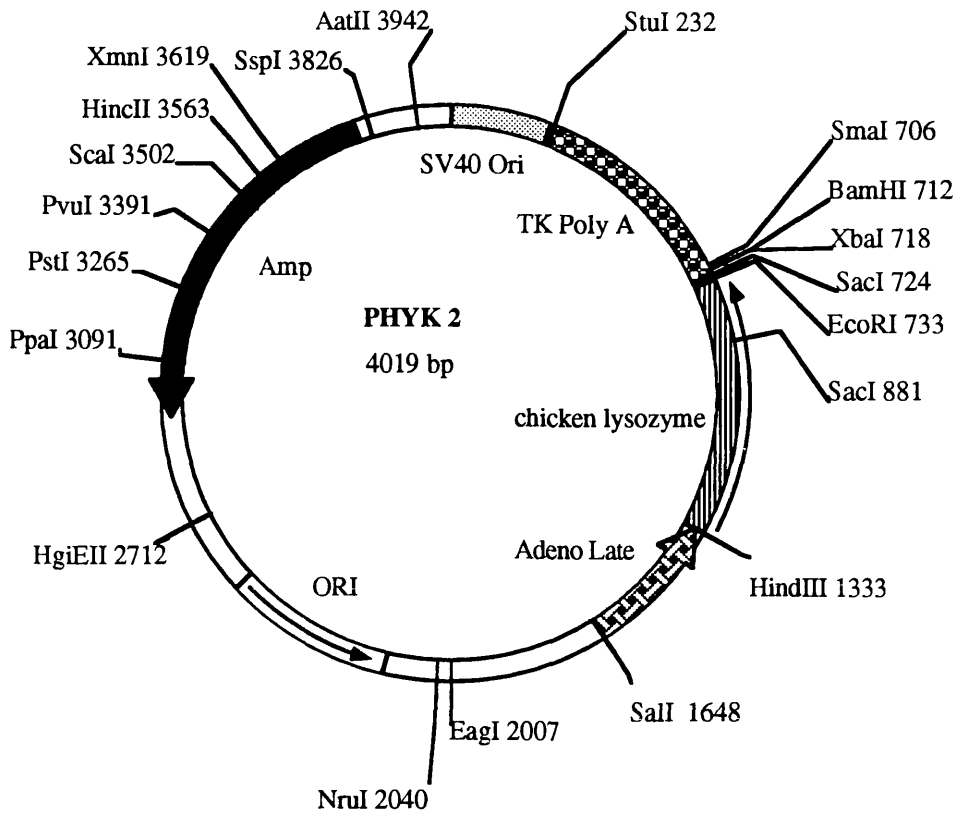orientation was confirmed by sequencing.

AatII 3942
XmnI 3619
SspI 3826
StuI 232
HincII 3563
ScaI 3502
PvuI 3391
PstI 3265
PpaI 3091
HgiEII 2712

SV40 Ori
TK Poly A
Amp
PHYK
4019 bp
chicken lysozyme
ORI
Adeno Late

SmaI 706
BamHI 712
XbaI 718
SacI 724
EcoRI 733
SacI 881
HindIII 1333
SacII 1648
EagI 2007
NruI 2040

Plasmid name: PHYK

Plasmid size: 4019 bp

Constructed by: Hugh Pelham

Construction date:

Comments/References: PBR322 deleted from 0 to 651 and 1063 to 2475 and minus AflIII + BspMI sites -- SV40 Ori 232bp -- TK poly A signal allowed 500bp -- Chicken lysozyme 600bp-- Adeno virus serotype 2 major late promoter 313bp.

XmnI 3619
AatII 3942
StuI 232
SspI 3826
HincII 3563
ScaI 3502
SmaI 706
PvuI 3391
BamHI 712
PstI 3265
XbaI 718
PpaI 3091
SacI 724
EcoRI 733
SacI 881
HgiEII 2712
HindIII 1333
EagI 2007
SalI 1648
NruI 2040

AatII 3942

StuI 232

SV40 Ori

TK Poly A

Amp

PHYK 2
4019 bp

chicken lysozyme

ORI

Adeno Late

Plasmid name: PHYK 2

Plasmid size: 4019 bp

Constructed by: changing PHYK SacII site to Sal I

Construction date:

Comments/References: PBR322 deleted from 0 to 651 and 1063 to 2475 and minus AflIII + BspMI sites -- SV40 Ori 232bp -- TK poly A signal allowed 500bp -- Chicken lysozyme 600bp-- Adeno virus serotype 2 major late promoter 313bp.
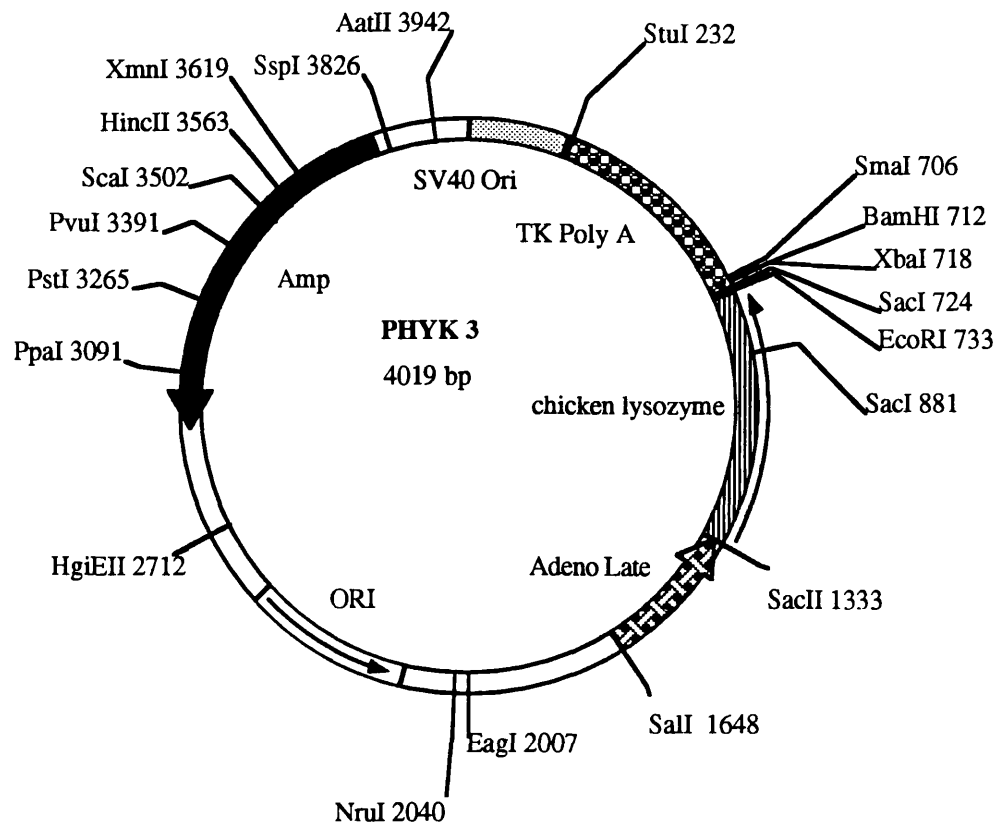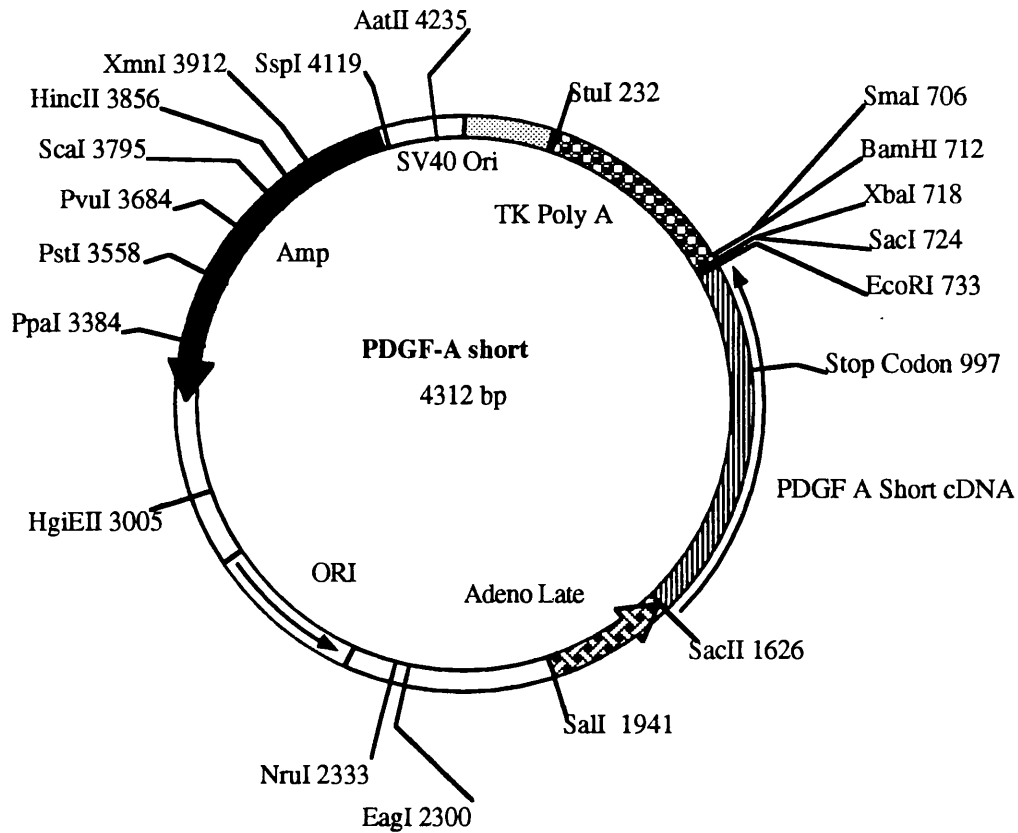
Plasmid name: PHYK 3

Plasmid size: 4019 bp

Constructed by: changing PHYK 2 HindIII to SacII

Construction date:

Comments/References: PBR322 deleted from 0 to 651 and 1063 to 2475 and minus AflIII + BspMI sites -- SV40 Ori 232bp -- TK poly A signal allowed 500bp -- Chicken lysozyme 600bp-- Adeno virus serotype 2 major late promoter 313bp.
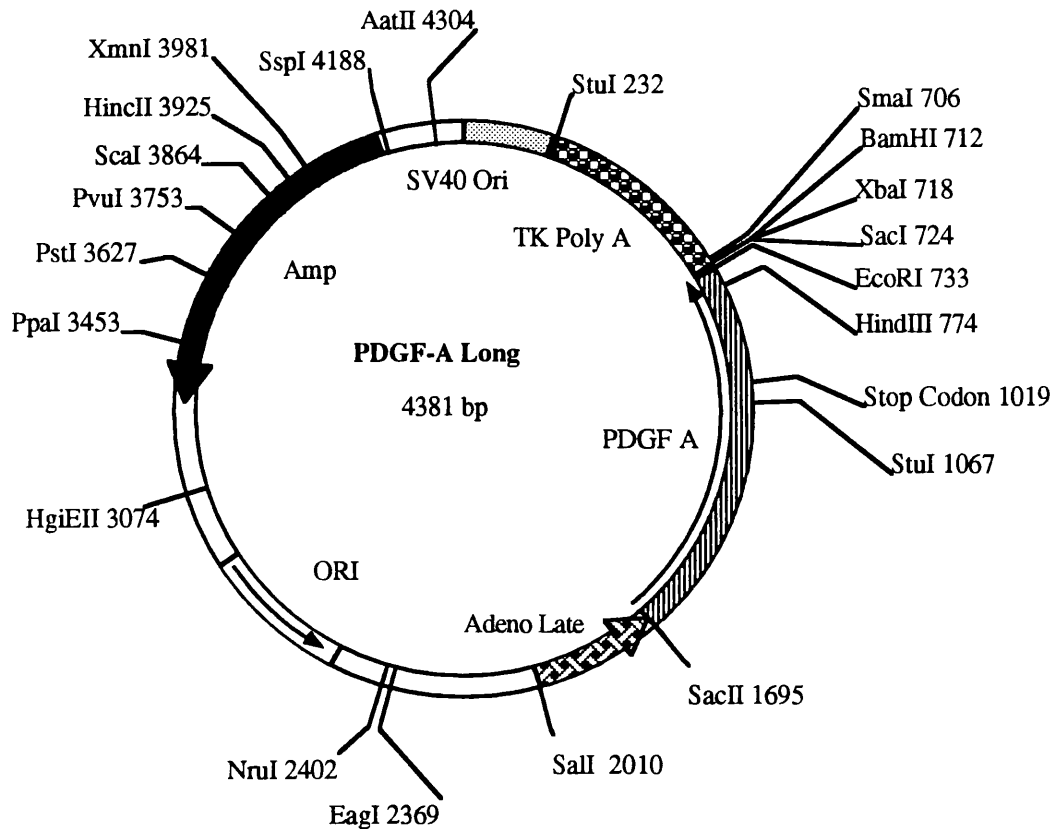
Plasmid name: PDGF-A short

Plasmid size: 4312 bp

Constructed by: inserting SacII / EcoRI fragment of human PDGF-A short form cDNA into PHYK 3 instead of chicken lysozyme

Construction date: 28 / 9 / 89

Comments/References: PBR322 deleted from 0 to 651 and 1063 to 2475 and minus AflIII + BspMI sites -- SV40 Ori 232bp -- TK poly A signal allowed 500bp -- Human PDGF A short cDNA 893bp -- Adeno virus serotype 2 major late promoter 313bp.
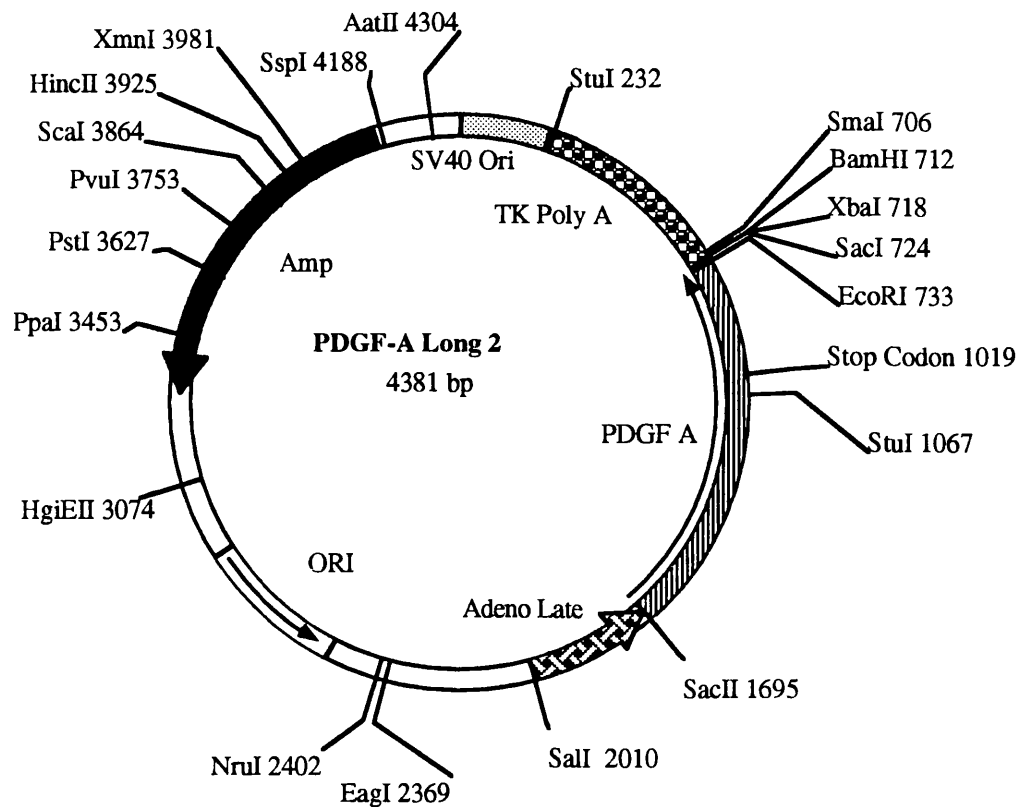
Plasmid name: PDGF-A Long

Plasmid size: 4381 bp

Constructed by: inserting SacII / EcoRI fragment of human PDGF-A Long form cDNA into PHYK 3 instead of Chicken lysozyme

Construction date: 19-2-88

Comments/References: PBR322 deleted from 0 to 651 and 1063 to 2475 and minus AflIII + BspMI sites -- SV40 Ori 232bp -- TK poly A signal allowed 500bp -- Huma n PDGF A (Long form cDNA) 962bp-- Adeno virus serotype 2 major late promoter 313bp.

Plasmid name: PDGF-A Long 2

Plasmid size: 4381 bp

Constructed by: killing the HindIII in "PDGF-A long "

Construction date: 29-3-88

Comments/References: PBR322 deleted from 0 to 651 and 1063 to 2475 and minus

AflIII + BspMI sites -- SV40 Ori 232bp -- TK poly A signal allowed 500bp -- Human

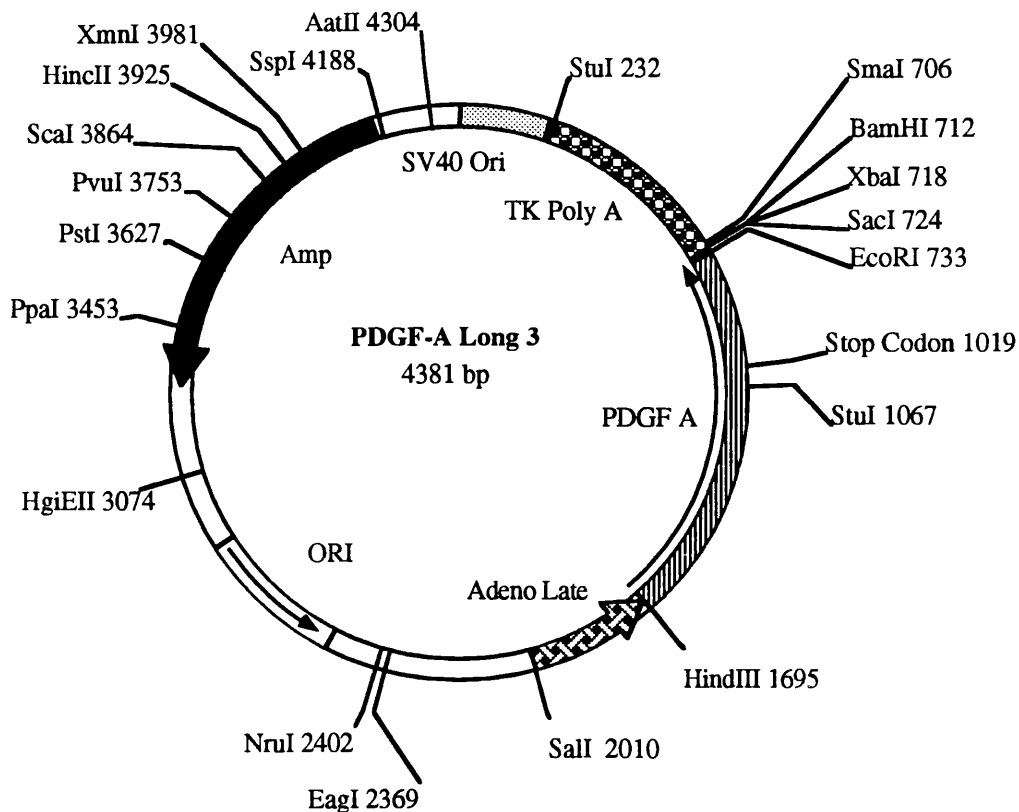PDGF A (Long form cDNA) 962bp-- Adeno virus serotype 2 major late promoter 313bp.

XmnI 3981
HincII 3925
ScaI 3864
PvuI 3753
PstI 3627
PpaI 3453

AatII 4304
SspI 4188
StuI 232

Amp

HgiEII 3074

ORI

NruI 2402
EagI 2369

SV40 Ori
TK Poly A

PDGF-A Long 3
4381 bp

PDGF A

Adeno Late

SalI 2010

SmaI 706
BamHI 712
XbaI 718
SacI 724
EcoRI 733

Stop Codon 1019
StuI 1067

HindIII 1695

Plasmid name: PDGF-A Long 3
Plasmid size: 4381 bp
Constructed by: changing "PDGF-A Long 2" SacII site to HindIII
Construction date: 29-3-88
Comments/References: PBR322 deleted from 0 to 651 and 1063 to 2475 and minus AflIII + BspMI sites -- SV40 Ori 232bp -- TK poly A signal allowed 500bp -- Human PDGF A (long form cDNA) 962bp-- Adeno virus serotype 2 major late promoter 313bp.
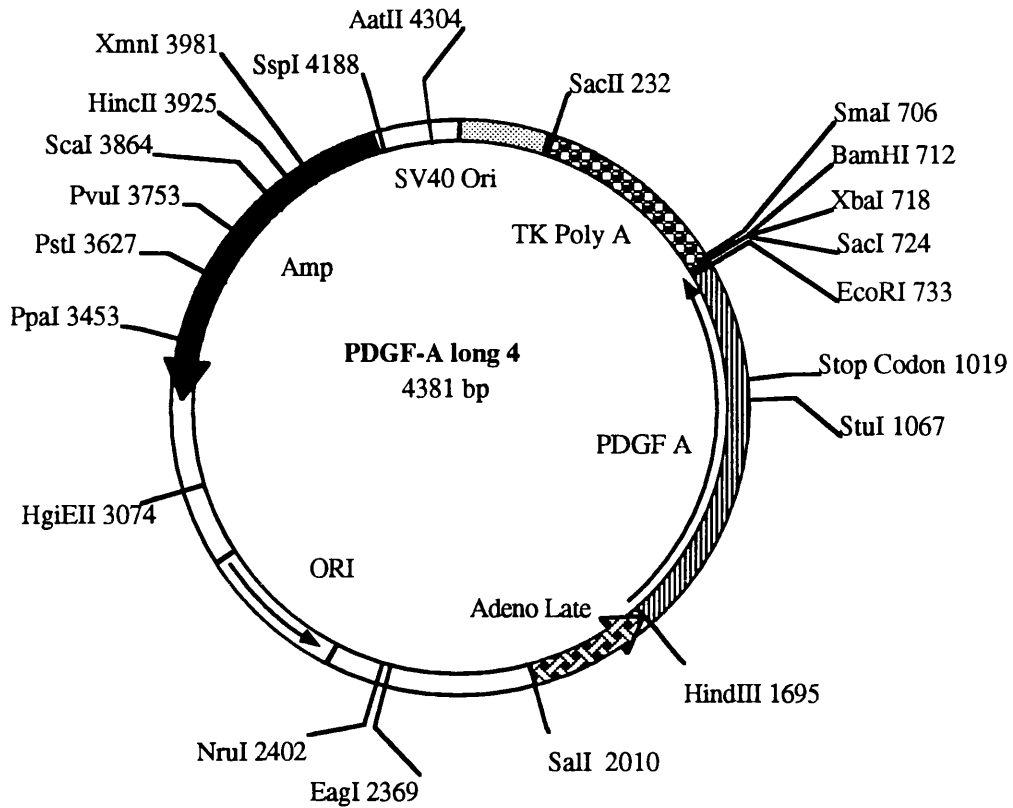
Plasmid name: PDGF-A long 4

Plasmid size: 4381 bp

Constructed by: changing "PDGF-A Long 3" StuI (232) to SacII

Construction date: 26-5-88

Comments/References: PBR322 deleted from 0 to 651 and 1063 to 2475 and minus AflIII + BspMI sites -- SV40 Ori 232bp -- TK poly A signal allowed 500bp -- Human PDGF A (Long form cDNA) 962bp-- Adeno virus serotype 2 major late promoter 313bp.
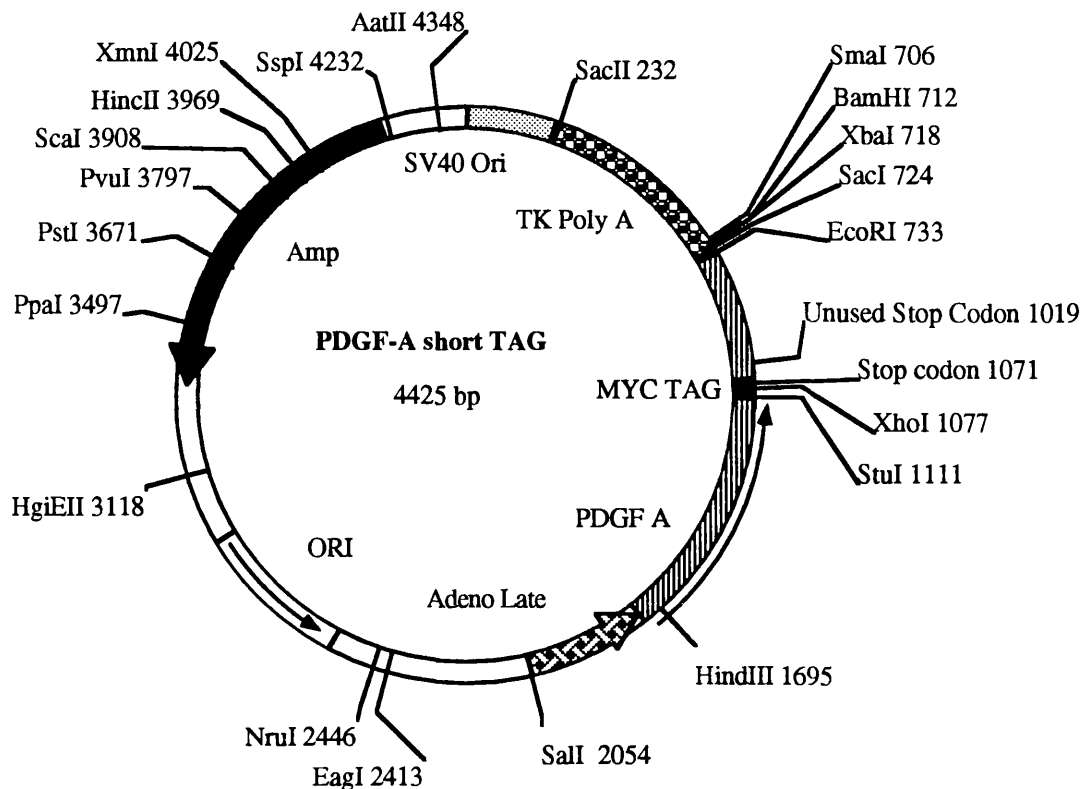
XmnI 4025
SspI 4232
AatII 4348
SacII 232
SmaI 706
BamHI 712
XbaI 718
SacI 724
EcoRI 733
HincII 3969
ScaI 3908
PvuI 3797
PstI 3671
PpaI 3497
HgiEII 3118
SV40 Ori
TK Poly A
Amp
PDGF-A short TAG
4425 bp
MYC TAG
Unused Stop Codon 1019
Stop codon 1071
XhoI 1077
StuI 1111
PDGF A
ORI
Adeno Late
NruI 2446
EagI 2413
SalI 2054
HindIII 1695

Plasmid name: PDGF-A short TAG

Plasmid size: 4425 bp

Constructed by: inserting 44bp MYC TAG at StuI site of "PDGF-A Long 4 "

Construction date: 25-5-88

Comments/References: PBR322 deleted from 0 to 651 and 1063 to 2475 and minus AflIII + BspMI sites -- SV40 Ori 232bp -- TK poly A signal allowed 500bp -- Human PDGF A (Long form cDNA) 962bp -- MYC TAG 44bp -- Adeno virus serotype 2 major late promoter 313bp.
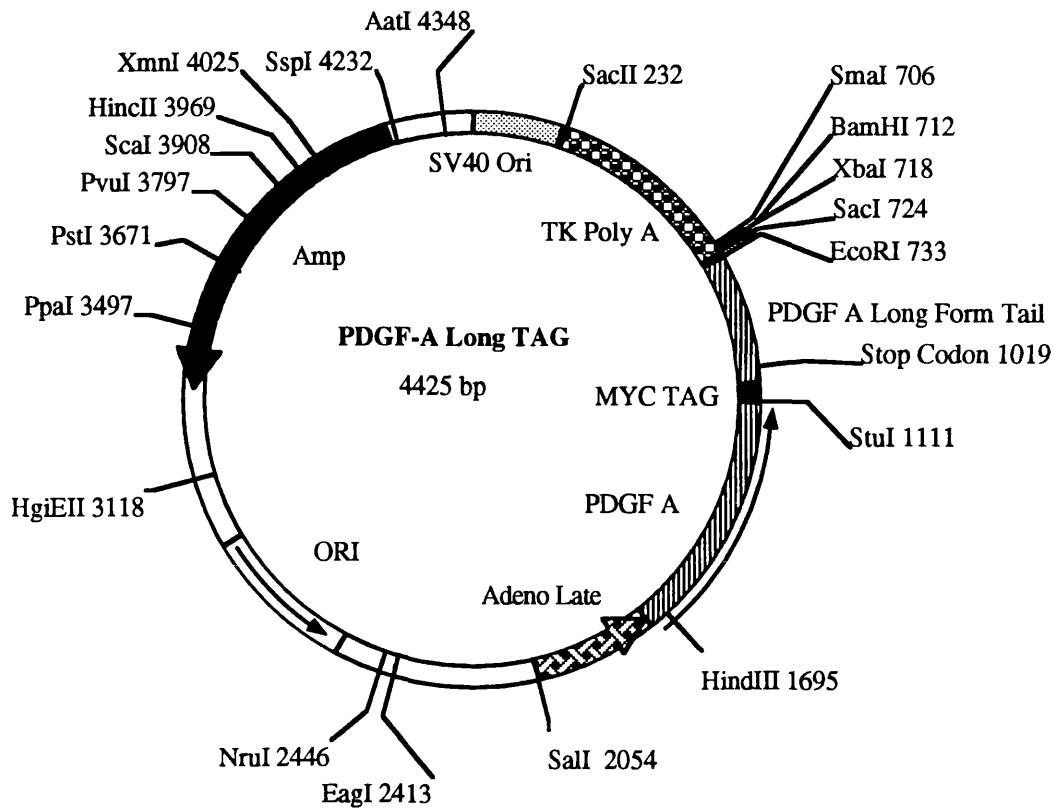
Plasmid name: PDGF-A Long TAG

Plasmid size: 4425 bp

Constructed by: filling in the XhoI site in the MYC TAG of PDGF-A short TAG

Construction date: 27-5-88

Comments/References: PBR322 deleted from 0 to 651 and 1063 to 2475 and minus AflIII + BspMI sites -- SV40 Ori 232bp -- TK poly A signal allowed 500bp -- Human PDGF A (Long form cDNA) 962bp -- MYC TAG 44bp -- Adeno virus serotype 2 major late promoter 313bp.