EXPRESSION OF FUNCTIONAL HUMAN GROWTH FACTORS IN INSECT CELLS.

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This thesis is dedicated to my Parents.

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ABBREVIATIONS.

AcNPV	Autographa californica nuclear polyhedrosis virus.
AEV	Avian erythroblastosis virus.
AR	Amphiregulin.
ATP	Adenosine 5'-triphosphate.
BSA	Bovine serum albumin.
CD	Circular dichroism.
cDNA	Complementary DNA.
CIP	Calf intestinal phosphatase.
CNS	Central nervous system.
cpm	Counts per minute.
CSF-1	Colony stimulating factor 1.
DAG	Diacyl glycerol.
DNA	Deoxyribose nucleic acid.
DS	Double stranded.
DTT	Dithiothreitol.
ECV	Extracellular virus.
EDAC	1-Ethyl-3-(3-dimethylaminopropyl)carbodimide-HCl.
EDTA	Ethylenediaminetetraacetic acid.
EGF	Epidermal growth factor.
EGF-BP	EGF binding protein.
EGTA	Ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid.
FCS	Foetal calf serum.
FeSV	Feline sarcoma virus.
FGF	Fibroblast growth factor.
Endo F	Endoglycosidase F.
FSBA	p-fluorosulphonyl 5'-benzoyl adenosine.
GM CSF	Granulocyte-macrophage colony stimulating factor.
h	Hours.
HMW-EGF	High molecular weight EGF.
^{1}H NMR	Proton nuclear magnetic resonance.
HPLC	High performance liquid chromatography.
IFN	Interferon.
IGF-1	Insulin-like growth factor 1.
IL-2	Interleukin 1.

IL-3	Interleukin 3.
IP3	Inositol 1,4,5-trisphosphate.
k b	Kilobase.
kD	Kilodalton.
Ki-MSV	Kirsten murine sarcoma virus.
LDL	Low density lipoprotein.
LTR	Long terminal repeat.
m i n	Minute.
Mo-MSV	Moloney murine sarcoma virus.
mRNA	Messenger RNA.
MRV	Malignant rabbit virus.
NRK	Normal rat kidney.
OV	Occluded virus.
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate buffered saline.
PDGF	Platelet derived growth factor.
PEG	Polyethylene glycol.
p.i.	post infection.
PIP2	Phosphatidylinositol 4,5-bisphosphate.
PLC	Phospholipase C.
PMA	Phorbol 12-myristate 13-acetate.
RF	Relicating form.
RNA	Ribose nucleic acid.
s	Second.
SDS	Sodium dodecyl sulphate.
Sf9	Spodoptera frugiperda.
SFV	Shope fibroma virus.
SGF	Sarcoma growth factor.
SSC	Saline sodium citrate.
SSV	Simian sarcoma virus.
SVGF	Shope virus growth factor.
TCA	Trichloroacetic acid.
TEMED	N,N,N',N'-tetramethylethylene diamine.
TGFα	Transforming growth factor α.
TGFβ	Transforming growth factor β.
TLC	Thin layer chromatography.
TNF	Tumour necrosis factor.
TPA	Tumour promoting agent.

TPR	Translocated promoter region.
tPA	Plasminogen activator.
Tris	Tris (hydroxymethyl) aminomethane.
uPA	urokinase.
UV	Ultraviolet.
v / v	Volume to volume.
VVGF	Vacinnia virus growth factor.
w / v	Weight to volume.

ABSTRACT.

Peptide growth factors are important in a wide variety of physiological and pathological processes as a consequence of their ability to generate a mitogenic signal via their interaction with and activation of a specific membrane-associated receptor. The subversion of the normal growth factor signalling pathway, albeit by the enhanced expression of the growth factor or receptor, the unregulated activity of the receptor protein-tyrosine kinase or the uncoupling of intracellular pathways can lead to uncontrolled cellular proliferation resulting in cellular transformation and the formation of tumours *in vivo*.

The major part of this thesis was devoted to the development of baculoviral expression systems producing large amounts of biologically active EGF and TGFα for the assessment of the structural and functional basis of ligand-receptor interactions. Insect cells infected with the recombinant baculoviruses expressed growth factors with the correct putative molecular weights which were immunologically indistinguishable from their authentic counterparts. The recombinant growth factors were able to bind to the EGF receptor expressed in insect cells and NR6+ fibroblasts and activate the receptor protein-tyrosine kinase activity, Furthermore, they induced a mitogenic response in NR6+ and Swiss 3T3 fibroblasts. It is anticipated that milligram quantities of these biologically important growth factors will be available for crystallisation. The elucidation of the three-dimensional structures of the growth factors by diffractional analysis of the crystals and structural mutational analysis will establish the nature of the receptor binding face. This information will lead to the design of clinically important EGF and TGFα antagonists. The recombinant ligands will allow the continued biophysical characterisation

of the conformation changes occuring in the extracellular domain of the EGF receptor in response to ligand binding. Cocrystallisation will ultimately lead to the determination of the ligand binding site of the receptor.

The biosynthetic precursors for EGF and TGFα are integral membrane glycoproteins suggesting that they may function to mediate physiological cell-cell recognition events. The TGFα precursor expressed in insect cells was able to stimulate the EGF receptor protein-tyrosine kinase activity. Characterisation of the precursor in insect cells will determine the relevance of this mechanism for the production of secreted growth factors and investigate further potential roles for their membrane-associated precursors.

The expression of c-erbB-2 in insect cells has allowed the evaulation of a putative growth factor receptor which is highly homologous to the EGF receptor. Furthermore, an *in vivo* association of the EGF receptor and p185$^{c-erbB-2}$ has been demonstrated. This system will provide a valuable tool for the determination of the mechanism responsible for the transmembrane translocation of the mitogenic signal.

It is anticipated that baculoviral coexpression of growth factor receptors and their cognate ligands, in conjunction with intracellular substrates will play an important role in the identification of their intracellular signalling pathways. The elucidation of the growth factor signalling pathway is crucial to the understanding of how oncogenic activation of any one of the components can result in cellular transformation.

CHAPTER 1.

INTRODUCTION.

1.1.   Growth factor receptor signal transduction.

Peptide growth factors are important in a wide variety of physiological and pathological processes as a consequence of their ability to generate a mitogenic signal via their interaction with and activation of a specific membrane-associated receptor.   The binding of a growth factor to its receptor promotes the generation of early signals in the membrane and cytoplasm and within minutes, the signal is relayed to the nucleus triggering the synthesis of DNA and cell division.   The early events induced by EGF and other growth factors include the stimulation of the Na^+/H^+ antiporter, elevation in intracellular Ca^{2+} concentration, stimulation of phospholipase C (PLC) and activation of amino acid and glucose transport pathways.   The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by $PLC\gamma$ produces inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which are responsible for the release of Ca^{2+} from intracellular stores (Berridge and Irvine, 1984) and the activation of protein kinase C (Nishizuka, 1986) respectively.   Each of these hydrolysis products may subsequently act to initiate other cascades of phosphorylation.

The generation of ionic signals by growth factors is amongest the earliest detectable mitogen-induced responses in quiescent cells.   The activation of the plasma membrane-bound Na^+/H^+ exchanger (Smith and Rozengurt, 1978) causes the extrusion of protons leading to a rapid alkalination of the cytoplasm (Schuldiner and Rozengurt, 1982).   This rise in intracellular pH may be important in the growth promoting

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effects of mitogens since treatment of cells with amiloride, an inhibitor of the Na^+/H^+ exchanger, can alter their proliferative response (Smith and Rozengurt, 1978). It has recently been observed that EGF treatment induces the phosphorylation of the Na^+/H^+ exchanger and hence the concomittant rise in intracelluar pH (Sardet et al., 1990).

It has been proposed that growth factors released by cells usually act in a paracrine manner on the adjacent cell population. However, growth factors secreted in an autocrine manner by malignant cells stimulate autonomous cell growth by interaction with these same producer cells (Todaro et al., 1977). The normal mechanism of growth factor signalling can be disrupted not only by this abnormal endogenous production of growth factors but also at a more distal stage in the pathway by the constitutive activation of the receptor, or by the uncoupling of intracellular growth regulatory pathways. Oncogenes, genes identified as transforming sequences of tumour cell DNA (Land et al., 1983) or as integral parts of the genome of tumour viruses (Bishop, 1983), have been implicated at each of the above stages of the signalling pathway. Not only can the expression of oncogenes lead to the endogenous production of growth factors, but the oncogenic products themselves can act as growth factors or growth factor receptor-related molecules or components of the intracellular signalling pathway (reviewed by Heldin and Westermark, 1984; Sporn and Roberts, 1985, 1988). In addition to the events in the membrane and cytoplasm, growth factors rapidly and transiently induce the expression in quiescent fibroblasts of the cellular oncogenes c-fos and c-myc, together with as many as one hundred other early genes (Kelly et al., 1983). Since some of these cellular oncogenes encode nuclear proteins (Abrams et al., 1983), it is plausible that their transient expression may play a role in

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the transduction of the mitogenic signal in the nucleus. Thus, the elucidation of the mechanism of action of growth factors is a crucial prerequisite for the understanding of the underlying causes of the unrestricted proliferation of malignant cells.

The identification of the extracellular and intracellular signals that stimulate cells to grow and divide provides insight into the multiple pathways that control cell growth. However, many aspects of the mechanism of mitogenic signal transduction through the activated receptor, the generation of regulatory signals and the subsequent modulation of other cellular mechanisms, remain unclear. The mechanism of cellular signal transduction and its subversion in the genesis of the malignant state will be discussed in the context of the epidermal growth factor (EGF), and the related transforming growth factor α (TGFα) and the activation of their specific receptor.

1.2. EPIDERMAL GROWTH FACTOR.

1.2.1. The initial identification of EGF.

EGF, a single chain polypeptide of 53 amino acids with a molecular weight of 6,043 daltons was first discovered in extracts of mouse submaxillary glands (Cohen, 1959). It was given its name because when injected into newborn mice, it induced precocious eyelid opening and incisor eruption as a consequence of growth stimulation of epithelial cells and the enhancement of keratinisation (Cohen, 1959, 1962). Subsequently, the human form of EGF was identified (Starkey, et al., 1975) and isolated from urine (Cohen and Carpenter, 1975). Gregory (1975) deduced the amino acid sequence of β urogastrone, a peptide isolated from human urine as an inhibitor of gastric acid secretion and, as a consequence of its amino acid sequence homology with murine EGF,

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(Savage et al., 1973) and its ability to induce mitogenesis in fibroblasts it was determined that β urogastrone was in fact, human EGF. Murine and human EGF share 37 amino acids at identical positions within the molecule, including the six Cys residues which help govern the tertiary structure of the molecule by virtue of their strict disulphide bonding (Savage et al., 1973). These disulphide bonds, together with the predicted β turns in the primary structure fold the molecule into an extremely compact structure (Holaday et al., 1976). This precise conformation determines the ability of EGF to bind to, and illicit its cellular responses through the EGF receptor, as demonstrated by the loss of activity associated with reduction of the disulphide bonds (Taylor et al., 1972) (Figure 1.1). The structure-function relationship of EGF will be discussed in detail in Chapter 3.

1.2.2. The biosynthesis of EGF.

The murine EGF cDNA sequence, isolated from a male mouse submaxillary gland cDNA library, predicted that EGF is synthesised as a precursor of 1,217 amino acids with a molecular weight of 133 kD (Scott et al., 198? ; Gray et al., 1983). Similarly the cDNA sequence for human EGF encodes a preprotein of 1,207 amino acids (Bell et al., 1986). The cDNA sequences predicted that the preproEGF molecule contained an internal hydrophobic segment characteristic of the transmembrane domain of many transmembrane proteins, suggesting that EGF is synthesised as a membrane-bound precursor. In addition to the mature EGF peptide, preproEGF contains a further eight Cys-rich sequences of 40 amino acids with distinct homology with EGF. A similar motif of repeats is displayed by many other proteins (for review see Engel, 1989), including the low-density lipoprotein (LDL) receptor (Russell et al., 1984). These structural similarities have led to the proposal that

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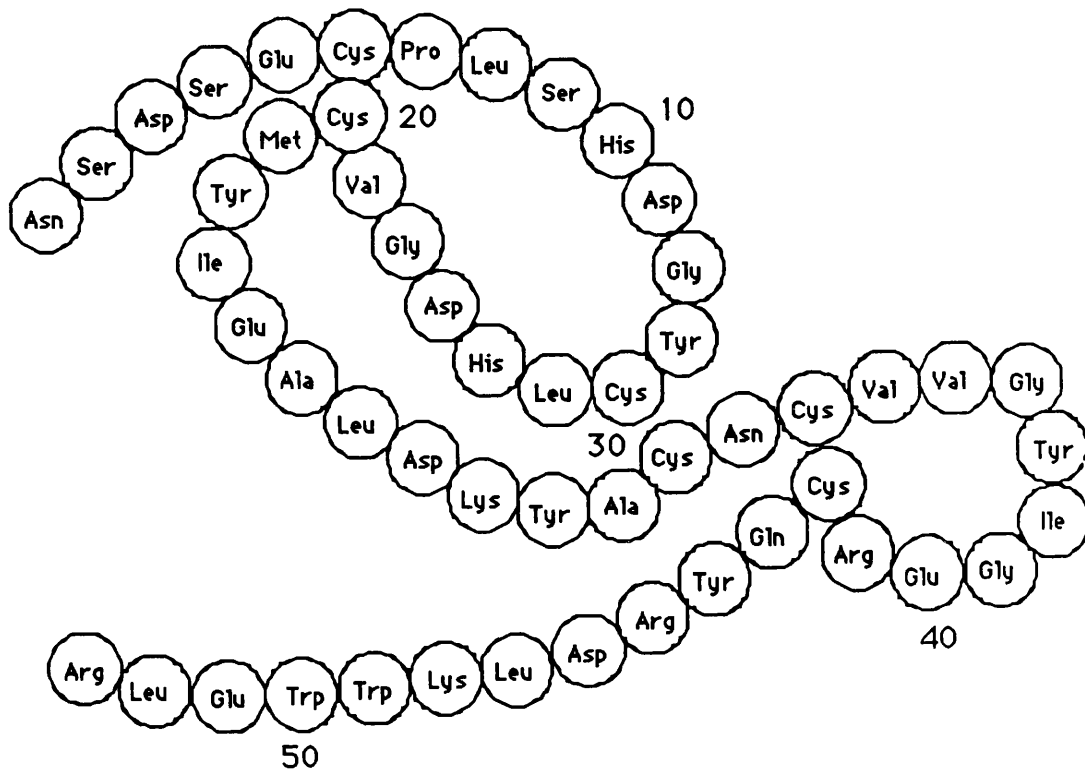
Figure 1.1. The amino acid sequence of human EGF.

preproEGF may function, not only as a precursor of EGF but also as a receptor for an as yet unidentified ligand (Pfeffer and Ullrich, 1985). The structure and potential functions of preproEGF and the role of the EGF-like repeats within the preprotein and other proteins will be discussed in Chapter 4. The membrane association of preproEGF suggested that it might exert its biological effects on a highly localised population of adjacent cells by directly interacting with their EGF receptors. Indeed, the human preproEGF cDNA when transfected into NIH 3T3 cells is expressed as a biologically active membrane-associated protein (Mroczkowski et al., 1988, 1989).

EGF has been isolated from mouse submaxillary glands as part of a high molecular weight complex (HMW-EGF), where each of the two EGF peptides are associated with a binding protein (EGF-BP) possessing Arg-specific esteropeptidase activity (Taylor et al., 1970). Upon association with the EGF-BP, the rest of the preprotein is cleaved away from the carboxyl terminus of the EGF peptide by the action of the esteropeptidase. Two EGF peptides and their EGF-BP then associate to form the stable HMW-EGF complex (Frey et al., 1979). PreproEGF is highly abundant in the kidney and submaxillary gland of the adult mouse, the level in the kidney only a factor of two lower than in the submaxillary gland (Rall et al., 1985). Surprisingly, the mature form of EGF is 2000-fold less abundant in the kidney than in the submaxillary gland, the reason for this lack of preproEGF processing remaining unclear.

1.2.3. Sources of EGF.

EGF can be detected in most human body fluids at concentrations of approximately 1 ng/ml (Dailey et al., 1978). The concentration of EGF

in the salivary gland is approximately 800 ng/ml, making it a particularly rich source of EGF (Cohen, 1959). It is present in a variety of tissues, including the placenta, kidney, stomach, bone marrow and duodenum but only at very low concentrations (Kasselberg et al., 1985). The circulating levels of EGF are low, less than 1 ng/ml in plasma, indicating that the circulatory system is unlikely to be an important source of EGF for target cells. A more physiologically relevant source of EGF is likely to be localised production, leading to the stimulation of adjacent cells in a paracrine fashion. The largest documented *in vivo* increase in EGF concentration occurs during the first two years of life and may be associated with the rapid rate of growth occurring at this stage (Mattilla et al., 1985).

1.2.4. *In vivo* activity of EGF.

EGF is the best characterised mitogen for epithelial cells, although its physiological function and the mechanism by which it induces cellular proliferation remain unclear. As stated earlier, mouse EGF was originally identified by its ability to stimulate precocious eyelid opening and incisor eruption in newborn mice (Cohen, 1962). Indeed, EGF can induce further changes in the somatic development of the mouse, including retarded growth rate and inhibition of hair growth (Tam 1985; Smith et al., 1985) and is a potent inducer of Ca^{2+} release from mouse calavariae (Stern et al., 1985). EGF treatment can directly stimulate cellular proliferation in a wide variety of tissues, such as the epidermal cells of the skin, lung, trachea, liver and kidney (Cohen and Taylor, 1974; Carpenter and Cohen, 1979; Gospodarowicz, 1983). Potential physiological roles for EGF have been proposed, including a role in foetal development (Thornburn et al., 1985), a role in the development of the mammary gland and in the control of milk production (Okamoto

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and Oka, 1984), an involvement in the functional maturation of the intestine (Oka and Orth 1983) and a participation in male reproductive function by the stimulation of the meiotic phase of spermatogenesis (Tsutsumi et al., 1986). The observation of an EGF-like activity in platelets (Oka and Orth 1983) and its production in keratinocytes (Rheinwald and Green, 1977) suggests the involvement of EGF in the wound healing response. EGF has both stimulatory and inhibitory effects on different cell populations within the same organ. During the process of tooth morphogenesis, EGF stimulates proliferation of the dental epithelium but inhibits proliferation in the mesenchyme (Partanen et al., 1985). Interestingly, the proliferation of dissociated mesenchymal cells is stimulated by EGF treatment, suggesting that the effects of EGF may be modulated by the interaction of the target cells with surrounding cells. The capacity of EGF to induce biological effects other than growth stimulation is illustrated by the fact that EGF inhibits gastric acid secretion (Gregory, 1975).

1.2.5. EGF-induced mitogenesis.

A variety of cells of ectodermal and mesodermal origin have been shown to respond to EGF in culture, including fibroblasts, keratinocytes, glial cells, chondrocytes, mammary epithelial cells, corneal and vascular endothelial cells, hepatocytes, kidney cells, HeLa and other transformed cells (Reviewed in Carpenter and Cohen, 1976). When cultured in the continuous presence of EGF, fibroblasts loose their contact inhibition and form high-density cell layers with a low serum requirement for growth (Carpenter and Cohen, 1976). The onset of DNA synthesis in response to EGF occurs approximately 15 hours after the addition of the growth factor, and reaches a maximum after 22-24 hours (Carpenter and Cohen, 1976). If EGF is removed after the onset of DNA

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synthesis, the maximum level is not affected, indicating that the cells are already committed. However, the continuous presence of EGF is required for approximately 6-8 hours in order to initiate even a minimum level of DNA synthesis (Heldin and Westermark, 1984).

1.3. TRANSFORMING GROWTH FACTORα

1.3.1. Initial identification of TGFα.

The initial observation that led to the identification of TGFα, was that fibroblasts transformed by murine sarcoma viruses (Mu-SV) displayed reduced numbers of EGF receptors (Todaro et al., 1976). As a consequence of this loss of EGF receptors, fibroblasts transformed by Mu-SV or feline sarcoma viruses (Fe-SV) were no longer able to bind EGF (Todaro et al., 1977). It was subsequently shown that these cells released an EGF-related peptide that bound to the EGF receptors, thereby rendering them unavailable for binding exogenous ligand. This factor was originally isolated from Moloney MuSV-transformed murine 3T3 cultures and was therefore termed sarcoma growth factor, SGF (DeLarco and Todaro, 1978a, b).

SGF preperations were able to induce profound morphological changes in rat fibroblasts, causing them to adopt a phenotype similar to that of virally transformed cells. It was shown that these preperations were able to promote colony formation in soft agar of normal anchorage-dependent fibroblasts. Furthermore, if these colonies were replated in the absence of sarcoma growth factors, they again grew as normal contact-inhibited fibroblasts (DeLarco and Todaro, 1978a, b; Todaro et al., 1980). The fact that this factor was synthesised by a number of transformed cells, in addition to its ability to transform

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normal rat kidney cells (NRK), led to the name transforming growth factor.

Extensive purification and characterisation of preparations of transforming growth factor showed that they in fact consisted of two structurally unrelated peptides, TGFα and β (Anzano et al., 1983). The EGF receptor binding capacity was an inherent property of TGFα alone, whereas the morphological effects observed arose as a consequence of its cooperativity with TGFβ (Anzano et al., 1983). TGFβ is a highly ubiquitous molecule produced by a variety of cell types, normal, neoplastic, mesenchymal and epithelial (reviewed by Goustin et al., 1986). The growth stimulatory effects of TGFβ have been observed only in fibroblast cells, possibly through an indirect mechanism involving the induction of endogenous growth factor synthesis resulting in autocrine growth (Loef et al., 1986). In fact, TGFβ is a potent growth inhibitor for most cell types tested, including monkey kidney cells (Tucker et al., 1984), several human carcinoma cell lines and secondary cultures of human foreskin keratinocytes (Moses et al., 1985). TGFβ may therefore play a role in the regulation of the cell cycle through the maintenance of the resting state via a negative feedback loop.

1.3.2. The isolation of cDNAs for human and rat TGFα.

TGFα species have been detected in the culture supernatants and extracts from a number of transformed human and rodent cells (DeLarco and Todaro, 1978a, b; Todaro et al., 1980; Marquardt et al., 1983). This population of TGFα molecules displays a heterogeneity in apparent molecular weight, ranging from the 6 kD species secreted by tumour cell lines to the 34 kD species isolated from the urine of some cancer patients (Sherwin et al., 1983). The 6 kD form has been purified to

homogeneity from a variety of cell sources (Marquardt et al., 1983; Massague, 1983), and subsequent amino acid sequencing led to the elucidation of the complete amino acid sequence for the 50 amino acid rat TGFα (Marquardt et al., 1984). Comparison of the amino acid sequences of rat TGFα and murine EGF indicated that they shared a 33% sequence homology (Figure 1.2). The structural and functional properties of TGFα will be discussed in Chapter 5.

The rat TGFα cDNA was isolated from a cDNA library prepared from mRNA extracted from FeSV-transformed Fisher rat embryo fibroblasts (Lee et al., 1985). The library was screened with a mixture of oligonucleotides based on the peptide sequence information and a cDNA clone encoding the TGFα peptide isolated. The cDNA sequence predicted that the growth factor was initially synthesised as part of a 159 amino acid precursor.

Synthetic oligonucleotides designed on the partial amino acid sequence of human TGFα were used to screen a complete human genomic library and a TGFα exon isolated (Derynck et al., 1984). The exon specifically recognised an extremely rare mRNA species of approximately 4.8 kb in a sample from a renal carcinoma cell line. A cDNA library was prepared using RNA from this cell line and a single clone isolated upon hybridisation with the TGFα exon. The clone was sequenced and found to encode the 50 amino acid TGFα form as part of a 160 residue precursor. Analysis of the human and rat cDNA sequences indicated the presence in the precursor of an amino terminal signal sequence and an extremely hydrophobic domain, characteristic of the transmembrane domain of membrane-spanning proteins, suggesting that the TGFα precursor is in fact synthesised as an integral membrane

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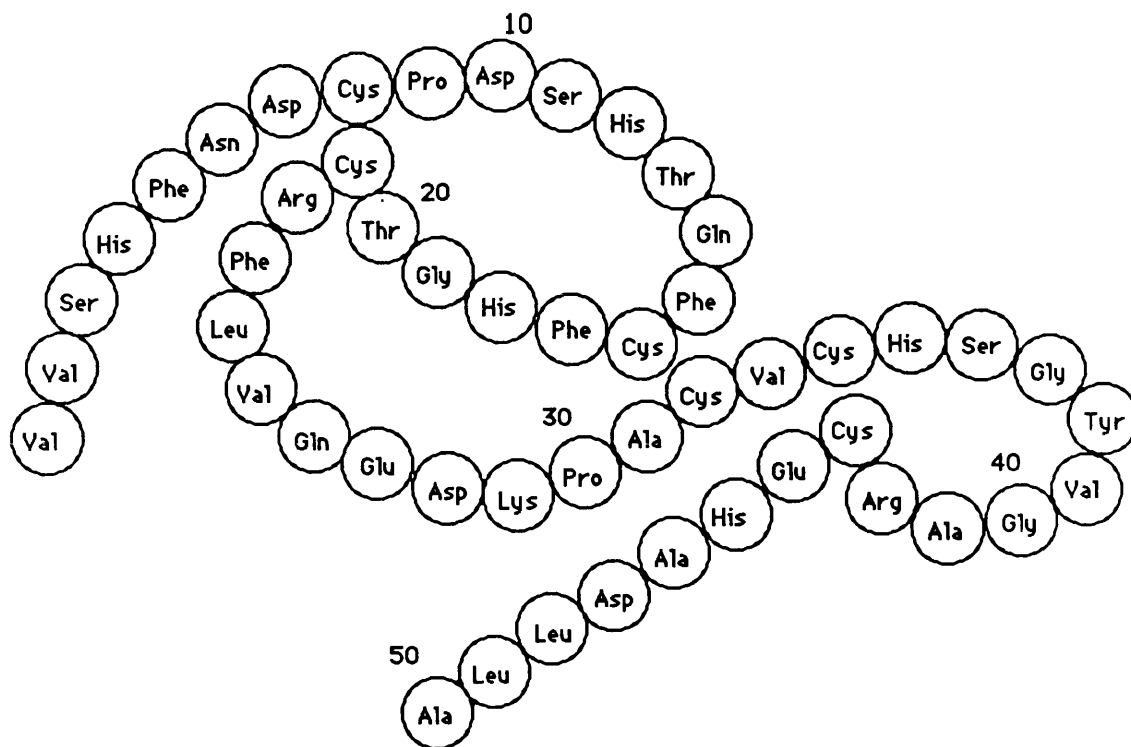
Figure 1.2. The amino acid sequence of human TGFα.

protein. The structure and potential functions of this precursor is discussed in greater detail in Chapter 4.

1.3.3. Cellular sources of TGFα.

TGFα was initially detected in the culture supernatants of rodent fibroblasts transformed with Moloney or Kirsten MSV (DeLarco and Todaro, 1978a, b; Todaro et al., 1977; Ozanne et al., 1982). Since those initial observations, it has been determined that cells transformed chemically (Moses et al., 1981) or by infection with SV40 (Kaplan et al., 1981) or polyoma virus (Kaplan et al., 1980) also secrete TGFα into their media. Furthermore, a variety of human tumour-derived cell lines synthesise TGFα (Todaro et al., 1980; Roberts et al., 1980; Marquardt and Todaro 1982). Derynck et al. (1986) examined a large number of different types of human tumours and tumour-derived cells lines for the presence of TGFα mRNA. No TGFα mRNA was detected in any cell lines of haematopoietic origin nor in the two normal fibroblast cell lines analysed. A mRNA species of 4.5-4.8 kb was clearly present in many cell lines of carcinoma or sarcoma origin. Indeed, TGFα mRNA was detected in surgically removed carcinomas and sarcomas, particularly in renal, mammary and squamous carcinomas and tumours of neuronal origin. The occurence of TGFα in such a wide variety of solid tumours suggests that the abnormal expression of TGFα may play a role in malignant transformation and tumour formation *in vivo*.

1.3.4. Further biological effects of TGFα expression during malignancy.

Malignancy-associated hypercalcaemia occurs relatively frequently in patients with renal, squamous and breast carcinomas and melanomas, tumours of this type most consistently displaying elevated

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expression of TGFα (Mundy et al., 1985). TGFα is a very potent inducer of Ca^{2+} release in mouse calvariae and in rat foetal long bones (Stern et al., 1985; Ibbotson et al., 1986). *In vitro* studies indicate that the induction of bone resorption by TGFα may be due to an inhibition of osteoblast activity as measured by the effects on collagen synthesis, and by the activation of the osteoclast population (Takahashi et al., 1986). These results led to the speculation that TGFα expression triggers or contributes in some way to malignancy-induced hypercalcaemia, a proposal supported by the observation that a cell line derived from an osteosarcoma contains highly elevated levels of TGFα mRNA (Derynck et al., 1986).

Angiogenesis is a prominent feature of several physiological and pathological processes, including tumour development. TGFα promotes neovascularisation as measured in the hamster cheek pouch assay, as a consequence of its mitogenic effect on endothelial cells (Schreiber et al., 1986). The endogenous production of TGFα by various solid tumours may therefore not only function to stimulate the growth of the tumour cells, but also to promote tumour-induced angiogenesis. Indeed, haematopoietic tumours which do not require neovascularisation for their development do not synthesise TGFα. TGFα exerts a potent activity on vascular tissue resulting in increased regional arterial blood flow in a variety of vascular beds. The synthesis of TGFα by tumour cells suggests a role for TGFα in the local vascular hyperdynamic state associated with malignancy (Derynck et al., 1986).

1.4. THE EXPRESSION OF TGFα IN NON-TRANSFORMED CELLS.

TGFα expression has been observed in a number of normal human tissues including the pituitary (Kobrin et al., 1986), where it

may be responsible for cellular proliferation under normal physiological conditions. TGFα expression has also been detected in rat kidney, liver, spleen and brain but at levels 10-20 fold lower than those seen in transformed fibroblasts (Lee et al., 1985). Recently, TGFα expression has been detected in RNA extracted from the adult human brain, a classically non-proliferating tissue (Wilcox and Derynck, 1988b). TGFα has subsequently been localised immunohistolochemically to the brain and furthermore, a cDNA clone has been isolated from a brainstem library (Kudlow et al., 1989). The TGFα secreted by brain cells may therefore have a paracrine effect on the proliferation of neurons and glial cells which appear to express EGF receptors (Libermann et al., 1984).

1.4.1. The role of TGFα in development.

The observation that mouse embryos express TGFα mRNA indicated that the expression of growth factors by tumour cells may reflect the abnormal production of such factors operating during the normal course of embryonic development (Twardzik et al., 1982). TGFα is differentially expressed during mouse foetal development, expression is detected at the stage of blastocyst formation, peaks at day 7 and then drops rapidly until it is no longer detectable at birth (day 21) (Twardzik, 1985). In situ analysis with TGFα-specific antibodies has indicated that TGFα is transiently expressed in several embryonic tissues, including the placenta, otic vesicle, oral cavity, pharyngeal pouch and kidney (Wilcox and Derynck, 1988a). This embryonic expression of TGFα and the detection of EGF receptor expression in mouse embryos as early as day 11 (Nexo et al., 1980) suggests that TGFα may function as a normal embryonic version of a family of EGF-related peptides.

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1.4.2. The role of TGFα in wound healing.

In vivo investigations demonstrated that topically applied EGF or TGFα promotes wound healing primarily by accelerating epidermal regeneration (Schultz et al., 1987). The infiltration of activated macrophages precedes epithelial and connective tissue proliferation indicating that they may be an important source of growth factors for these tissues. Indeed, it has recently been determined that cultures of activated macrophages express the gene for TGFα and secrete the growth factor into the medium (Madtes et al., 1988). Primary cultures of human keratinocytes, a further cell type involved in the wound healing response, have been shown to synthesise TGFα in a TGFα-inducible manner (Coffey et al., 1987). Furthermore, an EGF receptor-binding activity has been detected in platelets which could be EGF or TGFα (Oka and Orth 1983). The release of this EGF-like factor at a site of wound healing could induce enhanced TGFα expression in keratinocytes, thereby increasing their rate of proliferation. An imbalance between the amplification of TGFα expression and an inadequate mechanism of inhibition may be instrumental in the malignant conversion of keratinocytes into squamous carcinomas which consistently show elevated levels of TGFα expression. Interestingly, the exposure of keratinocytes to TPA, the most potent phorbol ester promoter of skin tumours, increases TGFα gene expression and protein synthesis (Pittelkow et al., 1989). Epidermal hyperplasia and angiogenesis are hallmarks of psoriasis, both characteristic physiological effects of elevated TGFα expression. It can therefore be proposed that the overexpression of TGFα in keratinocytes could be directly responsible for the initiation and maintenance of epidermal hyperproliferation in the psoriatic lesion (Elder et al., 1989).

1.4.3. Comparative biological activities of TGFα and EGF.

A comparitive binding study indicated that murine EGF and rat TGFα compete for human receptor binding with equal potency, although TGFα required a stringent pH optimum (Massague, 1983). Continued exposure of A431 cells to either EGF or TGFα induces down regulation according to the same kinetics (Massague, 1983). One of the first biological activities attributed to EGF was its ability to induce precocious eyelid opening in newborn mice. Comparison between human TGFα and murine and human EGF indicated that TGFα treatment induced a similar response (Tam, 1985). Both growth factors can induce other changes in the somatic development of the mouse, accelerated tooth eruption, retarded growth rate and inhibition of hair growth (Tam, 1985; Smith et al., 1985). Membrane ruffling is a very early response of cells in culture to treatment with various growth factors. Both TGFα and EGF are able to induce rapid and transient ruffling responses in sparsely cultured cells, the magnitude and duration of the response being greater with high doses of TGFα than with an equal dose of EGF (Myrdal, 1985).

TGFα is approximately 3-10 fold more potent than EGF in the promotion of Ca^{2+} release from mouse calavariae (Stern et al., 1985; Ibbotson et al., 1986). The difference is even more striking in the foetal rat long bone system where TGFα induces profound Ca^{2+} release in a dose-dependent manner, whereas EGF treatment does not trigger any statistically significant effect. Furthermore, TGFα was 10-100 fold more potent than EGF in stimulating the proliferation of osteoclast precursor cells (Ibbotson et al., 1986). EGF is a relatively poor inducer of angiogenesis, but TGFα is able to induce neovascularisation at low

concentrations that are without effect in the case of EGF (Schreiber et al., 1986).

These observations indicate that TGFα and EGF behave differently in certain biological systems, in many cases, TGFα appearing to be more potent than EGF. It is therefore important that TGFα and EGF should not be considered as mere analogues because they bind to the same receptor and cannot be discriminated by virtue of their biological effects in some assay systems. It is possible that TGFα and EGF interact with the receptor generating non identical conformational changes within the receptor or that the ligand-receptor complexes are processed differently within the cell.

1.5. RECEPTOR PROTEIN-TYROSINE KINASES.

A number of growth factors stimulate cell mitogenesis by their interaction with specific cell surface receptors that possess intrinsic protein-tyrosine kinase activity (for reviews; Yarden and Ullrich, 1988a,b; Schlessinger, 1988). Little is known about the cascade of biochemical events that the stimulation of the receptor protein-tyrosine kinase generates, although the cloning of cDNAs for several of the receptor protein-tyrosine kinases has made dramatic steps forward. The availability of the complete primary structure of several receptor protein-tyrosine kinases has suggested a common overall topology. The extracellular ligand-binding domain, designed to interact with a polypeptide growth factor, is connected via a hydrophobic transmembrane segment with the cytoplasmic catalytic domain which is capable of generating signals culminating in a plethora of cellular responses (Figure 1.3).

EXTRACELLULAR DOMAIN
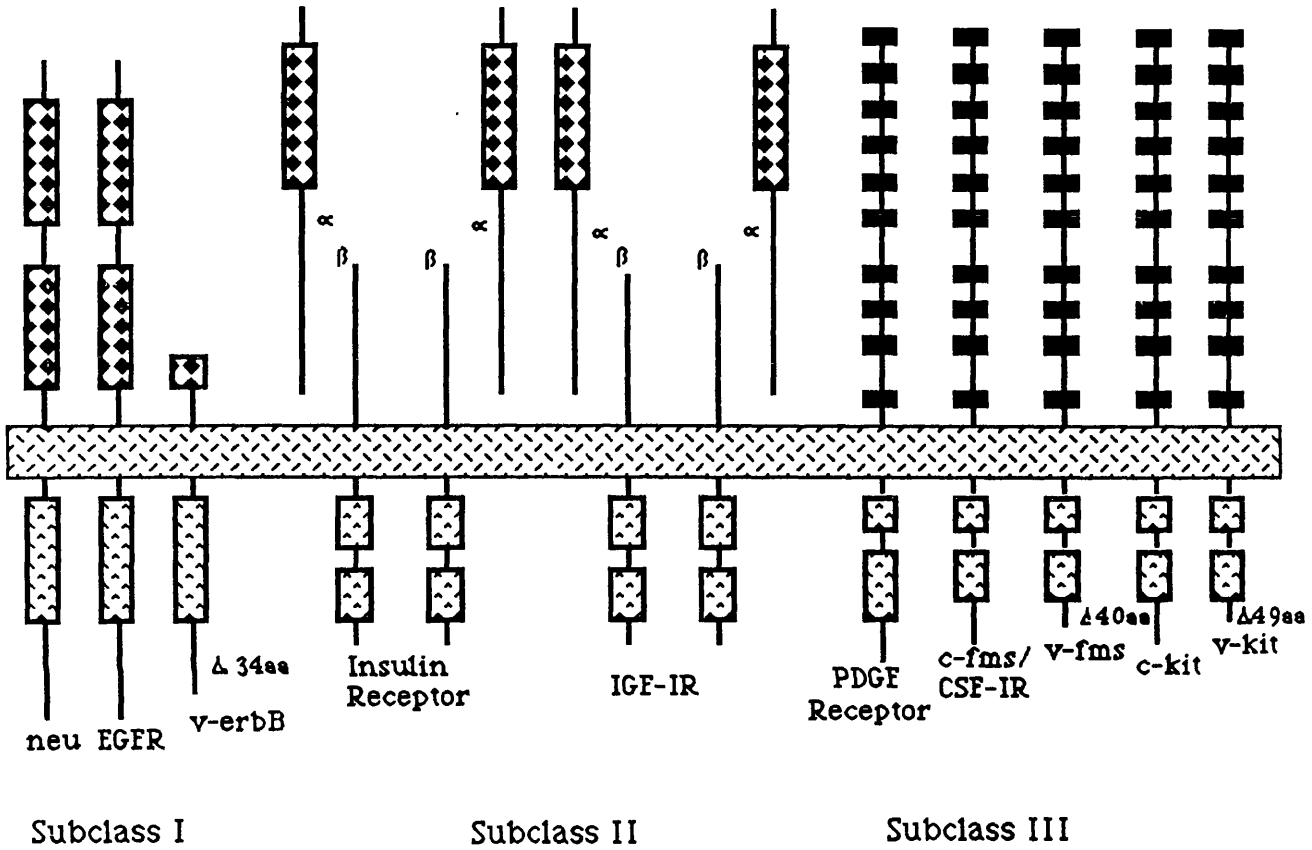
CYTOPLASMIC DOMAIN

FIGURE 1.3. Structural features of the receptor protein-tyrosine kinase family showing the suclasses

Cys-rich repeat regions

Kinase domain

Individual Cys residues

The large cytoplasmic domain of the receptor protein-tyrosine kinases (500-600 amino acids) readily distinguishes them from other cell surface receptors such as the Low Density Lipoprotein (LDL) receptor which displays a short cytoplasmic segment of only 50 residues (Yamamoto et al., 1984). Another characteristic of the protein-tyrosine kinase family of receptors is that they possess only a single transmembrane domain whereas the group of receptors, including the rhodopsin (Nathans and Hogness, 1983) and muscarinic (Kubo et al., 1986) receptors, contain seven membrane-spanning segments. Conversley, the putative Drosophila receptor protein-tyrosine kinase, sevenless (Hafen et al., 1987) contains two potential transmembrane sequences. However, it is unclear whether the polypeptide exists as a single moiety with two transmembrane domains or whether it is post translationally processed into two or more subunits.

1.5.1. CLASSIFICATION OF RECEPTOR PROTEIN-TYROSINE KINASES.

The receptor protein-tyrosine kinases can be classified into three structural subgroups.

1.5.1.1. Subclass I.

In the human and Drosophila EGF receptor, the extracellular ligand binding domain contains a gene-duplicated Cys-rich sequence (Ullrich et al., 1988; Livneh et al., 1985). A similar arrangement is also observed in the closely related c-erbB-2/neu gene product (Coussens et al., 1985; Bargmann et al., 1986a, b) and the recently identified c-erbB-3 gene product (Kraus et al., 1989; Plowman et al., submitted). Bajaj et al. (1987) proposed that the extracellular region of the human EGF receptor

and the c-erbB-2 product comprised two large homologous domains (L) each followed by several smaller Cys-rich domains (S) to give an arrangement of $L_1 S_1 S_2 S_3 L_2 S_1 S_2 S_3$. The Drosophila EGF receptor has a similar duplicated sequence of the L and S domains but the second S domain is further duplicated (Figure 1.4).

1.5.1.2.   Subclass II.

The Insulin receptor (Ullrich et al., 1985), its Drosophila homologue, DIR (Nishida et al., 1986) and the insulin-like growth factor 1 (IGF-1) receptor (Ullrich et al., 1986) function as heterotetrameric structures comprising two α and two β subunits connected by disulphide bonds.   The two α subunits form the ligand-binding domain and are disulphide linked to the two β subunits which traverse the membrane and contain the protein-tyrosine kinase domain.   Each of the α subunits possess a Cys-rich repeating motif and hence, the heterotetramer as a whole displays two such domains.   Further members of subclass II are c-ros (Matsushime et al., 1986), TRK (Martin-Zank et al., 1986) and MET (Park et al., 1987).

1.5.1.3.   Subclass III.

The receptors for the Platelet-derived growth factor (PDGF) family (α and β subtypes) (Yarden et al., 1986), Macrophage colony stimulating factor 1 (CSF-1) (Sherr et al., 1985) and the proto-oncogene, c-kit (Yarden et al., 1987) contain five Immunoglobulin-like segments in their extracellular domains.   Each of the Immunoglobulin-like repeats, except the fourth, possess the characteristic spacing of the two Cys residues and the Trp and Tyr residues which are a diagnostic feature of the Immunoglobulin domain (Williams and Barcley, 1988).   In addition, subclass III receptors are further discriminated by the

EXTRACELLULAR DOMAIN
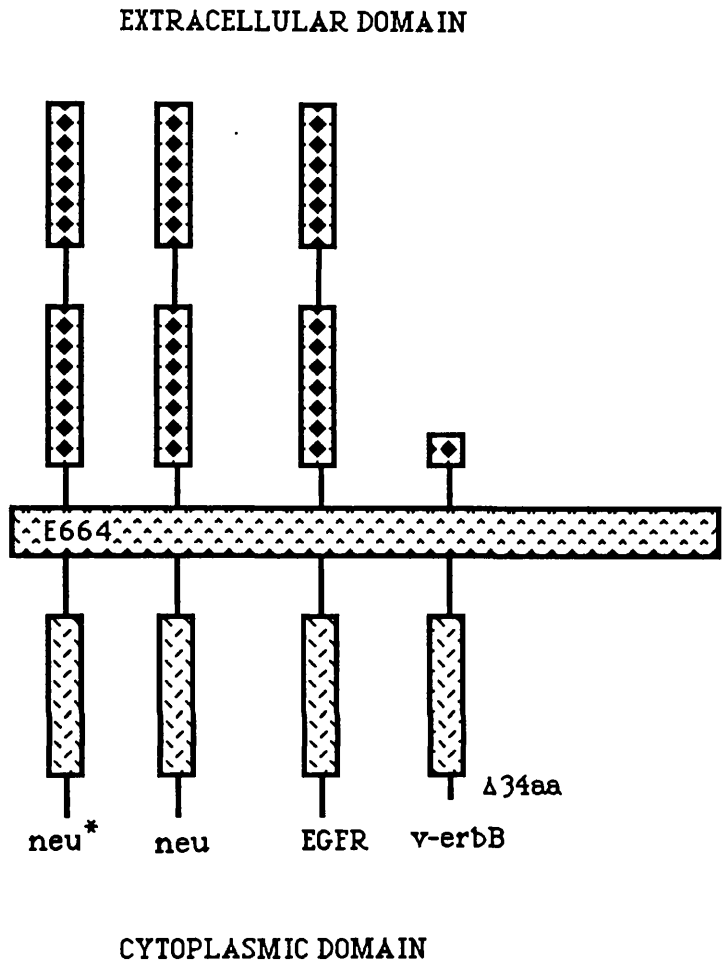
FIGURE 1.4. Members of the subclass I of the receptor protein-tyrosine kinase family showing the mutations giving rise to oncogenic activation

▣ Cys-rich repeat units

▣ Kinase domain

interruption of the protein-tyrosine kinase domain with a long structurally unique hydrophilic sequence of 70 amino acids for the CSF-1 receptor (Sherr et al., 1985) and 104 residues for the PDGF receptor (Yarden et al., 1986) (Figure 1.5). Recently, the receptor for chicken basic fibroblast growth factor (FGF) has been cloned (Lee et al., 1989). The presence of three Immunoglobulin-like domains in the putative extracellular region classified it as a member of the subclass III family. Interestingly, the kinase domain insertion sequence is only 14 amino acids, much shorter than those of the other members of the family.

The structure-function relationships of the receptor protein-tyrosine kinase family will be discussed in the context of the EGF receptor through which both EGF and TGFα illicit their cellular responses (for review see Carpenter, 1987) (Figure 1.6).

1.6. THE EGF RECEPTOR.

1.6.1. The extracellular domain.

The extracellular domain of the EGF receptor comprises 621 amino acids with a typical signal sequence preceding the ligand-binding region, which is heavily glycosylated at 11 or 12 Asn residues (Mayes et al., 1984). There is 50% amino acid sequence identity between the extracellular domain of the EGF receptor and the homologous c-erbB-2/neu translation product. Little is known about the three dimensional conformation that the extracellular domain adopts and which residues are important in maintaining the ligand-binding site and for generating the specific ligand-receptor interactions. Bajaj et al. (1987) proposed that the extracellular domain of the human EGF receptor

EXTRACELLULAR DOMAIN

Δ 40aa   Δ49aa

c-fms/   v-fms   c-kit v-kit
CSF-IR

CYTOPLASMIC DOMAIN
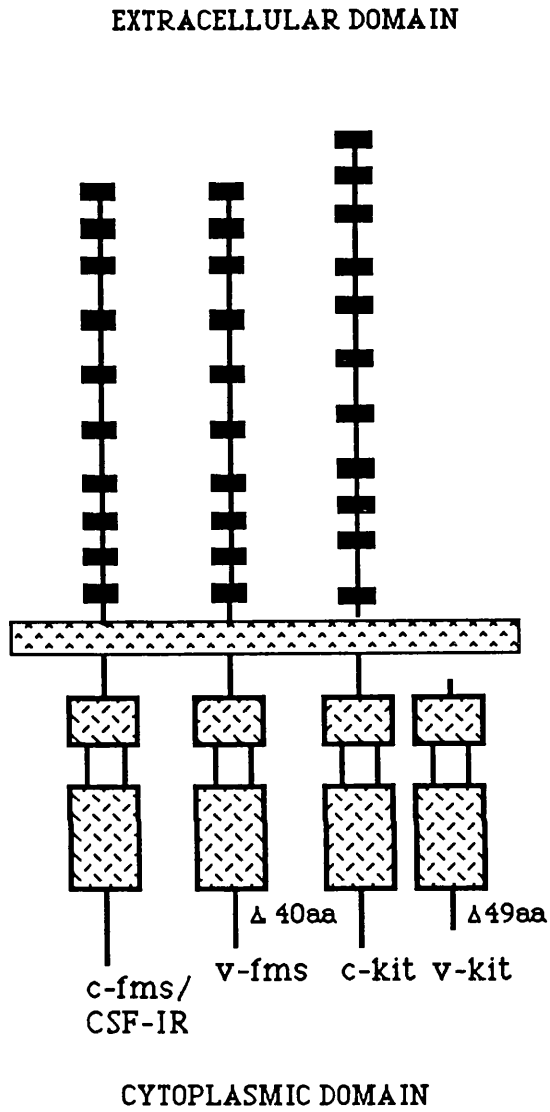
FIGURE 1.5. Members of the subclass III receptor protein-tyrosine
family showing the mutations giving rise to oncogenic activation

■ Individual Cys residues

▨ Kinase domain showing insertional sequences

EXTRACELLULAR DOMAIN   CYTOPLASMIC DOMAIN

FIGURE 1.6. Schematic representation of the EGF receptor showing the
functionally important residues and the four domains of the extracellular domain

Signal peptide

Transmembrane domain

comprised two large homologous domains (L) each followed by several smaller Cys-rich domains (S). They predicted that the L domains were made up of alternate α helix/β sheet structures with each of the Cys-rich S domains arranged as three subdomains of eight Cys residues. The close proximity of the second S domain to the transmembrane sequence indicated that the S domains were spatially adjacent to the membrane and according to their model, the L domains were placed away from the membrane. They proposed that the close proximity of the S domains to the membrane made them less accessible for ligand binding and therefore less likely to contribute directly to ligand binding. They therefore concluded that the L domains were involved directly in the establishment of the ligand binding site.

Lax et al. (1988) divided the extracellular domain into four segments, the amino terminal sequence termed domain I (analogous to L_1), the two Cys-rich repeating segments II (S_1) and IV (S_2) and the intervening sequence; domain III (L_2) displaying significant sequence homology with domain I. The observation that two monoclonal antibodies generated against domain III competed with EGF for receptor binding, suggested that this domain is a major component of the ligand-binding site, confirming the proposal of Bajaj et al. (1987). Substantiating evidence for this proposal came from the use of chimeric receptors constructed from the extracellular domains of the human and chicken EGF receptors, based on the findings that murine EGF bound to the chicken receptor with 100-fold lower affinity than to the human receptor (Lax et al., 1989). The chimeric receptor, containing domain III of the human receptor on the chicken receptor background bound murine EGF with higher affinity than the chicken wildtype receptor indicating that the human domain III was responsible for the high

affinity ligand binding. Conversely, the chimera containing the chicken receptor domain III on the human receptor background displayed similar low affinity binding to the chicken wildtype receptor.

It has been determined that one mole of EGF receptor binds one mole of EGF (Weber et al., 1984). The interpretation of binding studies indicated the presence of two classes of EGF receptors with different affinities for ligand binding, those with high affinity for binding comprising 5-10% of the receptor population and the remaining 90-95% having a low affinity (King and Cuatrecasas, 1982).

1.6.2. The Transmembrane domain.

Subclass I receptor protein-tyrosine kinases display a conserved length in their membrane-spanning segments although there appears to be no primary structural conservation. The membrane-flanking regions, the cluster of basic residues at the carboxyl terminus and the Pro residue at the amino terminus characteristic of the majority of membrane-associated proteins, presumably reflect the mechanism of translocation of the receptor across and anchorage into the lipid bilayer (Blobel, 1980).

1.6.3. The Juxtamembrane domain.

There is 75% homology between the EGF receptor and the c-erbB-2/ neu gene product in the segment that seperates the kinase domain from the cytoplasmic face of the plasma membrane (Coussens et al., 1985; Bargmann et al., 1986a, b). The conserved Thr residue at position 654, ten amino acids from the transmembrane segment serves, along with several other Ser/Thr residues, as a substrate for phosphorylation by the Ca^{2+}-dependent protein kinase C (Schlessinger, 1986). The

activation of protein kinase C, achieved by the treatment of cells with the phorbol ester TPA, abolishes high affinity ligand binding and reduces the receptor protein-tyrosine kinase activity (Shoyab et al., 1979). Hence, phosphorylation at Thr^{654} allosterically regulates ligand binding ,affinity and protein-tyrosine kinase activity, a phenomenon known as receptor transmodulation (Whiteley and Gasar, 1986). Treatment of cells with PDGF or bombesin has the same regulatory effect on the EGF receptor kinase, although potentially through the activation of another distinct kinase, since PDGF treatment of protein kinase C deficient fibroblasts is able to stimulate phosphorylation of the receptor at Thr^{654} (Davis and Czech, 1987).

The role of Thr^{654} phosphorylation in the regulation of kinase function has been studied through its mutation to several other residues. Lin et al. (1986) introduced an Ala residue at position 654 and determined that the mutant receptor was unable to internalise in response to TPA, although upon ligand binding, the mutant receptor was internalised and degraded correctly. Thus, two independent mechanisms were proposed for the internalisation of the occupied receptor, one dependent upon ligand binding and the second regulated through phosphorylation at Thr^{654} mediated by protein kinase C. When Thr^{654} was mutated to a Tyr residue, the mutant receptor retained high and low affinity ligand binding sites (Livenh et al., 1987). Following TPA treatment, the high affinity sites were abolished and the receptor was phosphorylated on several Thr/Ser residues. However, TPA treatment did not block the mitogenic activity of the mutant receptor, highlighted by the observation that EGF treatment stimulated DNA synthesis even in the presence of TPA. These observations suggested that the phosphorylation of Thr^{654}, mediated through protein kinase C,

functions as a negative control mechanism for EGF-induced mitogenesis.

1.6.4. The kinase domain.

The kinase domain of the receptor protein-tyrosine kinases displays the highest degree of sequence conservation not only within the family, but also in comparison with other protein-tyrosine kinases and, to a lesser extent, with the Ser/Thr family of kinases (Hanks et al., 1988). The alignment of the catalytic domains of the receptor protein-tyrosine kinases led to the determination of conserved residues which were implicated to play a functionally important role. The consensus Gly-X-Gly-X-X-Gly motif (Wierenga and Hol, 1983) found in many nucleotide binding proteins, in addition to the protein kinases, was found at the amino terminus of the catalytic domain. The involvement of the invariant Lys^{721} residue in the phosphotransfer reaction was demonstrated by the binding of the ATP analogue, p-fluorosulphonyl-5'-benzoyl adenosine (FSBA) at this position resulting in the inhibition of the kinase activity (Russo et al., 1985).

The importance of functional kinase activity was established when Lys^{721} was mutated to an Ala residue and the mutant receptor expressed in NIH 3T3 cells (Honegger et al., 1987a, b). The mutant receptor was found to be processed normally, expressed at the cell surface and displayed both high and low affinity sites for ligand binding, the high affinity sites being abolished upon TPA treatment. However, this mutant receptor protein-tyrosine kinase was shown to be inactive. Furthermore, the mutant was defective in the transduction of the mitogenic signal generated in response to ligand binding. These observations suggested that, although a functional kinase activity is not

required for the binding of ligand to the receptor nor for the transmodulation of the receptor by protein kinase C, it is necessary to trigger the various cellular responses to ligand binding. Thus, binding of the ligand provides a necessary but insufficient step, the activation of the protein-tyrosine kinase is ultimately essential for signal transduction. Substantiating evidence for the role of the kinase in signal transduction was provided by the generation of a mutant receptor with an insertion of four residues at position 708 (Prywes et al., 1986). Similar loss of kinase function was observed but in addition, TPA treatment was unable to modulate the affinity of the mutant receptor for EGF or induce its phosphorylation at Thr^{654}. It was therefore proposed that this mutation altered the structure of the kinase domain in such a way that it abolished its specific interactions with protein kinase C, these interactions being maintained in the Ala^{721} mutation.

It was further observed that a functional kinase is essential for normal cell routing of the receptor (Honneger et al., 1987a, b). Occupied kinase-deficient receptors were internalised and the ligand delivered to lysosomes for degradation. However, the receptor itself was not downregulated but was recycled to the cell surface. It was proposed that, under normal conditions, the receptor is internalised and degraded continuously (Schlessinger, 1988). Upon activation by ligand binding, the receptor is delivered to lysosomes for degradation only after several rounds of recycling, the process of degradation operating at approximately 30% efficiency. In the case of the kinase-deficient receptor, the efficiency of receptor degradation decreases so that the receptor undergoes continuous recycling. The observed reduction in the rate of endocytosis of a receptor where the entire kinase domain has been deleted lent further support to this model (Livneh et al., 1986).

Thus as a consequence of the protein-tyrosine kinase activity, the receptor may be specifically targeted for degradation via autophosphorylation or by the phosphorylation of a specific substrate involved in receptor trafficking.

1.6.5. The autophosphorylation domain.

The carboxyl terminus of the receptor protein-tyrosine kinases displays the highest degree of sequence heterogeneity among st even the members of the same subclass. This segment is typically very hydrophilic and rich in small amino acids, suggesting that this portion of the receptor is highly flexible. Proteolytic cleavage experiments with the EGF receptor have defined a sensitive hinge region seperating this tail region from the protein-tyrosine kinase domain (Gullick et al., 1985; Ek and Heldin, 1986). These observations have lead to the proposal that the carboxyl tail may interact with and modify the activity of the protein-tyrosine kinase. Indeed, the major sites for autophosphorylation, Tyr^{1173}, 1068 and 1148 are located in this segment (Downward et al., 1984b). All three Tyr residues are conserved in the c-erbB-2/ neu gene product suggesting that their autophosphorylation plays an important role in receptor function. Betrics and Gill (1985) demonstrated that ligand-induced receptor autophosphorylation enhanced the protein-tyrosine kinase activity. They suggested that in the basal state, the carboxyl terminus of the receptor interacted with the kinase domain behaving as an alternative substrate, thereby reducing the phosphorylation of exogenous substrates. They argued that receptor autophosphorylation would remove the carboxyl terminus from the catalytic site, release the negative constraint and therefore enhance the protein-tyrosine kinase activity. The importance of the autophosphorylation sites in the regulation of the protein-tyrosine

kinase activity was further demonstrated through point mutation (Honneger et al., 1988) and carboxyl terminal truncations (Livneh et al., 1986). Such mutant receptors retained full kinase activity and, although some of the cellular responses mediated through the mutant receptor in response to EGF were impaired, the mitogenic capacity was retained. The loss of the autophosphorylation sites did not have a measurable negative effect on the kinase activity confirming the proposal that receptor autophosphorylation functions to regulate the kinase. The loss of certain cellular responses to EGF indicated that sites of interaction with other substrates may have been eliminated through truncation.

Recently, a C' domain has been defined that is distinct from the kinase domain and seems to be required for ligand-induced receptor internalisation, via a predominant ly high affinity pathway, and for the effective coupling of activated receptors to mechanisms triggering a rise in intracellular Ca^{2+} levels (Chen et al., 1989). The extreme C' terminal 164 amino acids constitutes an inhibitory region that, when deleted from a kinase-active receptor enhances the receptor kinase activity. Moreover, such a deletion permits the internalisation of a kinase-inactive mutant receptor via a low affinity pathway, although no concomitant cellular responses are observed. In contrast, kinase-active, internalisation-incompetent receptors effectively signal gene transcription, morphological transformation and growth as a consequence of their failure to down regulate. Chen et al. (1989) suggested therefore, that this segment normally interacts with the kinase domain to mask sequences required for internalisation and that autophosphorylation may result in a conformational change serving to release the inhibition of both the kinase and the internalisation

functions. Thus, the mitogenic response of cells to EGF treatment is mediated through the activation of the intrinsic receptor protein-tyrosine kinase with a ligand-induced internalisation event serving to terminate the signal.

1.6.6.   Transmembrane signalling.

Recent biophysical investigations in our laboratory have confirmed the original suggestion that the binding of ligand to the EGF receptor induces a conformational change in the extracellular domain (Greenfield et al., 1989). However, it is still unclear how this structural perturbation is translated into the activation of the intracellular protein-tyrosine kinase. That the subclass I and II receptor protein-tyrosine kinases employ closely related or identical mechanisms for transmembrane signalling was illustrated by the use of chimeric receptors constructed from the extracellular ligand-binding domain of the insulin receptor and the transmembrane and cytoplasmic sequences of the EGF receptor (Riedel et al., 1986). Binding of insulin to the chimera was able to stimulate the protein-tyrosine kinase activity of the EGF receptor.

Several models have been proposed for the activation of the protein-tyrosine kinases. The 'flush-chain' model (Biswas et al., 1985) requires that ligand binding alters the interactions between the extracellular domain of the EGF receptor and the membrane, forcing slight changes in the positioning of the membrane-spanning domain, thereby causing an indirect alteration in the kinase domain. Biswas et al. (1985) characterised the sedimentation behaviour of detergent-solubilised receptors and determined that the protein-tyrosine kinase activity of the monomeric form was EGF-independent, whereas that of

the dimeric form was dependent on EGF. It was proposed that EGF binding induced the dissociation of the inactive receptor dimers into the active monomeric components (Biswas et al., 1985; Basu et al., 1986).

An alternative allosteric oligomerisation model has been proposed in which receptor oligomerisation promotes the activation of the kinase via subunit interaction between adjacent cytoplasmic domains (Yarden and Schlessinger, 1987). In support of the oligomerisation model, the microaggregation of occupied EGF receptors in plasma membrane vesicles at $37^{\circ}C$ has been observed (Zidovetzki et al., 1986). According to the model, monomeric receptors are in equilibrium with oligomeric receptors, with the oligomeric form exhibiting the higher affinity for ligand binding. Consequently, receptor oligomerisation is driven by preferential binding of the ligand to oligomeric receptors. Experiments using detergent-solubilised EGF receptors showed that EGF-induced receptor autophosphorylation was dependent on the concentration of the receptor and that immobilisation of the receptor on a support prevented the EGF-induced kinase activation (Yarden and Schlessinger, 1987a, b). The use of chemical cross-linking agents with both purified EGF receptor and A431 membrane preperations showed that the receptor undergoes ligand-induced dimerisation (Cochet et al., 1988). Recent investigations performed on receptor mutants has yielded further supporting evidence. The kinase-deficient mutant receptor, with the Lys^{721} residue of the ATP binding site replaced with an Ala residue, was phosphorylated in an EGF-dependent manner by an enzymatically active mutant lacking two of the autophosphorylation sites suggesting that the kinase-deficient receptor was phosphorylated by the active kinase as a consequence of dimer formation (Honegger et al., 1989).

Experiments with site-specific anti-receptor antibodies showed that mutant receptors devoid of the epitopes recognised by the antibodies were co-immunoprecipitated with the wildtype receptor or with mutant receptors recognised by the antibodies indicating the formation of receptor dimers. Although much evidence exists in support of the oligomerisation model, there are still reservations associated with it, mainly how EGF can activate the protein-tyrosine kinase in detergent solution where the concentration of EGF receptor is low, arguing against dimer formation. Furthermore, the results obtained with site specific anti-EGF receptor antibodies are some what controversial since they seem to illicit enhanced protein-tyrosine kinase activity with or without receptor clustering (Defize et al., 1986; Beguinot et al., 1986; Das et al., 1984). My results in support of the oligomerisation model are presented in Chapter 6.

How the subversion of normal cellular signalling pathways, described above, can lead to unregulated cellular proliferation and oncogenesis has been the focus of much intensive investigation. Clues to the understanding of the mechanisms of oncogenesis came with the discovery that certain retroviral oncogenes were derived from cellular proto-oncogenes that may function in normal cellular growth control (for reviews Bishop 1985, 1987; Marshall 1987; Sporn and Roberts 1985, 1988). Some of these oncogenes have the ability to confer growth factor autonomy on cancer cells with the result that they require fewer exogenous growth factors for optimal growth (Holley et al., 1983; Waterfield et al., 1983; Doolittle et al., 1984). These cells may become malignant by the autocrine action of endogenous growth factors acting on the producer cells via functional receptors, thereby allowing the phenotypic expression of the growth factor by the same cells that

produced it (Todaro et al., 1977). Indeed, it has been observed that many types of tumour cells release growth factors into their conditioned medium and possess functional receptors for these peptides.

1.7. GROWTH FACTORS AND ONCOGENESIS.

1.7.1. TGFα.

The autocrine action of growth factors was first described in rodent cells transformed with Moloney (Mo-MSV) and Kirsten (Ki-MSV) murine sarcoma viruses (Delarco and Todaro, 1978a, b; Ozanne et al., 1980). It has since been observed that many human tumours and tumour-derived cell lines produce and release TGFα and possess functional EGF receptors (Todaro et al., 1980; Marquardt and Todaro, 1982; Halper and Moses, 1983). The intimate relationship between TGFα expression and transformation was demonstrated using mutants of rodent sarcoma viruses, temperature-sensitive for the maintenance of the transformed phenotype (Kaplan et al., 1982b). At the permissive temperature, Rat-1 and NRK fibroblasts became transformed and continued to grow in serum-free medium without the addition of exogenous growth factors as a consequence of the release of TGFα into the medium. However, at the non-permissive temperature, cellular growth was dependent upon exogenous growth factors or medium conditioned by their transformed counterparts.

Further evidence supporting a role for the aberrant expression of growth factors in transformation was supplied by experiments involving the transfection of NRK fibroblasts with a retroviral construct carrying the rat TGFα coding sequence. In the presence of TGFβ, the NRK cells displayed the transformed phenotype and formed colonies in soft agar as a consequence of the secretion of large amounts

36

of TGFα into the medium (Watanabe et al., 1987). Indeed, Rat-1 fibroblasts transfected with a human TGFα cDNA expression vector displayed the transformed phenotype and induced tumour formation in nude mice, anti-TGFα monoclonal antibodies preventing these transfectants forming colonies in soft agar (Rosenthal et al., 1986). The observation that many solid tumours synthesise TGFα and also possess functional EGF receptors supports the suggestion that TGFα synthesis could act in an autocrine fashion in these tumours.

1.7.2. Platelet-derived growth factor, PDGF.

Compelling experimental evidence for the autocrine hypothesis was provided by the determination of the relationship between $p28^{v-sis}$, the oncogenic product of the simian sarcoma virus (SSV), and PDGF (Devare et al., 1983; Johnson et al., 1984). The amino terminal 109 residues of the B chain of PDGF is almost identical with the sequence of $p28^{v-sis}$ (Waterfield et al., 1983; Doolittle et al., 1984). PDGF-like peptides are produced by human osteosarcomas (Heldin et al., 1980; Graves et al., 1983) and gliomas (Nister et al., 1984) and by several SV40-transformed cells and mouse 3T3 fibroblasts transformed with Mo-MSV and Ki-MSV (Bowen-Pope et al., 1984). With the exception of those cells transformed by SSV, the PDGF-like activity seems to be of cellular origin. That these SSV-transformed cells possess functional PDGF receptors was demonstrated by the selective blocking effect of antibodies against PDGF on the incorporation of thymidine into the DNA of 3T3 and NRK fibroblasts transformed with SSV (Huang et al., 1984). However, the mitogenic blocking effect was dependent on cell type and density. Furthermore, the observed ability of SSV-transformed cells to grow in nude mice correlates with their ability to produce and release PDGF-like peptides.

1.7.3. The involvement of other growth factors in transformation.

Other growth factors have been implicated in the establishment and maintenance of the transformed phenotype, the genes for the haematopoetic growth factors interleukin-2 and 3 (IL-2, IL-3) are activated as a consequence of insertional activation in certain leukemia cells (Chen et al., 1985). Transfection of a haematopoietic precursor cell line with a retroviral construct carrying the coding sequence for granulocyte-macrophage colony stimulating factor (GM-CSF) resulted in malignant transformation (Lang et al., 1985). Furthermore, these transfectants were able to induce leukaemias in athymic mice.

The enhanced cellular response to growth factors may arise, not only as a result of the elevated expression of the genes encoding the growth factors, but also at steps further along the signal transduction pathway. The discovery of the structural relationships between receptor protein-tyrosine kinases and retroviral oncogenic products of the protein-tyrosine kinase family offered a significant insight into the understanding of growth factor receptor function and the role of specific domains in the generation and regulation of mitogenic signals. As a consequence of their ability to generate a mitogenic signal, receptor protein-tyrosine kinases harbour great oncogenic potential which, when activated, can result in the subversion of the normal regulation of signalling pathways. Two criteria must be realised in order to unlease receptor protein-tyrosine kinase-mediated transforming potential, the activation of the receptor protein-tyrosine kinase and the deregulation of the signal generating activity.

1.8. MUTATIONAL ACTIVATION OF RECEPTOR PROTEIN-TYROSINE KINASES.

Activation of receptor protein-tyrosine kinases can be triggered by a variety of mutations occurring at specific residues within the receptor sequence. Truncation of, or point mutations within the external domain may mimic ligand-stimulated kinase activation. In addition, mutations involving the carboxyl terminus may abolish the regulatory control of the kinase activity (Figure 1.7). Several specific examples are discussed in the following section.

1.8.1. EGF receptor/v-erbB.

The amino acid sequence homology between the chicken v-erbB oncogene product and the human EGF receptor provided the first indication that the v-erbB oncogene product of the avian erythroblastosis virus (AEV) had arisen as a consequence of the transduction of chicken EGF receptor sequences into the retroviral genome (Downward et al., 1984). The recombination event arising in the AEV-H strain led to the deletion of most of the extracellular domain and of 34 amino acids, including Tyr ^{1173}, from the carboxyl terminus. Other v-erbB variants have been described that display variation in their carboxyl truncations, although all involve the loss of at least one of the potential autophosphorylation sites (Nilsen et al., 1985). The involvement of the carboxyl truncations in the transforming potential of v-erbB was investigated by the reconstruction of the ligand-binding domain by *in vitro* recombination with the human EGF receptor. When expressed in Rat-1 and NIH 3T3 fibroblasts, the construct conferred partial ligand responsiveness, but did not abolish the oncogenic potential (Riedel et al., 1986). Khazaie et al. (1988) observed that the

EXTRACELLULAR DOMAIN
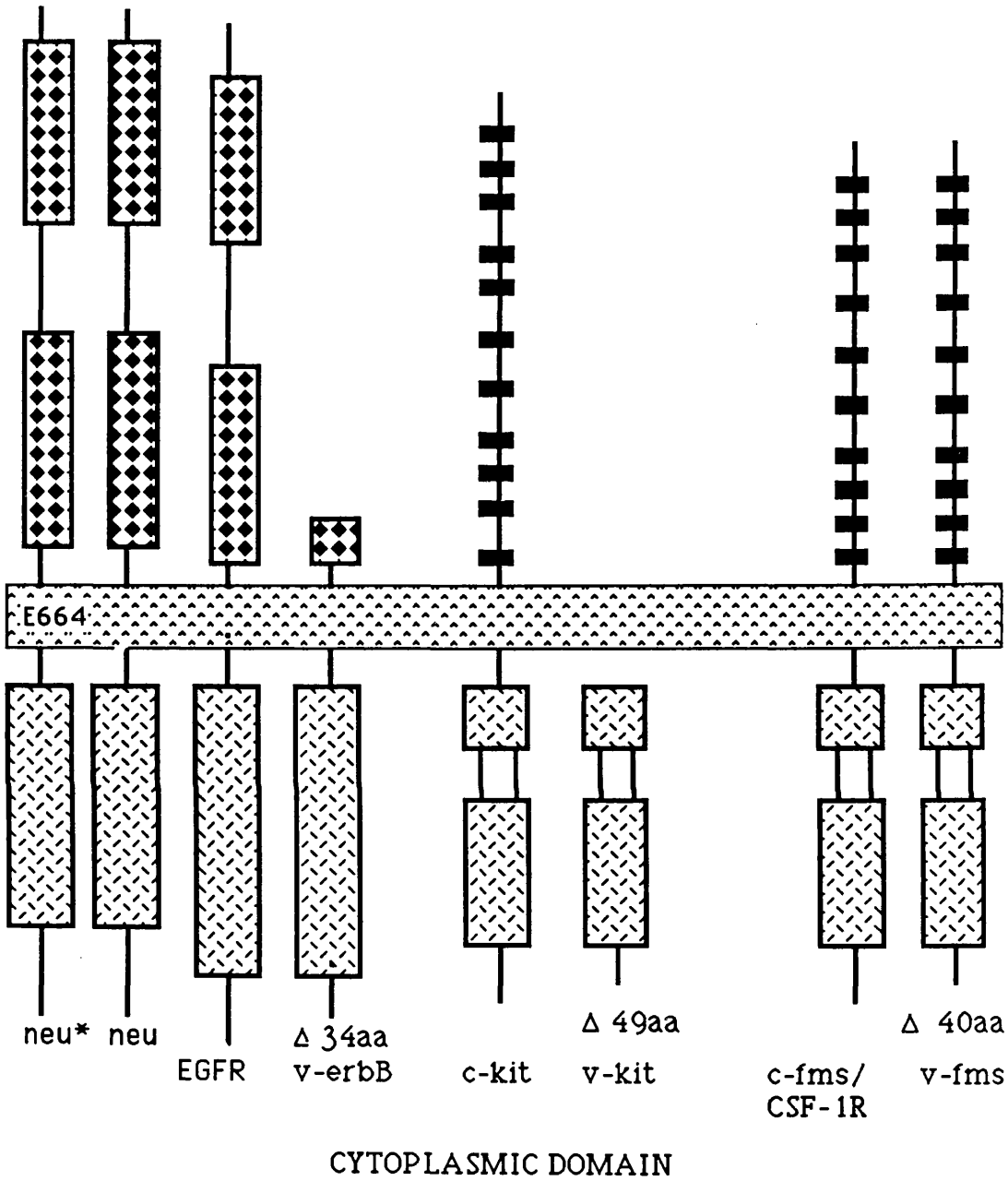
FIGURE 1.7. Comparison of the schematic structures of proto-oncogene and oncogene products of the receptor protein-tyrosine kinase family

◆ Cys-rich repeat regions

■ Cys residues

▨ Kinase domain

introduction of the full length EGF receptor into primary chicken fibroblasts and erythroblasts induced phenotypic transformation in the presence of EGF.   In chicken fibroblasts, the deletion of the entire ligand-binding domain lead to constitutive transformation, carboxyl terminal truncations serving to magnify the effect.   However, none of the mutants were as potent as v-erbB, suggesting that additional mutations in the viral protein contribute to its oncogenic potential.   In chicken erythroblasts, the truncation of the ligand-binding domain was sufficient to induce self-renewal and the removal of 32 residues from the carboxyl terminus enhanced transformation regardless of the presence or absence of the ligand-binding domain (Khazaie et al., 1988). Interestingly, a more extensive carboxy-terminal deletion abolished erythroblast transformation capacity, whereas it was enhanced in fibroblasts.   Khazaie et al. (1988) therefore proposed, that mitogenic signalling in erythroblasts may proceed via a different pathway than that operating in fibroblasts, the carboxyl terminus of the receptor being instrumental in the substrate specificity of its protein-tyrosine kinase.   Substantiating evidence was derived from the observation that different AEV strains induce distinct types of neoplasias in chickens as illustrated by the strain bearing a single amino acid deletion, arising from the insertion of AEV into the c-erbB gene, which transforms only cells of the erythroid origin (Nilsen et al., 1985).   Progressive carboxyl terminal deletions of sequences in AEV-H rendered the virus incapable of transforming erythroblasts but did not markedly effect its ability to transform fibroblasts (Yamamoto et al., 1983b).

1.8.2.   neu/ onc-neu.

The carcinogen-induced activation of neu, the rodent homologue of c-erbB-2, involves only a single point mutation rather than an extensive deletion (Bargmann et al., 1986a, b). The best characterised example of the activation of a proto-oncogene by a point mutation is that of the ras oncogene. The c-H-ras proto-oncogene was mutated so that each of the 20 amino acids were present at position 12 (Seeburg et al., 1984). Eighteen of the mutants were transforming, only the natural Gly and Pro were nontransforming. These results suggested that these mutations act by disrupting a regulatory activity of the ras protein. The mutation activating neu involves the replacement of Val^{664} in the transmembrane segment with a Glu residue, which is sufficient to confer oncogenicity (Coussens et al., 1985, Bargmann et al., 1986a, b). It is unlikely that this activation arises as a consequence of loss of negative regulatory control, but rather that an enhanced activity is ascribed to the presence of Glu at position 664. The mutational activation of neu is discussed in detail in Chapter 6.

1.8.3.   c-fms/v-fms.

The v-fms oncogene of the McDonaugh strain of feline sarcoma virus (FeSV) is the oncogenic counterpart of the CSF-I receptor/c-fms (Sherr et al., 1985; Haype et al., 1984;). The v-fms product contains the complete extracellular domain of the c-fms proto-oncogene product and retains the ability to bind CSF-I (Coussens et al., 1986). The only identifiable major alteration in the v-fms product is the deletion of 40 amino acids from the carboxyl terminus and their replacement with eleven residues encoded by the retroviral env gene, thereby removing the putative autophosphorylation site at Tyr^{969} (Coussens et al., 1986;

Roussel et al., 1987). Most of the point mutations arising in the v-fms sequence are likely to be due to species-specific divergence, although their potential involvement in v-fms oncogenic capacity cannot be excluded. Indeed, mutations in the external domain confering ligand-independency may be partly responsible for v-fms oncogenic potential (Wheeler et al., 1986). The involvement of carboxyl-terminal truncations in the establishment of the transforming ability of v-fms was illustrated by the findings that the replacement of this portion with the corresponding segment from c-fms reduced transforming potential (Browning et al., 1986). Furthermore, when Tyr^{969} of the chimera was mutated, full transforming capacity was restored (Roussel et al., 1987). However, it was shown that mutation of Tyr^{969} in c-fms was not sufficient to induce transformation, since the mutated c-fms product was only able to confer transformation when activated by ligand. Thus, the mutation of Tyr^{969} activates the oncogenic potential of c-fms only in conjunction with exogenous ligand or mutations confering ligand independency.

1.8.4. c-kit/v-kit.

The transduction of the c-kit gene by the Hardy-Zuckerman 4 strain of FeSV causes extensive structural deletions at both termini, including the entire external domain, the transmembrane domain and part of the juxtamembrane segment. At the carboxyl terminus, 49 residues, including a potential autophosphorylation site at Tyr^{936}, are replaced by five unrelated residues (Yarden and Schlessinger, 1987).

It has been demonstrated that the W mutant strain of mice display severe macrocytic anaemia, lack of hair pigmentation and sterility as a consequence of a mutation arising within the W locus (Russell, 1979).

These observations suggested that the W locus was intimately involved in the developmentally important processes of gametogenesis, melanogenesis and haematopoesis. The discovery that the c-kit proto-oncogene shared chromosomal localisation with the W locus suggested that the two genes were in fact one and the same (Chabot et al., 1988). The putative involvement of c-kit in haematopoeisis can be further inferred from its high degree of homology with the receptor for CSF-1 (Sherr et al., 1985), a known haematopoetic growth factor and the observation that c-kit is expressed in haematopoetic tissues including bone marrow and spleen (Qiu et al., 1988). Thus the mutational activation of the c-kit proto-oncogene in the germ line may effect the migration and/or proliferation of cells during early embryogenesis resulting in an intrinsic defect in the haematopoetic stem cell lineage.

1.8.5. The mutational activation of other putative protein-tyrosine kinases.

The cDNA sequence of the MET proto-oncogene predicted a gene product of 157 kD displaying structural features characteristic of the growth factor receptor protein-tyrosine kinase family (Park et al., 1987). Treatment of a human osteogenic sarcoma cell line with the chemical carcinogen N-methyl-N'-nitro-N-nitrosoguanidine resulted in the activation of the MET proto-oncogene (Cooper et al., 1984) as a consequence of genetic rearrangement (Park et al., 1986). This rearrangement fused sequences from the MET locus on chromosome 7 to sequences from the translocated promoter region (TPR) on chromosome 1 resulting in the generation of a 65 kD fusion protein lacking the putative external domain of the proto-oncogene.

The TRK oncogene was identified as a result of a DNA transfection experiment employing the DNA from a human colon carcinoma (Martin-Zanca et al., 1986). The TRK proto-oncogene, a putative member of the receptor protein-tyrosine kinase family, is activated by a gene rearrangement between the TRK proto-oncogene and the gene for the non-muscle form of tropomyosin resulting in the truncation of the putative extracellular domain of TRK.

The UR2 sarcoma virus, an acutely transforming chicken retrovirus (Balduzzi et al., 1981) encodes a fusion protein p68 $^{gag-ros}$ (Feldman et al., 1982) derived from the c-ros proto-oncogene (Shibuya et al., 1982). Both c- and v-ros encode a protein-tyrosine kinase activity with extensive homolgy to the human insulin receptor within their kinase domains (Ullrich et al., 1985) and it was therefore suggested that the ros gene encodes a putative growth factor receptor (Neckameyer et al., 1986). The cloning of the human homologue of v-ros, indicated that the oncogenic activation of c-ros results in the truncation of the extracellular domain of the gene product (Matsushime et al., 1986).

It seems likely therefore, that one of the mechanisms responsible for the activation of the proto-oncogenes MET, TRK and c-Ros operates through the truncation of the extracellular domain of the receptor protein-tyrosine kinase resulting in deregulation of the protein-tyrosine kinase activity in an analogous fashion to v-erbB.

1.9. AMPLIFICATION OF GENES ENCODING RECEPTOR PROTEIN-TYROSINE KINASES.

1.9.1. Amplification of the EGF receptor gene.

Another mechanism leading to an elevation in the level of protein-tyrosine kinase activity involves increasing the number of the specific cell surface receptors and/or the affinity of receptor binding to the growth factor. Elevated levels of the EGF receptor have been observed in human gliomas, meningiomas (Libermann et al., 1985), squamous carcinomas of the lung (Hendler and Ozanne, 1984) and ovary, cervical, and renal carcinomas, certain cases associated with the amplification of the EGF receptor gene (Xu et al., 1984). The intensively studied cell line A431 (derived from a squamous carcinoma), possesses approximately 2.5×10^6 EGF receptors per cell displaying high affinity binding for EGF (Haigler et al., 1978). Monoclonal antibodies to these high affinity receptors inhibit proliferation of A431 cells *in vitro* and *in vivo* when transplanted into athymic mice. The importance of overexpression of the EGF receptor was further demonstrated by transformation and colony formation in soft agar of NIH 3T3 fibroblasts, transfected with a retroviral construct carrying the EGF receptor coding sequence, when cultured in the presence of EGF (Velu et al., 1987). Furthermore, when transplanted into nude mice, these cells formed tumours in an EGF-dependent manner. Indeed transfection of the EGF receptor into NR6 fibroblasts which have lost EGF receptor expression responded to EGF stimulation in a comparable manner (DiFiore et al., 1987a).

1.9.2. Amplification of the c-erbB-2 gene.

Amplification and overexpression of the EGF receptor related gene c-erbB-2 has been observed in primary breast tumours (Slamon et al., 1987), cell lines derived from breast tumours (Van der Nover et al., 1987), adenocarcinomas (Yukota et al., 1986) and a single gastric cancer cell line (Fukushige et al., 1986). Indeed, overexpression of the c-erbB-2 coding sequence in NIH 3T3 cells induced the malignant phenotype suggesting a role for the overexpression of the c-erbB-2 gene product in transformation (DiFiore et al., 1987b). Furthermore, markedly elevated levels of c-erbB-3 mRNA have been reported in certain human mammary carcinoma cell lines (Kraus et al., 1989). The neu/ c-erbB-2/ c-erbB-3 sequences will be discussed in detail in Chapter 6.

The above examples illustrate that both qualitative and quantitative mechanisms, resulting in either the overexpression of a functionally normal receptor or the generation of a constitutively active receptor, may be involved in the contribution of certain growth factor receptors to abnormal cellular growth control and transformation.

1.10. Aims of this study.

From this introduction, it can be seen that the subversion of a normal growth factor signalling pathway, albeit by the enhanced expression of the growth factor or its receptor or by the unregulated activity of the receptor protein-tyrosine kinase can lead to uncontrollable cellular proliferation and oncogenesis. Recent biophysical investigations in this laboratory have confirmed the original suggestion that the binding of ligand to the EGF receptor causes

a conformational change in the extracellular domain (Greenfield et al., 1989). However, the question as to how this structural perturbation is translocated across the membrane, thereby activating the protein-tyrosine kinase remains unresolved. Two models have been proposed to explain the mechanism of signal transduction, both models require that a conformational change in the extracellular domain of the receptor is generated upon ligand binding. The 'flush chain' model proposes that the binding of EGF promotes the dissociation of inactive dimers into the active monomeric form (Biswas et al., 1985). Conversely, the oligomerisation model argues that ligand binding directs the association of the inactive monomers into the active dimeric unit (Yarden and Schlessinger, 1987). The determination of the interactions existing in the ligand-receptor complex would be fundamental to the elucidation of the signal transduction mechanism.

The aim of this thesis is to produce various tools for detailed biochemical and biophysical investigations of different aspects of the signal transduction pathway. The generation of large quantities of native and mutant EGF and TGFα will allow the determination of their complete three-dimensional conformation through continued ^{1}H NMR analysis and diffractional analysis of crystals. The determination and comparison of the receptor binding sites of EGF and TGFα will generate information as to the ligand binding site of the EGF receptor, co-crystallisation between the EGF receptor and EGF and TGFα further characterising the ligand binding site. The large scale production of the ligands could be used to continue the circular dichroism (CD) and spectroscopic analyses performed in this laboratory on the conformational changes occurring in the receptor upon ligand binding. The elucidation of the tertiary structures of EGF and TGFα will

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enable the design of antagonists that would bind to the EGF receptor without triggering cellular proliferation. These antagonists would be pharmaceutically important for the treatment of certain malignancies known to overexpress the EGF receptor.

The large scale expression of the c-erbB-2/ neu gene product would allow investigation into the mechanism of signal transduction in another putative subclass I protein-tyrosine kinase. Furthermore, affinity chromatography of the conditioned medium from transformed cells would help identify the as yet unknown ligand for this putative receptor protein-tyrosine kinase.

It has been proposed that the membrane-associated precursor form of TGFα, preproTGFα may play a role in cell-cell signalling by virtue of its homology with cell surface proteins displaying EGF-like repeats. The ability of a membrane-associated TGFα to bind to and activate the EGF receptors on adjacent cells could play an important role in many *in vivo* systems known to express elevated levels of preproTGFα such as in solid tumours and in proliferating skin keratinocytes.

In order to produce large quantities of these components of the growth factor signalling pathway, the baculovirus expression system was employed since it has been shown to perform many of the post-translational modifications occurring in higher eukaryotes, thereby producing biologically active proteins (Lucklow and Summers, 1988). This system is briefly reviewed below.

1.11. THE BACULOVIRUS EXPRESSION SYSTEM.

The helper independent baculovirus expression system has been developed for the expression of a wide variety of heterologous genes

(for reviews see Lucklow and Summers, 1988; Miller, 1988; Miller et al., 1986). Autographa californica nuclear polyhedrosis virus (AcNPV) is the prototype virus of the family Baculoviridae which has an extensive host range infecting more than 30 species of Lepidopteran insects. AcNPV replicates abundantly in several well-established insect cell lines, including the Spodoptera frugiperda cell line, Sf9 (Figure 1.8). The major advantage of this system is that it allows the abundant expression of recombinant proteins which are antigenically, immunogenically and functionally similar to their authentic counterparts. In addition, AcNPV is non-pathogenic to vertebrates and plants and does not require transformed cells or transforming elements as do other mammalian expression systems. Indeed, insect cells employ many of the protein modification, processing and transport systems which occur in higher eukaryotes which may play an essential role in producing a fully biologically active recombinant protein.

The baculovirus expression system utilises the highly expressed and regulated AcNPV polyhedrin promoter. The AcNPV genome is composed of double-stranded, circular supercoiled DNA of approximately 128 kb in length. The size of the genome and the extendable nature of the rod-shaped viral capsid ensure that the vectors are able to accommodate large inserts, the largest to date comprising approximately 10 kb (Carbonell et al., 1985). During infection, two forms of viral progeny are produced, extracellular virus (ECV) is released after 12 hours and occluded virus (OV) after 4-5 days (Figure 1.8). The latter type of particle is embedded in proteinaceous viral occlusions called polyhedra. The polyhedron is composed of a 29 kD subunit, polyhedrin which accumulates up to approximately 25% of the total infected-cell protein, with as many as 100 polyhedra per cell. This type of temporal

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Figure 1.8. Baculovirus Lytic Cycle. The schematic represents the unique biphasic life cycle of a typical baculovirus. In the environment, a susceptible insect ingests the viral occlusions from a food source. The crystal dissociates in the gut of the susceptible host to release the infectious viral particles which invade the gut cells, penetrate to the nucleus and uncoat. Viral DNA replication is detected by 6 hours. By 10-12 hours post infection extracellular virus buds from the surface to infect other cells and tissues. Late in infection, (18-24 hours post infection) the polyhedrin protein assembles in the nucleus of the infected cell and virus particles become embedded in the proteinaceous occlusions. The viral occlusions accumulate to large numbers and the cells lyse. The viral occlusions are responsible for the horizontal transmission among susceptible insects, the extracellular virus is responsible for secondary and cell to cell infection in cultured cells or the insect host. The polyhedrin gene is not essential for virus infection or replication.

THE LIFE CYCLE OF BACULOVIRUS INFECTION
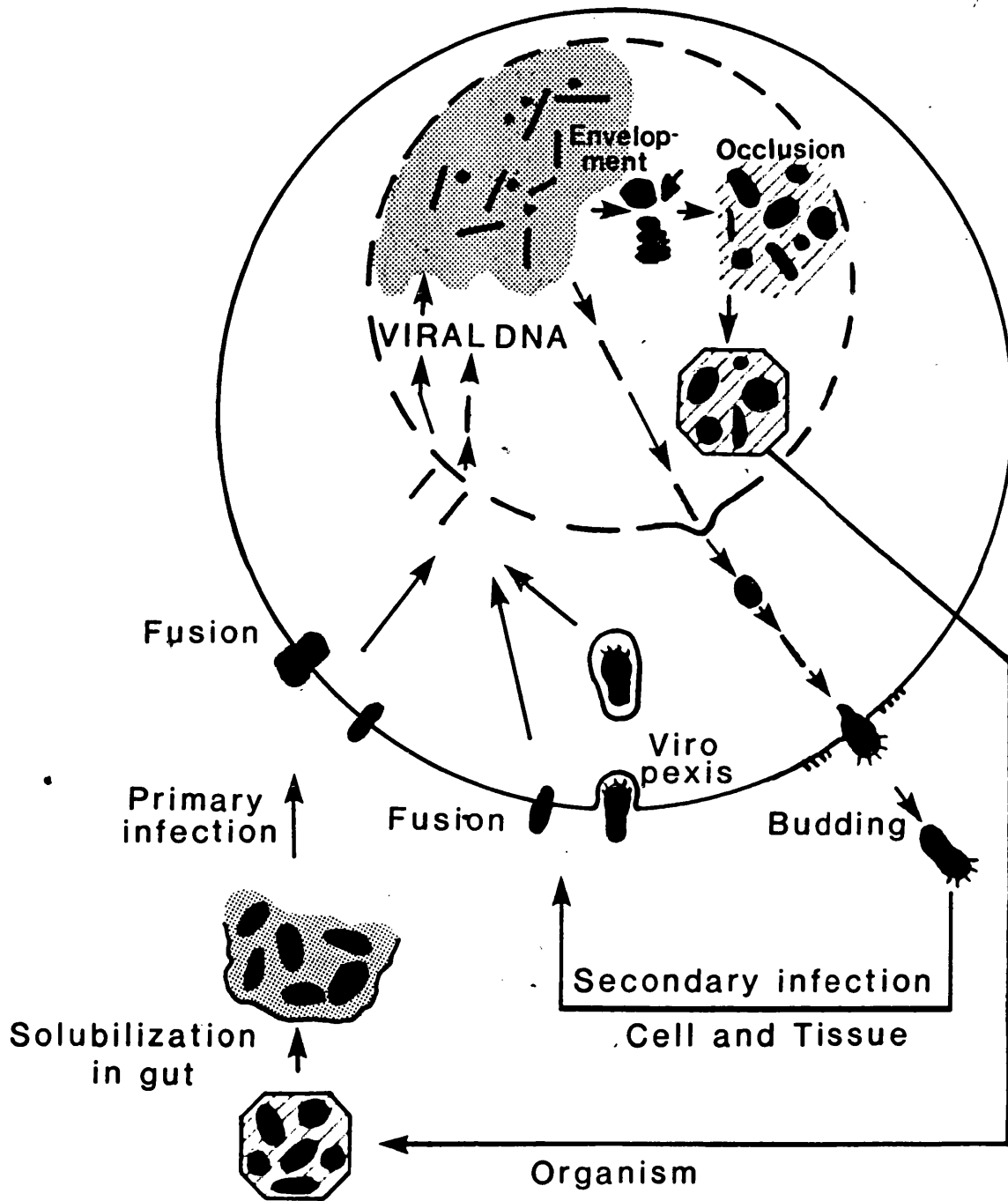
Modified from C.P. Van der Beek 1980

regulation ensures that the expression of foreign gene products, which may have adverse effects on the cell, will not diminish the production of ECV for subsequent infection. Virus occlusions play an important role in the natural virus life cycle, providing the means for the horizontal transmission of the virus in the wild. When infected larvae die, millions of polyhedra are left in the decomposing tissue, the occlusions protecting the embedded virus from inactivation by environmental conditions. When larvae feed on the contaminated plant, the polyhedra are ingested and dissolved in the alkaline environment of the gut, thereby releasing the virus which invade and replicate in the cells of the midgut. The OV form enters the cell by absorptive endocytosis or fusion, and once inside the cell, the nucleocapsids are released from the envelope at nuclear pores and accumulate in the nucleus. DNA replication is initiated at 6 hours p.i and by 10 hours post-infection (p.i), ECV are released from the cell by budding, resulting in secondary infection throughout other tissues. Polyhedrin can be detected by 12 hours p.i although occlusions are not visible until 18-24 hours p.i at which time, they continue to accumulate for 4 or 5 days until the cells lyse.

The polyhedrin gene has been shown to be nonessential for replication and production of ECV in cultured cells. Deletion or insertional inactivation of the polyhedrin gene results in the production of occlusion negative particles forming plaques which are distinctly different from those of the wild type occlusion positive plaques. These distinctive plaques morphologies provide a means for the identification of recombinant virus particles. The foreign gene is inserted into the AcNPV genome by the process of cell-mediated homologous recombination. The vector, containing the foreign gene

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under the control of the polyhedrin promoter flanked by viral sequences, is cotransfected with wild type AcNPV DNA resulting in recombination between the viral sequences flanking the passenger gene and the homologous sequences in the wild type DNA. As a consequence, the wild type polyhedrin sequence between the cross over points is replaced by the corresponding plasmid sequence and the foreign gene becomes stably integrated into the AcNPV genome. The frequency of recombination is dependent on several factors, including the total length of the wild type DNA to be replaced, the size of the insertion and the length of the homologous flanking regions although generally, a recombination frequency of between 0.1 and 1.0% can be achieved.

The expressed gene or gene product may influence expression levels whether it be as a consequence of an intrinsic property of the protein or the pathway through which the protein is processed. The factors which determine how well a foreign gene will be expressed are not yet well characterised and therefore, it is difficult to predict how efficiently different genes will be expressed. However, utilising the baculovirus system, a wide range of proteins, both intra- and extracellular, have been expressed efficiently. Insect cells have the ability to perform a wide range of post-translational modifications which confer correct folding, biological activity and antigenicity including, phosphorylation, glycosylation, myristylation, palmitylation, signal sequence recognition, proteolytic processing, disulphide formation and oligomerisation. Human IL-2 (Smith et al., 1985) and α - and β-IFN (Maeda et al., 1985; Smith et al., 1983) are all secreted as a result of the precise removal of the signal sequence. Heterologous proteins are targeted to the correct cellular compartment, c-myc to the

nucleus (Miyamoto et al., 1985) and the human EGF receptor (Greenfield et al., 1987), human CD4 (Webb et al., 1989) and the influenza virus haemagglutinin complex (Kuroda et al., 1986) to the cell surface. Insect cells are able to glycosylate proteins although they contain negligible amounts of galactosyl and sialyl transferases (Butter et al., 1981). It is assumed that the N-linked core glycosylation step is similar among vertebrates and invertebrates but that terminal glycosylation differs, hence the observed reduction in molecular weight of the influenza virus haemagluttinin complex (Kuroda et al., 1986), human tissue plasminogen activator (tPA) (Furlong et al., 1988) and β-IFN (Smith et al., 1983) when expressed in the baculovirus system. It is not clear what effect, if any, this difference in terminal glycosylation may have on the biological activity of the individual foreign gene products. Indeed, the activity of the above mentioned proteins are indistinguishable from their authentic counterparts. Human c-myc produced by the baculovirus expression system is phosphorylated (Miyamoto et al., 1985) and human β-IFN (Smith et al., 1983), α-IFN (Maeda et al., 1985), IL2 (Smith et al., 1985), tPA (Furlong et al., 1988), PDGF-BB (Giese et al., 1989) and influenza virus haemaglutinin (Kuroda et al., 1986) are all correctly proteolytically processed, liberating fully biologically active molecules. The baculovirus system is also capable of generating disulphide-linked dimers, as illustrated by the production of active glutathione S-transferase (Cheng-Hsieh et al., 1989), PDGF-BB (Giese et al., 1989) and tPA (Furlong et al., 1988) and multimeric complexes such as the oligomeric complexes of the influenza virus polymerases (St. Angelo et al., 1986). Several proteins with authentic enzyme activity have been produced, human terminal transferase (Chang et al., 1988), tyrosine hydroxylase (Ginns et al., 1988) and tPA (Furlong et al., 1988). In addition, the EGF receptor has been expressed as a membrane-associated

glycoprotein (although with a lower molecular weight as a result of incomplete terminal glycosylation), with ligand-stimulatable autophosphorylation ocurring at the same tyrosine residues as those of the receptor isolated from A431 membranes (Greenfield et al., 1987). Indeed, the cytoplasmic protein-tyrosine kinase domain of the EGF receptor (Wedegaertner and Gill, 1989) and that of the insulin receptor (Herrera et al., 1988) have been expressed both of which are capable of autophosphorylation.

CHAPTER TWO.

2.1. MATERIALS.

2.1.1. General chemicals.

Foetal calf serum (FCS), fungizone and gentamycin were obtained from Gibco BRL (Paisley, Scotland, UK). Protein A and G Sepharose CL-4B, bovine serum albumin (BSA) salmon sperm DNA, ATP, CHAPS and Tween 20 were obtained from Sigma Chemical Co (Poole, Dorset, UK). Low melting point seaplaque agarose was obtained from ICN Biochemicals (High Wycombe, Bucks, UK). Tunicamycin was obtained from Boehringer Mannheim (Lewes, East Sussex, UK). Protein and DNA molecular weight size markers were obtained from Gibco BRL. The Sequenase DNA Sequencing Kit was obtained from United States Biochem. Corp. (Cambridge Biosciences, Cambridge, UK) and the *in vitro* Mutagenesis Kit from Amersham International (Aylesbury, Bucks, UK). All other chemicals were obtained from BDH Chemicals Ltd or Sigma and were of analytical grade (AnalaR).

2.1.2. Growth factors.

Recombinant human EGF, was kindly provided by Dr. Carlos-George Nascimento (Chiron Corp., San Francisco, USA).

2.1.3. Radiochemicals.

$[\gamma\text{-}^{32}P]$ adenosine 5' triphosphate [ATP] and $[\alpha\text{-}^{32}P]$ ATP (10 mCi/ml), $[^{35}S]$cysteine (1300 Ci/ml), $[^{3}H]$Thymidine (5 Ci/mmol), $[^{125}I]$Protein A (150 μCi/μg), $[^{125}I]$Streptavidin (33 μCi/μg) and $[^{125}I]$murine EGF (100 μCi/μg) were obtained from Amersham International.

2.1.4. Enzymes.

All restriction endonucleases, DNA ligase, T4 kinase, klenow fragment, calf intestinal phosphatase (CIP), RNase 1 'A', proteinase K and endoglycosidase F were obtained from Boehringer Mannheim. Elastase and lysozyme were obtained from Sigma.

2.1.5. Antibodies.

The anti-21N antibody was raised against the predicted C-terminus of p185 (residues 1243-1255 in $p185^{c-erbB-2}$) and recognises both $p185^{c-erbB-2}$ and $p185^{c-erbB-2}$ (Gullick et al., 1987). The rat monoclonal antibodies, ICR9 (anti-EGF receptor) and, ICR13 (anti-$p185^{c-erbB-2}$) were kindly provided by Prof. B. Gusterson. The mouse anti-IgG human EGF monoclonal antibody (Ab-2) and mouse anti-IgG human TGFα monoclonal antibody (Ab-1) were obtained from Oncogene Science Inc. (Cambridge Bioscience, Cambridge, UK). The anti-phosphotyrosine polyclonal was raised against the phosphopeptide, phospho Tyr-Ala-Gly (1:1:1) according to Kamps and Sefton (1988) and was kindly provided by Dr. M. Fry.

2.1.6. Insect cell media.

Cysteine-free and 2 x Grace's Insect media were obtained from Gibco BRL. IPL41 was obtained from JR Scientific (San Francsico, USA) and kindly prepared by Annette Waugh (Table 2.1).

Basic nutrients

sugars

amino acids

organic acids

vitamins

salts

trace elements

Yeast extract-ultrafiltered (high MW contaminants)

di- and tri-peptides

trace elements

metal chelating ability

Lipids

Tween 80

Pluronic polyol F-68

cod liver oil

cholesterol (sterol)

Vitamin E (antioxidant and free-radical scavenger)

Table 2.1. Components of IPL 41 insect cell culture medium (JR. Scientific, USA).

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METHODS.

2.2. Precipitation of nucleic acids with ethanol.

Nucleic acids were precipitated by the addition of 0.1 volume of 3M NaOAc, pH 4.8 and 2.5 volume of ethanol for DNA and 3 volume of ethanol for RNA. The contents were mixed thoroughly and the nucleic acids precipitated by incubation on a dry-ice, ethanol bath for 10 min or at -20°C overnight. The nucleic acids were collected by centrifugation for 10 min, washed with 70% ethanol, dried under vacuum and resuspended in TE to the required concentration.

2.3. Phenol: chloroform extraction of DNA.

Phenol and chloroform denature protein and can therefore be used singly or in combination to extract protein from DNA solutions. TE-saturated phenol was made according to Man iatis (1988) and a 1:1 (v/v) mix with chloroform: isoamylalchohol 24:1 (v/v) prepared. The volume of the DNA solution was adjusted to 400 μl and an equal volume of phenol &/or chloroform added. The phases were mixed well by vortexing and separated by centrifugation for 5 min. The upper aqueous phase was transferred to a fresh tube leaving behind the material at the interface. In the case of the phenol extraction, the aqueous phase was then extracted with chloroform. The DNA was precipitated with ethanol, washed with 70% ethanol, dried under vacuum and resuspended in TE to the required concentration.

2.4. THE PREPARATION OF PLASMID DNA.

2.4.1. Maxipreparation of plasmid DNA.

A single colony was used to inoculate 5 ml of L-broth, supplemented with the appropriate antibiotic(s) and incubated with shaking at 37°C overnight. This culture was added to 250 ml of L-broth plus antibiotic(s) and incubated at 37°C for 36 h with moderate shaking. The bacteria were harvested by centrifugation for 20 min at 4500g, resuspended in 7 ml of solution 1 (25 mM Tris-HCl pH 8.0, 50 mM glucose, 10 mM EDTA, 5 mg/ml lysozyme) and incubated at room temperature for 10 min. 14 ml of solution 2 (0.2 M NaOH, 1% (w/v) SDS) were added and the tube placed on ice for 10 min with periodic inversion to ensure proper mixing. 10.5 ml of ice cold solution 3 (5 M NaOAc, pH 4.8) was added, the tube inverted several times and incubated on ice for a further 10 min. The cell debris was pelleted by centifugation for 20 min at 4500g and the supernatant filtered through gauze. Nucleic acids were precipitated by the addition of 0.6 volume of isopropanol and centrifugation for 10 min at 5800g. The pellet was resuspended in 3 ml of TE/CsCl solution containing CsCl at 1.05 g/ml, 500 μl of 10 mg/ml ethidium bromide was added per ml and the solution centrifuged for 10 min at 9000g. The supernatant was loaded into a 1/2 x 2 in Beckman polyallomer quick-seal ultracentrifuge tube, the tubes topped up with the TE/CsCl solution, weighed (ideal weight 9.3-9.9 g to ensure the correct density gradient is achieved) and centrifuged overnight at 200,000g. Closed circular plasmid DNA was isolated by drawing out, with a 19-gauge needle, the lower of the two fluorescent bands visible on UV illumination (the upper band contains chromosomal DNA). The plasmid DNA was rebanded to remove any contaminating chromosomal DNA by

centrifugation for 4 h at 275,000g. The ethidium bromide was extracted with an equal volume of water-saturated isopropanol, the upper (pink) organic phase discarded and the extraction repeated until the aqueous phase was colourless. The DNA was precipitated at -20°C on the addition of 3 volumes of water and 3 volumes of ethanol. The DNA was collected on centrifugation for 20 min at 1100g, washed with 70% ethanol, dried under vacuum and resuspended in TE at a concentration of 1 mg/ml and stored at -20°C

2.4.2. Miniprep ration of plasmid DNA.

A single colony was used to innoculate 2 ml of L-broth supplemented with the appropriate antibiotic(s), and the culture incubated with shaking at 37°C overnight. 1.5 ml was decanted into an eppendorf tube and the bacteria pelleted upon centrifugation for 2 min. The supernatant was aspirated, the pellet resuspended in 100 µl solution 1 (25 mM Tris-HCl pH 8.0, 50 mM glucose, 10 mM EDTA, 5 mg/ml lysozyme) and incubated at room temperature for 5 min. 200 µl solution 2 (0.2 M NaOH, 1% (w/v) SDS) was added and the tubes placed on ice for 5min at which time, 150 µl 5M NaOAc pH 4.8 was added and the incubation time extended for a further 5 min. The cell debris was pelleted by centrifuging for 5 min, the supernantant transfered to a fresh tube and extracted with a 1:1 (v/v) phenol: chloroform solution. The plasmid DNA was ethanol precipitated, washed with 70% ethanol, dried under vacuum and resuspended in 25 µl TE.

2.5. PREPARATION OF M13 DNA.

2.5.1. Maxipreparation of double-stranded M13.

2 ml of L-broth was inoculated with 200 µl of an overnight culture of the host bacteria and a single plaque transferred with a sterile loop. The culture was incubated with shaking at 37°C overnight and 1.5 ml decanted into an eppendorf tube. The bacteria was pelleted upon centrifugation for 5 min and the supernatant used to innoculate 100 ml of L-broth containing a 1 ml aliquot of an overnight culture of the host bacteria. The culture was incubated, with shaking at 37°C for 4-5 h. The cells were harvested by centrifugation at 7600g for 20 min and the double-stranded (RF) M13 DNA isolated according to the plasmid DNA maxipreperation method.

2.5.2. Minipreparation of double-stranded M13 DNA.

Double-stranded (RF) M13 DNA was prepared from the bacterial pellet obtained during the isolation of single-stranded M13 DNA according to the method for minipreperation of plasmid DNA.

2.5.3. Minipreparation of single-stranded M13 DNA.

2 ml of L-broth was inoculated with 200 µl of an overnight culture of the host bacteria and a single plaque transferred with a sterile loop. The culture was incubated with shaking at 37°C for 6 h and 1.5 ml decanted into an eppendorf tube. The bacteria was pelleted upon centrifugation for 5 min and the supernatant transferred to a fresh tube. 200 µl of PEG/NaCl solution (20% (w/v) PEG 6000, 2.5 M NaCl) was added and the tubes incubated at room temperature for 30 min. The phage DNA was precipitated upon centrifugation for 5 min, the

supernatant aspirated, ensuring all of the PEG solution was removed and the pellet resuspended in 100 μl TE. 500 μl of phenol was added, the tubes vortexed for 15 s, rotated on a wheel at room temperature for 15 min and then vortexed for a further 15 s. The phases were seperated by centrifugation for 3 min and the aqueous phase extracted with chloroform. Both organic phases were re-extracted with 100 μl of TE, the aqueous phases combined and the DNA ethanol precipitated, washed with 70% ethanol, dried under vacuum and resuspended in 25 μl of TE.

2.6. PREPERATION OF COMPETENT *E.COLI*.

2.6.1. Rubidium chloride method.

This method is based on that of Hanahan (1985). Several colonies were picked off a freshly streaked SOB (2% (w/v) bactotryptone, 0.5% (w/v) bactoyeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) plate and dispersed in 1 ml SOB medium. 1 colony was used per 10 ml of culture medium and inoculated into a flask containing SOB medium with a medium volume to flask volume of between 1:30 and 1:100. The culture was incubated at 37°C with moderate shaking until the cell density reached 4-7 x 10^7 cells/ml and then chilled on ice for 15min. The cells were pelleted by centifugation for 10 min at 1100g and the pellet drained thoroughly. The pellet was resuspended by moderate vortexing in 0.3 x volume of RF1 solution (100 mM RbCl, 50 mM manganese chloride, 10 mM calcium chloride, 30 mM potassium acetate, 15% (w/v) glycerol, pH 5.8) and incubated on ice for 60 min. The cells were pelleted as before, resuspended in 0.08 x volume of RF2 (10 mM MOPS pH 6.8, 10 mM RbCl, 75 mM calcium chloride, 15% (w/v) glycerol) and incubated on ice for 15 min. The cells were then used straight away

or aliquoted and flash frozen in liquid nitrogen and placed at -70°C for storage.

2.6.2. Calcium chloride method.

5 ml of L-broth supplemented with the appropriate antibiotic(s) was innoculated with a single colony and incubated with moderate shaking overnight at 37°C. 40 ml of L-broth plus antibiotic(s) was innoculated with 200 μl of the overnight culture and incubated with shaking at 37°C until an OD_{580} of 0.3 was achieved. The cells were pelleted by centrifugation for 10 min at 1100g, resuspended in 0.5 x volume of ice-cold 100 mM calcium chloride and incubated on ice for 30min. The cells were then pelleted, as before, resuspended in 0.02 x volume of ice-cold 100 mM calcium chloride and incubated on ice for a further 15 min. The cells were then competent for transformation as described.

2.7. THE TRANSFORMATION OF *E.COLI*.

2.7.1. Transformation with plasmid DNA.

The DNA was added to competent cells, mixed by gentle pipetting and incubated on ice for 30 min to allow the DNA to absorb on to the cells. The cells were then induced to take up the DNA by heat-shocking at 42°C for 90 s, at which time, 0.8 ml of L-broth was added and the cells incubated at 37°C, with shaking, for 30 min. The cells were then gently spread onto a pre-dried agar plate, containing the relevant antibiotic selection and incubated at 37°C overnight.

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2.7.2. Transformation with phage DNA.

The DNA was added to competent cells, mixed with the cells by gentle pipetting and the tube incubated on ice for 30 min. The cells were then heat-shocked at 42°C for 90 s and returned to ice for 5 min. 200 μl of a log phase culture of *E. coli* was added followed by 4 ml of molten top L-agarose, preincubated at 45°C and the culture poured immediately onto a prewarmed L-agar plate. The plate was left to set and then incubated at 37°C overnight.

2.8. Digestion of DNA with restriction endonucleases.

DNA was digested according to the manufacturer's specifications. Generally, restriction enzymes can be divided into three categories based on their buffer requirements:

BUFFER	NaCl	Tris-HCl pH 7.5	$MgCl_2$	DTT
Low	0	10 mM	10 mM	1 mM
Medium	10 mM	10 mM	10 mM	1 mM
High	100 mM	50 mM	10 mM	1 mM

DNA was digested in a volume of 20 μl as follows:

DNA (approx. 1 μg)

10 x Restriction Buffer   2 μl

water   to 20 μl

Restriction enzyme   1-5 units

DNA was allowed to digest for at least 1 h at the specified temperature. For digestion with more than one enzyme with different buffer requirements, the first digest was extracted with chloroform, ethanol precipitated and the DNA then digested with the second enzyme.

2.9. SUBCLONING RESTRICTION FRAGMENTS.

2.9.1. Ligation of DNA restriction fragments.

Ligation of DNA fragments was generally performed in 20 μl. The DNA was ligated in a 1:1 ratio when cloning a restriction fragment into a vector, mixed with 2 μl of 10 x Ligation buffer (660 mM Tris-HCl pH 7.6, 66 mM $MgCl_2$, 100 mM DTT, 6.6 mM ATP) and the volume adjusted to 20 μl with water. 0.1 units of T_4 DNA ligase was then added for sticky-end ligations and 1 unit for blunt-end ligations and the reaction incubated at room temperature for a minimum of 3 h. The reaction was stopped by chloroform extraction and the DNA ethanol precipitated prior to further processing.

2.9.2. 'Shotgun' cloning restriction fragments.

1 μg of plasmid or DS M13 DNA was digested with the required restriction endonuclease(s) and the enzyme heat-inactivated at 75°C for 10 min. One complete minipreparation of the plasmid or DS M13 DNA, containing the insert, was digested and the enzyme heat-inactivated as before. 0.1 x volume of the digests were combined and ligated over night in a 20 μl ligation volume. 0.25 x and 0.1 x volumes of the ligation mix were then used to transform aliquots of competent cells.

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2.10. Phosphatasing of restriction fragments.

The vector was digested with the relevant restriction enzyme, extracted with chloroform, ethanol precipitated and resuspended in 45µl of water. 5 µl of 10 x calf intestinal alkaline phosphatase (CIP) buffer (0.5 M Tris-HCl pH 9.0, 10 mM MgCl$_2$, 1 mM ZnCl$_2$, 10 mM spermidine) was added, followed by 0.01 units of CIP. The reaction mix was incubated at 37°C for 30 min and then shifted to 55°C for a further 30 min at which time, another 0.01 units of CIP were added and the incubations repeated. The enzyme was inactivated by the addition of SDS to 0.5% (w/v) and EGTA to 20 mM and incubation for 15 min at 65°C. The DNA was extracted with chloroform, ethanol precipitated, dried under vacuum and resuspended in TE to the required concentration.

2.11. THE ELECTROPHORESIS OF DNA FRAGMENTS.

2.11.1. Agarose gel electrophoresis.

DNA and RNA molecules are fractionated according to size when run on an agarose gel. 0.8% (w/v) agarose gels were generally used although 1.5% (w/v) gels were employed for the sizing of DNA fragments larger than 10 kb. The appropriate volume of 0.8% agarose (w/v) in TAE buffer was made and boiled to allow the agarose to dissolve. The agarose solution was cooled to 60°C and ethidium bromide added to a concentration of 1 µg/ml. 0.1 x volume of loading buffer (50% (w/v) glycerol, 1 mM EDTA, 0.4% (w/v) bromophenol blue) was added to the samples and electrophoresis performed in TAE at 5V/cm against standard molecular weight markers (BRL kb markers).

2.11.2. Polyacrylamide electrophoresis (PAGE).

DNA fragments of less than 1 kb in length were purified by polyacrylamide gel electrophoresis (PAGE), in general, at an acrylamide concentration of 9% (w/v). 15 ml of a solution of acrylamide:bis 30:1 (w/v) was added to 5 ml of 10 x TBE and the volume adjusted to 50 ml with water. Polymerisation was initiated on the addition of 50 μl of ammonium persulphate 25% (w/v) and 10 μl of TEMED. Electrophoresis was performed against BRL DNA size markers until the samples had migrated the required distance.

2.12. THE PURIFICATION OF DNA.

2.12.1. Electroelution of DNA from agarose gels.

The required DNA fragment was visualised under UV illumination and excised from the gel with as little of the surrounding agarose as possible. The gel slice was placed in a piece of dialysis tubing which was clipped at one end, 400 μl of TE added, the excess air expelled and the top of the dialysis bag clipped. The dialysis bag was submerged in TAE and the DNA electroeluted out of the gel slice at 100V for 1-2 h. The DNA was released from the wall of the tubing by reversing the direction of the current for 90 s and transferred to an eppendorf tube. The dialysis bag was washed with 100 μl of TE and the total elution volume extracted with phenol: chloroform 1:1 (v/v). The DNA was ethanol precipitated, dried under vacuum and resuspended in TE at the required concentration.

2.12.2. Electroelution by PAGE.

Following electrophoresis, the gel was soaked in TBE containing 1 µg/ml ethidium bromide for 10 min and the DNA fragments visualised upon UV illumination. The required fragment was then electroeluted from the acrylamide using the same procedure as for agarose gels.

2.12.3. Oligonucleotide purification by denaturing PAGE.

Oligonucleotides can be purified from denaturing acrylamide gels for use as radioactively labelled probes, primers and for *in vitro* mutagenesis. A 15% (w/v) acrylamide, 8M urea gel was prepared as follows:

acrylamide:bis (38:1), 8M urea	15 ml
10 x TBE	10 ml
8 M urea	75 ml
ammonium persulphate 30% (w/v)	100 µl
TEMED	10 µl

The gel was pre-electrophoresed in 1 x TBE at 200V for 30 min. The oligonucleotide was resuspended in formamide loading buffer (99% (w/v) formamide, 0.25% (w/v) bromophenol blue and xylene cyanol, 1mM EDTA) and loaded onto the gel at approximately 20 µg per well. Electrophoresis was performed at 300V until the bromophenol blue front had migrated two thirds of the length of the gel. The oligonucleotide was visualised by UV-shadowing against a thin-layer chromatography plate (Merck 60F254, catalogue number 5554) illuminated with a long wave UV light source. The band was cut out and

placed in a siliconised eppendorf tube and 300μl of elution buffer (0.5 M ammonium acetate, 1 mM EDTA pH 8.0) added. The tube was incubated at 37°C overnight, at which time the tube was centrifuged at room temperature for 10 min. The supernatant was transferred to a fresh tube avoiding any acrylamide fragments and the pellet washed with a further 0.5 volume of elution buffer. The pellet was vortexed and recentrifuged and the supernatants combined. The DNA was precipitated with ethanol for 15 min in a dry-ice, ethanol bath, pelleted by centifugation at 4°C for 20 min, washed with 70% ethanol, dried briefly and resuspended at the required concentration in TE.

2.13. THE RADIOACTIVE LABELLING OF DNA.

2.13.1. Random priming of DNA fragments.

The klenow fragment of DNA Polymerase I possesses the polymerase function and the 3'-> 5' exonuclease activity, but lacks the 5'-> 3' exonuclease activity of the intact enzyme. According to the method of Feinberg and Vogelstein (1983), the klenow fragment is used to copy a single-stranded DNA template using random oligonucleotides as primers, thereby generating a stable probe of high specific activity. DNA fragments were labelled as follows:

DNA (20-100 ng)   1 μl

random primer (1 μg)   1 μl

water   8 μl

The components were mixed well, incubated at 95°C for 5 min and then placed on ice. 3 μl of 10 x Messing buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl) was added, followed by dATP, dTTP and dGTP each

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at a final concentration of 30 mM and 100 μCi of [α-^{32}P] dCTP (10 mCi/ml, Amersham). 1 μl of klenow fragment was added and labelling performed at 37°C for at least 1 h. Unincorporated [α-^{32}P] dCTP was removed by passing the rection mix through a Sephadex G50 column as described.

2.13.1B. Removal of unincorporated radionucleotide from a random priming reaction.

Unincorporated radioactively labelled nucleotides can be removed from a random priming reaction by passing the reaction mix through a spun Sephadex G50 column. The nucleotides are retarded in the Sephadex matrix whereas, the labelled DNA molecules are collected in the eluate. The Sephadex G50 beads were pre-swollen in column running buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl). The bottom of a 1 ml syringe was blocked with a small quantity of siliconised glass wool and the syringe filled to capacity with the G50 suspension. The column was packed by centrifugation for 5 min at 500g and equilibrated by the addition of 100 μl of column running buffer, 0.1% (w/v) SDS and centrifugation as before. This procedure was repeated until 100 μl of buffer eluted. The volume of the reaction mix was increased to 100 μl by the addition of column running buffer, 0.1% (w/v) SDS, loaded on the column and the radioactively labelled DNA molecules collected upon centrifugation. The DNA was denatured by incubation at 95°C for 5 min and stored on ice until required.

2.13.2. 5' end labelling of oligonucleotides.

T$_4$ Polynucleotide kinase catalyzes the transfer of the γ-phosphate of ATP to the 5'-hydroxyl terminus of DNA. Oligonucleotides

synthesised using the Applied Biosystems DNA Synthesizer were 5'-end labelled according to the following protocol:

dephosphorylated DNA	1-5 pmol of 5'-ends
10 x kinase buffer (500 mM Tris HCl	5 μl
pH 7.6, 100 mM MgCl$_2$, 50 mM DTT,	
1 mM spermidine, 1 mM EDTA)	
100 μCi [γ-^{32}P] ATP (10 mCi/ml. Amersham)	10 μl
T$_4$ polynucleotide kinase	20 units

The above components were mixed and incubated at 37°C for 30min. The reaction was stopped by the addition of 1 μl of 0.5 M EDTA and the DNA purified by chloroform extraction and ethanol precipitation. The oligonucleotide was then analysed by denaturing PAGE.

2.14. HYBRIDISATION OF DNA IMMOBILISED ON NITROCELLULOSE FILTERS.

2.14.1. Radioactively labelled restriction fragments.

Bacteria grown on nitrocellulose were lysed by laying the filter colony-side up for 2 min on a piece of Whatmann 3MM soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl). The filter was then neutralised by incubation for 5 min on a piece of Whatmann 3MM soaked in neutralising solution (1.0 M Tris-HCl pH 7.4, 1.5 M NaCl). The DNA was irreversibly bound to the filter by baking under vacuum at 80°C for 2 h and the filter washed in 3 x SSC, 0.1% (w/v) SDS at 65° C for 30 min to remove any agar. The filter was then prehybridised in

prehybridisation buffer (50% (v/v) formamide, 5 x SSC, 5 x Denhardt's solution, 5 mM NaPO4 pH 6.8, 1 mM sodium pyrophosphate, 0.1 mM ATP, 0.1% (w/v) SDS, 0.1mg/ml sheared, sonicated salmon-sperm DNA) to block any sites on the nitrocellulose that would bind the probe non-specifically. The filter was sealed in a plastic bag with the minimum volume required to cover the filter, air bubbles removed and incubated at 42°C for 4 h. The radioactively labelled probe was added and the incubation extended overnight. The filters were washed 3 or 4 times for 5 min at room temperature in 3 x SSC, 0.1% (w/v) SDS and then for 30min at 65°C with one change of wash buffer. The filters were then monitored and if the background was still high, the stringency of the wash buffer was increased to 1 x SSC, 0.1% (w/v) SDS and the incubation continued for a further 30 min with one buffer change. The stringency was increased further to 0.1 x SSC, 0.1% (w/v) SDS if the background was still high. After washing, the filters were air-dried and exposed to Hyperfilm (Amersham) overnight at -70°C against an intensifying screen.

2.14.2. End-labelled oligonucleotides.

The oligonucleotide was labelled to a high specific activity with [γ-^{32}P]-ATP and T4 polynucleotide kinase. Unincorporated nucleotide was removed by centrifugation through a Sephadex G-25 column. The filters were prehybridised for at least 1 h at the hybridisation temperature in prehybridisation buffer (6 x SSC, 10 x Denhardt's solution). The probe was added and the incubation continued overnight at which time, the filters were washed in 6 x SSC, 0.1% (w/v) SDS for 10min at room temperature. The filters were then washed at 5-10°C below the Td for 2 min and exposed to Hyperfilm (Amersham) at -70°C overnight.

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2.15. M13 DI-DEOXY SEQUENCING.

2.15.1. The sequencing reaction.

The chain-termination method of sequencing DNA involves the *in vitro* synthesis of a DNA strand by a DNA Polymerase I, using a single-stranded DNA template. Synthesis is initiated from a unique site in the template where the oligonucleotide primer anneals. The synthesis reaction is terminated when a nucleotide analogue is incorporated. These analogues are 2',3'-dideoxynucleoside 5'-triphosphates (ddNTPs) that do not support continued DNA synthesis, since they lack the 3'-OH which is involved in the elongation reaction. When the correct ratio of dNTPs and one of the four ddNTPs is achieved, enzyme-catalysed polymerisation will terminate in the proportion of the population of DNA molecules where the ddNTP was incorporated. Four separate reactions, each involving a different ddNTP, will generate complete sequence information. A radioactively labelled nucleotide is included in the reaction so that the population of labelled molecules can be visualised by autoradiography following seperation by electrophoresis.

The Sequenase DNA Sequencing Kit (United States Biochemical Corporation) was employed for all the sequencing reactions with a single-stranded M13 DNA template. The DNA synthesis is performed in two steps. In the first step, the relevant oligonucleotide primer is annealed to the template and is extended using limiting concentrations of the dNTPs, including radioactively labelled dATP. This step continues to almost complete incorporation of labelled dATP into DNA molecules of

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lengths varying from several nucleotides to hundreds of nucleotides. In the second step, the concentration of all of the dNTPs is increased and a ddNTP is added. DNA synthesis continues until all the growing molecules are terminated on the incorporation of ddNTP. In this reaction, the molecules are only extended further by several dozen nucleotides. Briefly, for each sample, a single annealling (and subsequent labelling) reaction was prepared as follows:

primer	1 μl
10 x sequencing buffer	2 μl
(200 mM Tris-HCl, pH 7.5,	
50 mM MgCl$_2$, 250 mM NaCl)	
template DNA (1 μg)	7 μl

The tube was incubated at 65°C for 2 min and slow-cooled to 33°C by wrapping the heating-block in silverfoil and placing it at 4°C. This step allows the oligonucleotide primer to anneal to the template DNA. 5x Labelling Mix (7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP) was diluted 5-fold with water and the Sequenase enzyme diluted 1:8 in ice-cold TE. The following solutions were added to the annealled template-primer:

template-primer	10 μl
0.1 M DTT	1 μl
diluted labelling mix	2 μl
[α–^{35}S] dATP	0.5 μl
diluted Sequenase	2 μl

The components were mixed well and incubated for 5-10 min at room temperature. For each labelling reaction, 4 tubes were labelled G, A, T and C. 2.5 μl of the ddGTP Termination Mix (80 μM of all four dNTPs, 8 μM ddGTP) was added to the tube labelled G. With a fresh tip for each, the tubes labelled A, T and C were filled with 2.5 μl ddATP Termination Mix (80 μM all four dNTPs, 8 μM ddATP), 2.5 μl ddTTP Termination Mix (80 μM all four dNTPs, 8 μM ddTTP) and 2.5 μl ddCTP Termination Mix (80 μM all four dNTPs, 8 μM ddCTP) respectively. The tubes were pre-warmed at 37°C for 1 min and 3.5 μl of the labelling reaction added to each of the corresponding four tubes, using a fresh tip for each transfer. The contents were mixed thoroughly, centrifuged, returned to the 37°C waterbath and the incubation extended for a total of 5 min. 4 μl of stop solution (95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF) was added to each of the termination reactions, heated to 75-80°C for 2 min and 2-3 μl loaded onto a 6% (w/v) polyacrylamide 7.6 M urea sequencing gel.

2.15.2. The preparation of 7.6M urea sequencing gels.

The sequencing reactions were analysed on 6% (w/v) acrylamide, 7.6 M urea gels. A stock 40% (w/v) acrylamide solution (acrylamide:bis 38:2) was prepared in 7.6 M urea and 50 ml of gel mix prepared as follows:

40% (w/v) acrylamide 7.6 M urea	7.5 ml
10 x TBE	5.0 ml
7.6 M urea	37.5 ml

100 µl of 25% (w/v) ammonium persulphate and 50 µl of TEMED was added to initiate polymerisation and the gel poured immediately between the glass plates. The gel was pre-electrophoresed in TBE at 40W for 30 min. The samples were boiled for 2 min so as to seperate the labelled sequenced strand from the template strand and 1-2 µl loaded into the wells. Electrophoresis was performed at 40W until the bromophenol blue in the sample buffer had migrated to 2.5 cm from the bottom of the gel. The gel was then fixed for 20 min in 10% (v/v) acetic acid, 10% (v/v) methanol, dried under vacuum for 20 min at 80°C and autoradiographed against Amersham (Hyperfilm) overnight.

2.16. OLIGONUCLEOTIDE-DIRECTED *IN VITRO* MUTAGENESIS.

The oligonucleotide *in vitro* mutagenesis system (Amersham) was used for all of the mutagenesis procedures performed. The system is based on the method of Sayers et al. (1988) and has been designed to be up to 95% efficient. This method involves a strand-specific selection step which eliminates the non-mutant sequence, *in vitro*, avoiding the utilisation of the host-mediated repair system which can also remove the mutant sequence. A nitrocellulose filtration step is employed to remove any contaminating template, thereby reducing the non-mutant background considerably. The system can be used with high efficiency for insertion and deletion mutagenesis due to its integral exonuclease step which digests the non-mutant strand. The oligonucleotide is annealed to the single-stranded template and extended by klenow fragment in the presence of T_4 DNA ligase which generates a mutant heteroduplex. The non-mutant strand can be selectively removed by the incorporation of a thionucleotide into the mutant molecule. NciI cannot cleave if its recognition site contains a thionucleotide and thus,

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single-stranded nicks are generated on the treatment of DNA containing one phosphorothioate and one non-phosphorothioate strand (non-mutant) with NciI. Exonuclease III recognises these nicks and digests away the non-mutant strand leaving the mutant strand to act as a template in the construction of a homoduplex mutant molecule. Briefly, the annealing, priming and extension reactions are typical, except that dCTP is replaced by $[\alpha\text{-}^{35}S]$dCTP.

Single-stranded recombinant M13 template DNA was prepared and resuspended in TE at a concentration of 1 μg/μl. The mutant oligonucleotide was prepared as a stock solution of 5 OD_{260} units/ml and 5'-phosphorylated as follows:

oligonucleotide stock solution	2.5 μl
10 x kinase buffer (1 M Tris-HCl pH 8.0,	3.0 μl
1 mM EDTA, 70 mM DTT, 10 mM ATP)	
water	25 μl

Two units of T4 polynucleotide kinase were added and the tube incubated at 37°C for 15 min at which time, the kinase was inactivated by incubation at 70°C for 10 min. The mutant oligonucleotide was annealed to the single-stranded DNA template as follows:

single-stranded DNA template (1 μg/μl)	5.0 μl
phosphorylated mutant oligonucleotide	2.5 μl
Buffer 1	3.5 μl
water	6.0 μl

The tube was incubated at 70°C for 3 min and then at 37°C for 30min at which time, the following reagents were added:

MgCl$_2$ solution	5 μl
Nucleotide mix 1	19 μl
water	6 μl
klenow fragment	6 units
T$_4$ DNA ligase	6 units

The tube was incubated at 16°C overnight at which time, a 1 μl sample was removed for analysis on an agarose gel. Any single-stranded non-mutant DNA was removed by filtration through a nitrocellulose filter. The following reagents were added to the reaction mix:

water	170 μl
5M NaCl	30 μl

The sample was applied to the top reservoir of the filter unit and centrifuged for 10 min at 500g. 100 μl of 500 mM NaCl was applied and the column spun as before, so as to wash through any remaining RF DNA. The DNA was ethanol precipitated, collected by centrifugation for 15 min, washed with 70% ethanol, dried briefly and resuspended in 25 μl of Buffer 2. A 10 μl aliquot was dispensed into a fresh tube for digestion of the non-mutant strand by NciI. 65 μl of Buffer 3 and 5 units of NciI were added and the tube placed in a 37°C waterbath for 90 min. A 10 μl sample was removed for analysis on an agarose gel and the remainder

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subjected to exonuclease III digestion to remove the nicked non-mutant strand. The following reagents were added:

500 mM NaCl	12 µl
Buffer 4	10 µl
Exonuclease III (25 units/µl stock)	2 µl

and the tube incubated at 37°C for 30 min. The enzyme was inactivated by heating at 70°C for 15 min at which time, a 15 µl sample was removed for analysis. Double-stranded closed circular mutant homoduplex molecules were produced by treatment with DNA polymerase I and T4 DNA ligase.

Nucleotide mix 1	13 µl
$MgCl_2$ solution	15 µl
DNA polymerase I	3 units
T4 DNA ligase	2 units

The above reagents were added to the reaction mix and the tube incubated at 16°C for 3 h. An aliquot of fresh or frozen competent cells was transformed with a 20 µl sample of the reaction mix, plaques picked and single-stranded DNA prepared. The DNA was sequenced by the M13-dideoxynucleotide method and the mutant sequence recognised. The entire length of the insert was then sequenced so as to ensure that no unwanted changes had occurred.

2.17. Culture of Fibroblasts.

NR6 (Pruss and Herschmann, 1977) and NR6+ (DiFiore et al., 1987) fibroblasts were grown in DMEM, 10% (v/v) FCS, 1% (v/v) penicillin and streptomycin and incubated at 37°C at 10% CO_2. A confluent 9 cm dish of cells was passaged 1 in 5 by trypsinisation, every 3 days for routine maintenance.

2 17B. Cell counting.

A hemocytometer (Improved Neubauer) was used to determine the concentration of a cell suspension by counting the number of cells in the 1 mm^2 area. The concentration of the sample was derived as follows:

$$c = n/v$$

where c = cell concentration (cells/ml), n = number of cells counted and v = volume counted (ml). The depth of the chamber is 0.1 mm, the area 1 mm^2 and therefore, $v = 10^{-4}$ ml.

2.18. CULTURE OF INSECT CELLS.

Insect cells (Sf9) were cultured according to the protocols established by Summers and Smith (1987). All culture medium was incubated at 27°C prior to use and the cells routinely incubated at 27°C. The culture medium used throughout was IPL41 (JR Scientific) supplemented with 10% (v/v) FCS. Routine culture was performed in the absence of antibiotics although fungizone and gentamycin were added for large culture volumes at 1% and 0.1% (v/v) respectively. Sf9 cells have a doubling time of 18-24 h in IPL41, 10% (v/v) FCS and thus were subcultured at least three times a week. The cells from a nearly

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confluent culture were dislodged by tapping the flask gently against a surface, minimising any foaming and 0.5-1.0 ml (2.0-2.5 x 10^6 cells) transferred to a new 25 cm^2 flask containing 4 ml of fresh medium. The flask was rocked gently to distribute the cells evenly and incubated at 27°C.

2.18.1. Freezing Sf9 cells.

Sf9 cells were grown to log phase, pelleted by centrifugation for 10 min at 1000g, resuspended in 10 ml IPL41, 10% (v/v) FCS and counted. The culture volume was increased to give a cell density of 8.0 x 10^7/ml and the cells placed on ice. An equal volume of freezing solution (15% (v/v) DMSO, 1% (v/v) fungizone, 0.1% (v/v) gentamycin in IPL41,10% (v/v) FCS) was added and 1 ml aliquots dispensed into NUNC cryostore tubes. The tubes were placed in a polystyrene box, incubated at -70°C overnight and then placed in liquid nitrogen for long term storage.

2.18.2. Thawing Sf9 cells.

An aliquot of frozen Sf9 cells was removed from liquid nitrogen storage and thawed in a 27°C waterbath for 2-3 min. The cells were then transferred to a 25 cm^2 flask containing 10 ml IPL41, 10% (v/v) FCS, prewarmed to 27°C and incubated for 1 h at 27°C. The medium was then removed and replaced with 10 ml of fresh medium and the cells incubated at 27°C overnight and passaged the following day.

2.18.3. Purification of extracellular virus (ECV) DNA.

The following procedure was adapted from that of Smith et al. (1982). Relatively pure viral DNA can be obtained from extracellular virus particles (ECV) separated from infected culture medium by centrifugation. Sf9 cells infected with AcNPV (at 2.0 x 10^6 cells/ml) will

yield approximately 1 μg of purified virus DNA per ml of culture medium harvested at 48 h post infection. Duplicate 50 ml cultures of Sf9 cells, set up in 250 ml flasks, were infected with AcNPV as described previously and the infected cells pelleted at 36-48 h post infection by centrifugation for 10 min at 1000g. The supernatant was transferred to ultracentrifuge tubes and the ECV pelleted by centrifugation for 30 min at 100,000g. The medium was decanted and the tubes inverted to drain off all residual liquid. The ECV was resuspended overnight in 500 μl of 0.1 x TE and 4.5 ml of extraction buffer (100 mM Tris-HCl pH7.5, 100 mM EDTA, 20 mM KCl) was then added. The suspension was transferred to a 15 ml polypropylene centrifuge tube, 200 μg of Proteinase K added and the mixture digested for 1-2 h at 65°C. 500 μl of 10% (w/v) sarkosyl was then added and the incubation extended for a further 2 h at which time, the DNA was extracted twice with phenol: chloroform. Due to the sensitivity of the DNA to shearing, the extraction was performed on a rotating wheel at room temperature for 5 min and the phases seperated by low speed centrifugation. The aqueous layer was carefully transferred to a fresh tube using a wide mouth pipette and the extraction repeated. The DNA was ethanol precipitated, washed in 70% ethanol, dried briefly, resuspended in 0.5 ml of 0.1 x TE and incubated for 15 min at 65°C to help the DNA resuspend. The DNA was stored at 4°C.

2.18.4. Transfection of Sf9 cells.

Plasmids containing foreign genes can be transfered to the AcNPV genome by *in vivo* recombination using a modification of the calcium phosphate precipitation technique. Sf9 cells were seeded in $25 cm^2$ flasks at a density of 2.0×10^6 cells per flask and allowed to attach for 1 h at 27°C. 1 μg of AcNPV DNA was mixed with 2 μg of plasmid DNA in an eppendorf tube. In addition, controls with AcNPV DNA alone and

plasmid DNA alone were prepared. The medium was removed from the flasks and replaced with 0.75 ml IPL41, 10% (v/v) FCS, 1% (v/v) fungizone, 0.1% (v/v) gentamycin and the flasks left at room temperature. 0.75 ml of transfection buffer (25 mM HEPES pH 7.1, 140mM NaCl, 125 mM $CaCl_2$) was added to the DNA and the tube vortexed gently. A control tube of transfection buffer alone was included. The DNA solution was added dropwise to the medium in the flask and a calcium phosphate precipitation formed between the $CaCl_2$ in the transfection buffer and the phosphate in the medium. The solution was distributed evenly across the surface of the cells and the flasks incubated for 4 h at 27°C. The medium was then removed carefully and the cells washed 4 times with 5 ml of IPL41, 10% (v/v) FCS, 1% (v/v) fungizone, 0.1% (v/v) gentamycin and then incubated in a further 5 ml of medium at 27°C for 4-6 days. After this time, the cultures were inspected with an inverted microscope for positive signs of infection. These signs include the appearence of polyhedra, a 25-50% increase in cell diameter, most easily discerned as nuclear expansion and cell lysis late in infection (after 4-6 days). The virus was then plated on fresh monolayers and the recombinant virus identified by plaque hybridisation and purifed by plaque assay as described below.

2.18.5. THE IDENTIFICATION OF RECOMBINANT VIRUS.

2.18.5.1. Plaque assay.

Sf9 cells were seeded in IPL41, 10% (v/v) FCS in 60 x 150 mm grided dishes at a density of 2.5×10^6 cells per dish. The cells were allowed to attach on incubation at 27°C for 1 h. A series of 10-fold dilutions of virus stock in 1 ml of medium was prepared, 10^{-4} to 10^{-7} for transfection mixes and 10^{-1} and 10^{-2} for virus picked from plaques.

After 1 h, the medium was removed from the cells, the virus i noculum added and the dishes rocked gently to distribute the virus. In addition, a wild-type virus infection and an uninfected control were performed. The dishes were then incubated at 27°C for 1 h. Meanwhile, the 1.5% (w/v) low melting-point agarose (Seaplaque) overlay was prepared. The volume of overlay required was calculated (4 ml per dish) and, for every 100 ml needed, 1.5 g of agarose was dissolved in 50 ml of water and autoclaved for 15-20 min. For each 100 ml of overlay, 50 ml of 2 x Graces medium (Gibco), 20% (v/v) FCS, 2% (v/v) fungizone and 0.2 % (v/v) gentamycin was prepared. Both solutions were allowed to equilibrate in a 37°C waterbath at which time, they were combined and returned to the waterbath until required. After 1 h, all of the medium was removed from the cells by leaning the dishes against the edge of a 25 ml pipette and aspirating with a fresh pasteur pipette for each dish. 4 ml of overlay was added slowly from a pipette resting on the side of the dish and the dish rocked immediately to distribute the agarose evenly. The dishes were then left undisturbed for at least 1 h to allow the agarose to solidify. The dishes were then transferred to a sandwich box containing damp tissues, the box sealed and incubated for 3-5 days at 27°C. After this time, the plaques were either screened visually or by hybridisation depending on the round of screening.

2.18.5.2. Plaque hybridisation.

This procedure is based on that described by Villareal and Berg (1977). After the plaque assay was performed, the dishes were allowed to dry overnight at room temperature. A sample plate was tested for dryness by lifting the edge of the agarose overlay with a sterile microspatula, if a milky smear of cells collect at the interface of the dish and overlay the dishes are too wet. When the dishes were sufficiently

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dry, 5 orientation marks were made in the agarose with a pasteur pipette and corresponding marks made on the bottom of the dish with a marker pen. The edge of the overlay was gently loosened with a sterile microspatula, the dish inverted over a 90 x 150 mm dish and the overlay eased out and dropped into the larger dish. The overlays were then stored at 4°C in a closed container containing damp towels to prevent them drying out. The four blotting solutions (A,B,C,D) were prepared as below:

solution A, 0.05 M Tris-HCl pH 7.4, 150 mM NaCl

solution B, 0.5 M NaOH, 1.5 M NaCl

solution C, 1.0 M Tris-HCl pH7.4, 1.5 M NaCl

solution D, 0.3 M NaCl, 30 mM sodium citrate

A dry 47 mm nitrocellulose filter (Millipore) was placed on top of the cells remaining on the dish and secured with a drop of solution A. The dish was held up to a light source, and the orientation marks traced onto the filter with a ball-point pen. A 47 mm circle of Whatman 3MM paper was saturated with solution A and placed on top of the nitrocellulose filter in the dish. The filter was tamped down firmly with the top of a 50 ml polpropylene Falcon tube to bring the nitrocellulose in contact with the cells and to expel any air pockets. After 1 min, the filter was tamped down again, the Whatman filter discarded and the nitrocellulose filter placed cell side up on a piece of Whatman 3MM saturated in solution B. After 2-3 min, the filter was dried on paper towels and transferred to a Whatman filter saturated in solution C for 2-3 min. The nitrocellulose filter was then dried on paper towels, immersed in solution D and dried on paper towels. When all of the fiters

had been processed, they were baked for 2 h at 80°C under vacuum. The filters were then hybridised with a suitable radioactively labeled probe and the areas of positive hybridisation on the autoradiograph aligned on the original agarose overlay. An area of several mm around each positive was picked, transferred to 1 ml of medium and plaque purified again.

2.18.5.3. Visual screening.

Plaques were examined after 3-4 days at a magnification of 30X. After 3 or 4 rounds of radioactive hybridisation screening, the plaques were well separated and therefore relatively easy to visualise. The wild-type plaques (occlusion positive) contain polyhedra which are bead-like and very refractile to light (Figure 2.1A), whereas the recombinant plaques do not contain polyhedra and thus are light grey and less refractile (occlusion negative) (Figure 2.1B). The location of the occlusion negative plaques were marked on the grid on the bottom of the dish, the area of the overlay picked with the cut-off end of a Gilson yellow tip and transferred to 1 ml of medium. A second round of plaque purification was performed on a 10^{-2} dilution of the plaque so as to ensure that no contaminating wild-type virus was present. When the plaque was completly pure, 0.5 ml of the innoculum was used to infect 2.5×10^6 cells in 5 ml of IPL41, 10% (v/v) FCS in a 25 ml flask and incubated at 27°C for 3-4 days. After this time, the cells were pelleted by centrifugation at 1000g for 10 min and the virus stored at 4°C.

2.18.6. Determination of virus titre by plaque assay.

Virus stocks were titered using a procedure adapted from Volkman et al. (1976). A series of dilutions from 10^{-1} to 10^{-9} was prepared in a final volume of 1 ml of IPL41, 10% (v/v) FCS and duplicate

Figure 2.1. 2.5 x 10^6 Sf9 cells were plated to 60 x 150 mm dishes and the cells allowed to attach upon incubation at 27°C for 1 h. The medium was then removed and the cells infected with wildtype (Panel A) or recombinant virus (Panel B) for 1 h at 27°C at which time, the medium was removed and the cells overlaid with agar. The dishes were then incubated at 27°C for 3 days to allow the plaques to develop.

Panel A. Infection of cells with wildtype virus generates occluded or occlusion positive plaques in which the polyhedra are clearly visible.

Panel B. Infection of cells with recombinant virus generates non-occluded or occlusion-negative plaques.
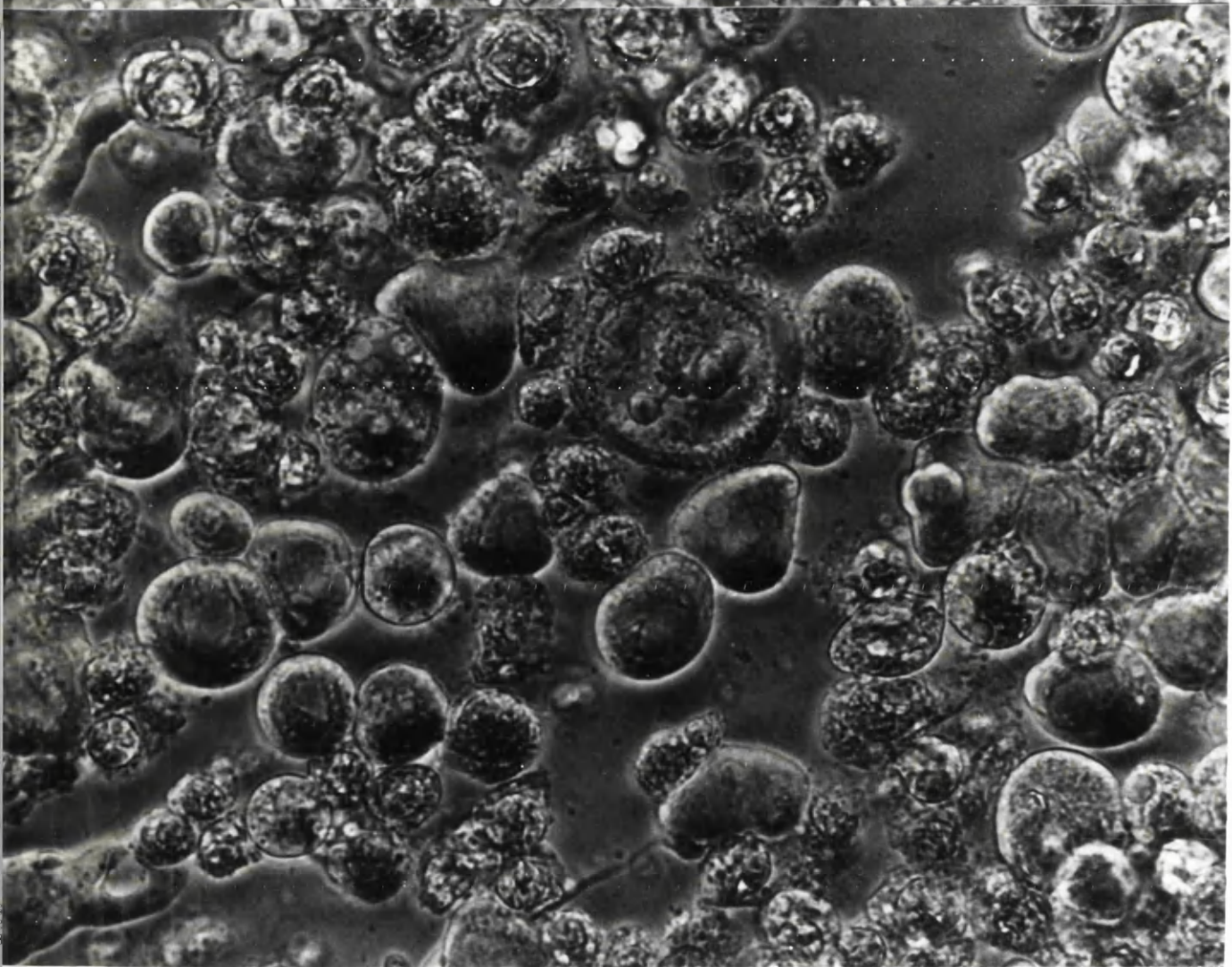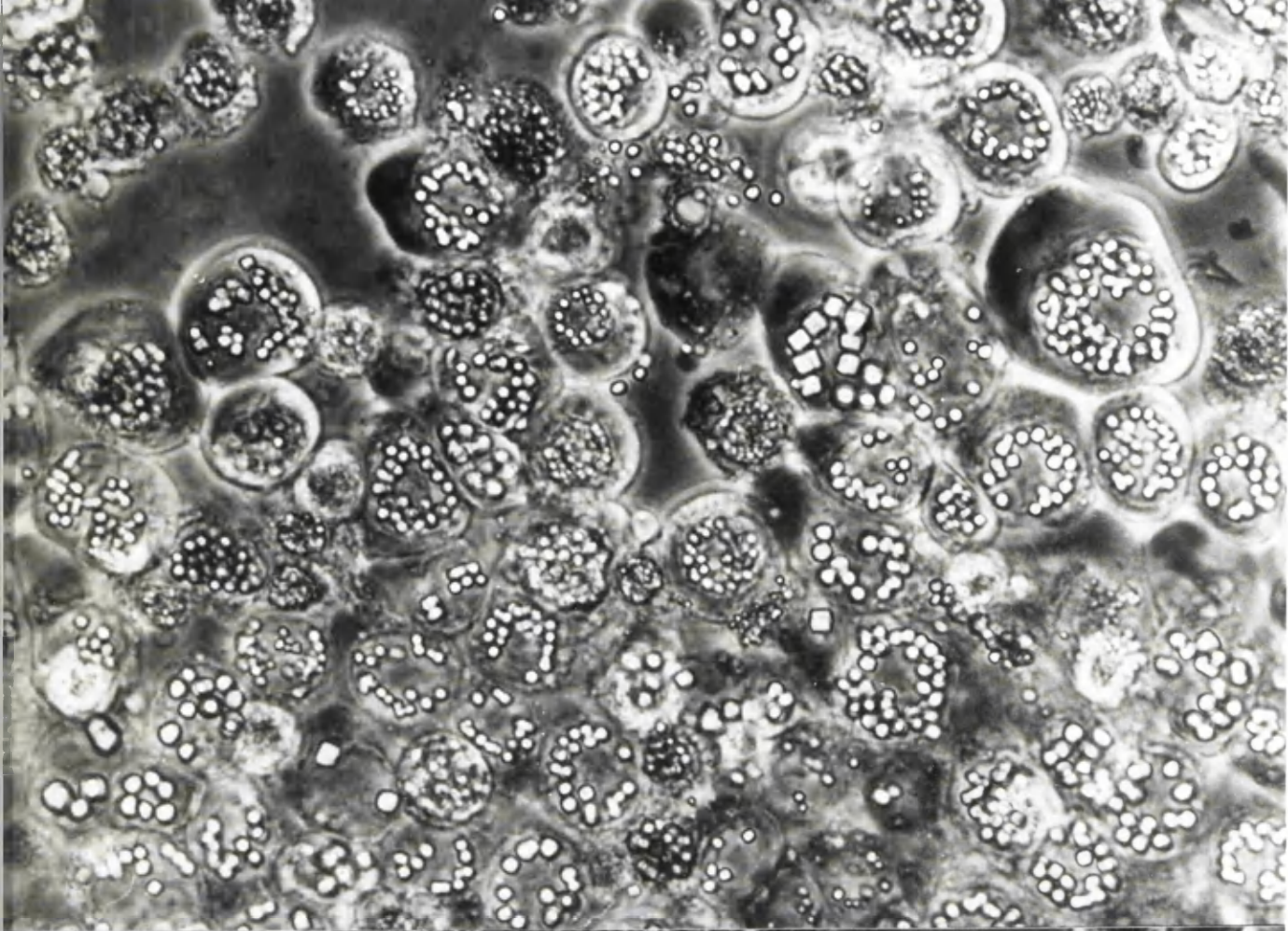
dishes were set up for each dilution for a plaque assay as described earlier. The plaques were counted, and the virus titre (PFU/ml) calculated as follows:

PFU/ml = 1/dilution x number of plaques x 1/(ml inoculum/plate)

2.18.7. Infection of Sf9 cells with virus.

Type of vessel	Cell Density	Minimum Virus vol	Incubate in final volume
60 mm^2 dish	2.5 x 10^6/dish	1 ml	3 ml
25 cm^2 flask	3.0 x 10^6/flask	1 ml	5 ml
75 cm^2 flask	9.0 x 10^6/flask	2 ml	10 ml
150 cm^2 flask	1.8 x 10^7/flask	4 ml	20 ml

Table 2.2. Approximate seeding densities for typical vessel sizes.

The cells were counted and seeded into flasks or dishes at the appropriate density according to Table 2.2 and allowed to attach for 1 h at 27°C. After the cells had attached, the medium was removed and the appropriate amount of virus added (see Table 2.2). Unless a specific multiplicity of infection (MOI) was required, the minimum volume of virus stock required to cover the cells was used.

ml of innoculum required = MOI(PFU/cell) x number of

cells/virus titre (PFU/ml)

In general, it was seen that an MOI of 10 gave a synchronous infection and high ECV titres.

The cells were incubated for 1 h at 27°C at which time, the innoculum was replaced with 5 ml of IPL41, 10% (v/v) FCS and the culture incubated at 27°C for a further 3 days. The medium was collected for protein purification or virus stocks and the cell pelleted analysed for protein production as described.

2.19. THE ANALYSIS OF CELLULAR PROTEINS.

2.19.1. LYSIS OF CELLS.

2.19.1.1. Preparation of whole-cell lysates.

Cells were washed twice with PBS and resuspended in 0.5 ml of 1 x sample buffer (2% (w/v) SDS, 5 mM sodium phosphate pH7.0, 10% (w/v) glycerol, 0.1 M DTT, 0.01% (w/v) bromophenol blue) previously heated to 95°C and transferred to an eppendorf tube. The pellet was disrupted by repeated passage through a 21-gauge needle followed by a 23-gauge needle, β-mercaptoethanol added to 5% (v/v), the samples heated to 95°C for 3 min and analysed by SDS-PAGE.

2.19.1.2. Triton lysis of cells.

Cells were washed twice with PBS and resuspended in 0.1 ml of ice-cold lysis solution (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) triton x 100, 2 mM EDTA), transferred to an eppendorf tube and incubated on ice for 30 min. The lysate was then centrifuged for 20 min in a refridgerated eppendorf centrifuge so as to pellet the cell debris. The supernatant was transferred to a fresh eppendorf tube, 20 μl of 5 x SDS sample buffer (10% (w/v) SDS, 50% (v/v) glycerol, 500 mM Tris-HCl

pH 6.8, 1 M DTT, 0.05% (w/v) bromophenol blue) added and incubated at 95°C for 3 min. The sample was then analysed by PAGE as described.

2.19.2. Immunoprecipitation of protein.

The required amount of antibody and 30 µl of Protein Sepharose-A or G, preswollen in triton x 100 lysis buffer, was added to the lysate or 1 ml of conditioned medium in an eppendorf tube. The tubes were placed on a rotating wheel and incubated for 2 h at 4°C. The immunoprecipitate was collected upon centrifugation for 3 min and washed twice with 1 ml of ice-cold lysis buffer and twice with 1 ml PBS, 1% (v/v) triton. After the final wash, the supernatant was discarded, the pellet resuspended in 50 µl of 1 x SDS sample buffer, β-mercaptoethanol added to 5% (v/v) and incubated for 3 min at 95°C. 0.5 x sample volume was analysed by SDS-PAGE.

2.19.3. SDS-PAGE analysis of proteins.

SDS-PAGE is performed in two layers, an upper stacking gel and a lower resolving gel. The stacking gel is at a lower percentage of acrylamide than the resolving gel and at pH 6.8, whereas the resolving gel and the running buffer are at pH 8.8. This ensures that the proteins run through the stacking gel as tight bands and are only separated when they begin to migrate through the higher pH of the resolving gel. The percentage of acrylamide used depends on the size range of the protein of interest. In general, 7.5% (w/v) resolving and 5% (w/v) stacking gels were used for high molecular weight proteins (100-200 kD) and 12.5-15% (w/v) resolving and 7.5% (w/v) stacking gels for low molecular weight proteins (5-30 kD). The resolving gel was mixed from a stock solution of 30% (w/v) acrylamide (acrylamide:bis 30:0.8) and Tris-HCl pH 8.8 and SDS were added to a final concentration of 375 mM

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and 0.1% (w/v) respectively. Polymerisation was initiated by the addition of 100 μl of 10% (w/v) ammonium persulphate and 30 μl TEMED. The gel was then poured quickly between the plates to approximately 3/4 of the height of the plates and overlayed with water-saturated N-butanol. When the gel was set, the butanol was poured off and the surface of the gel washed well with water. The stacking gel was prepared in the same way but with Tris-HCl pH 6.8, layered over the resolving gel and the comb inserted. Following polymerisation, the comb was removed, the wells flushed out and the samples applied and electrophoresis performed in running buffer (200 mM glycine, 25 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS) typically at 40-100V depending on the size of the protein and the duration of the run. After electrophoresis, the gel was either immunoblotted, as described, or stained by soaking in Coomassie blue stain for 30 min (45% (v/v) methanol, 10% (v/v) acetic acid, 0.25% (w/v) Coomassie blue) followed by destaining in 5% (v/v) methanol, 5% (v/v) acetic acid. The gel was then soaked in water for 15 min and dried under vacuum for 2 h at 80°C.

2.19.4. Transfer-hybridisation of proteins.

Proteins separated by SDS-PAGE can be selectively identified by specific antisera by the process known as immunoblotting. The gel was immersed in transfer buffer (20% (v/v) methanol, 40 mM glycine, 50mM Tris-base) and covered with a piece of nitrocellulose presoaked in transfer buffer. The gel/nitrocellulose was sandwiched between several layers of presoaked Whatman 3MM and placed on the transfer apparatus (Biorad) with the nitrocellulose between the anode and the gel. The proteins were transferred from the gel onto the nitrocellulose by applying for 90 min, a current which is dependent on the dimensions of the gel:

mA= area of gel in cm^2 x 0.8

The gel was then stained, as before, to check that the transfer was complete and the nitrocellulose hybridised with the specific antisera.

2.19.5.1. Immunoblotting with [^{125}I]Streptavidin.

All incubations and washes were carried out at room temperature with all antibody incubations performed in sealed plastic bags rotated on a wheel in order to evenly distribute the antibody solution. The membrane was blocked for 60 min with TBST (10 mM Tris-HCl pH 8.0, 150mM NaCl, 0.05% (v/v) Tween-20), 3% (w/v) milk powder using approximately 1 ml per cm^2 and washed twice for 10 min in TBST. The membrane was then incubated for 60 min with 10 μg of the primary antibody diluted in 10 ml of TBST, 3% (w/v) milk powder and washed as before. The membrane was then incubated for 60 min with 2 μg of biotinylated second antibody (Amersham) diluted in 10 ml of TBST, 3% (w/v) milk powder after which time, the washing procedure was repeated and the membrane incubated for 30 min in 10 ml of 1:400 dilution of [^{125}I]streptavidin (specific activity of 33 μCi/μg, Amersham) in TBST. Unbound [^{125}I]streptavidin was removed by three 10 min washes in TBST and the membrane subjected to autoradiography against Hyperfilm (Amersham).

2.19.5.2. Immunoblotting with [^{125}I]protein A.

The proteins were transferred from the gel to the nitrocellulose as described. The filter was immersed in blocking buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.5% (v/v) triton x 100, 0.1% (w/v) BSA) and incubated overnight at 4°C. The filter was washed twice in blocking buffer at room temperature and then incubated for 1 h at room

temperature with a 1:500 dilution of antibody in blocking buffer. Excess antibody was removed by subjecting the filter to five 5 min washes in blocking buffer prewarmed to 37°C. The bound antibody was detected using 10 μCi [^{125}I]protein A (specific activity of 150 μCi/μg, Amersham) in blocking buffer for 1 h at room temperature. The filter was washed as before, air dried and exposed to Hyperfilm (Amersham) overnight at

-70°C.

2.19.5.3. Phosphotyrosine immunoblotting.

Proteins were immunoblotted with anti-phosphotyrosine antibodies according to the method of Kamps and Sefton (1988). The proteins were immunoblotted as before, but with the addition of 7.5% (w/v) sodium orthovanadate to the transfer buffer. The blot was then washed for 1 h at room temperature in rinse buffer (10 mM Tris-HCl, pH7.4, 0.9% (w/v) NaCl, 0.01% (w/v) NaN$_3$), 5% (w/v) BSA, 1% (w/v) sodium orthovanadate. The blot was incubated with a 1:50 to 1:300 dilution of the antisera (dependent on the titre of the serum) for 2 h at room temperature in rinse buffer, 5% (w/v) BSA, 1% (w/v) sodium orthovandate. Unbound antibody was removed by 2 x 10 min washes in rinse buffer, 1 x 10 min wash in rinse buffer, 0.05% (v/v) NP40 and a further two 5 min washes in rinse buffer. The blot was incubated for 1h with 10 μCi [^{125}I]protein A (specific activity of 150 μCi/μg, Amersham) and then washed as before. The blot was then exposed to Hyperfilm (Amersham).

2.19.6. *In vitro* phosphorylation assays.

The EGF receptor was immunoprecipitated, washed twice with ice-cold triton x 100 lysis buffer, twice with ice-cold kinase buffer (50 mM

Tris HCl pH 7.5, 150 mM NaCl, 0.1 mM MnCl$_2$, 1 mM MgCl$_2$, 0.2% (w/v) triton x 100, 100 μM sodium orthovanadate) and resuspended in 50 μl kinase buffer. 1 μCi [γ-^{32}P]ATP (specific activity 10 mCi/ml, Amersham) was added and the reaction incubated at 4°C for 10 min, at which time, the reaction was terminated by the addition of 10 μl of 5 x SDS sample buffer and incubation at 95°C for 2min. The products of the reaction were analysed by SDS-PAGE.

2.19.7. Metabolic labelling of Sf9 cells.

2.5 x 10^6 cells were plated to 6 cm dishes and infected with the recombinant virus. After 48-72 h, the medium was replaced with 1 ml of labelling medium (Cys-free Graces insect medium (Gibco) supplemented with 10% (v/v) complete medium and 1% (v/v) dialysed FCS). [^{35}S]Cys (Amersham 1300 Ci/mmol) was added to a final concentration of 100μCi/ml and the cells incubated for varying lengths of time (20 min for pulse-chase labeling or 4 h for normal labelling). The labelling medium was removed and stored at 4°C and the cells cultured in 1 ml of complete medium for a further 2 h (or various times for pulse-chase analysis). The medium was removed and combined with the labelling medium, the cells were washed twice in ice-cold PBS and lysed in 100 μl triton x 100 lysis buffer. The medium and cell lysates were analysed by immunoprecipitation.

2.19.8. [^{125}I] EGF competition assay.

2 x 10^4 NR6+ fibroblasts (DiFiore et al., 1987a) and NR6 (Pruss and Herschman, 1977) were plated in DMEM, 10% (v/v) FCS to 24 well plates and incubated at 37°C for 2-3 days until just subconfluent. The medium was carefully aspirated and the cells washed twice in ice-cold binding buffer (PBS/ Ca^{2+}/ Mg^{2+}, 0.1% (w/v) BSA). The EGF standards (0-10 mM)

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and the test samples were prepared at the required concentrations in binding buffer in a sufficient volume to allow duplicate 250 µl aliquots to be taken for each sample. [^{125}I]EGF (specific activity of 100 µCi/µg, Amersham) was added so that approximately 5 x 10^4 cpm would be added to each well and each of the standards and samples dispensed in duplicate. The cells were incubated at 4°C for 4 h, at which time the labelling buffer was aspirated and the cells washed twice with binding buffer. 500 µl lysis buffer (0.5 M NaOH, 0.1% (w/v) SDS) was added to each well and the cells lysed by incubation at room temperature for 10min. The lysates were then transferred to scintillation vials and counted on the γ channel of a scintillation counter for 1 min.

2.19.9. [^{3}H]Thymidine incorporation assay.

2 x 10^4 NR6 (Pruss and Herscman, 1977) and NR6+ (DiFiore et al., 1987a) fibroblasts were plated to 24 well plates in DMEM, 10% (v/v) FCS and incubated for 2-3 days until confluent. The cells were then made quiescent by incubation at 37°C in starving medium (DMEM supplemented with 10% (v/v) original medium) for 5 days. The EGF standards, the test samples or 10% (v/v) FCS were added in duplicate and the cells incubated for a further 18 h, at which time, 1 µC i [^{3}H]Thymidine (specific activity of 5 Ci/mmol) was added to each well. Incubation was extended for a further 4 h and the cells washed once with ice-cold PBS. 500 µl of ice-cold 5% (w/v) TCA was dispensed into each well and incubated at 4°C for 20 min, at which time, the cells were washed with 5% (w/v) TCA. The cells were lysed by the addition of 300 µl of 0.5 M NaOH and incubation at room temperature for 60 min. The DNA was precipitated by the addition of 2 x volume 10% (w/v) TCA and the incubation at 4°C overnight. The DNA was immobilised on glass scintered filters (Millipore) arranged on a vacuum pump assembly

(Millipore). The filters were washed twice with 5% (w/v) TCA and twice with ethanol. The filters were air dried and then placed in scintillation vials containing scintillant (Beckman Ready Save). After storage for at least 1 h at room temperature, to reduce the background, the filters were counted on the [^{3}H] programme of the scintillation counter.

2.19.10. Tunicamycin treatment.

Tunicamycin was prepared at a stock solution of 1 mg/ ml in 50mM sodium carbonate, pH 10.0 and stored at -20°C. 2.5 x 10^6 cells were plated on 6 cm dishes and incubated at 27°C for 36 h at which time, tunicamycin was added to a final concentration of 5 μg/ ml and the incubation extended for a further 2 h. The cells were labelled with [^{35}S]Cys as before, but in the continued presence of 5 μg/ml tunicamycin. The cells were lysed with triton x 100 lysis buffer, the labelled protein immunoprecipitated and analysed by SDS-PAGE.

2.19.11. Endoglycosidase F treatment.

The infected cells were labelled with [^{35}S]Cys and lysed with triton x 100 lysis buffer. The labelled protein was immunoprecipitated, 150 μl of endoF buffer (100 mM sodium acetate pH 5.5, 50 mM EDTA, 1% (w/v) triton x 100, 0.2% (w/v) SDS, 1% (v/v) β-mercaptoethanol) added and the sample heated to 95°C for 2 min. 10 mU of Endoglycosidase F was added to a 50 μl aliquot of the supernatant and incubated at 37°C for 24 h. 10 μl of 5 x SDS sample buffer was added and the reaction mixture analysed by SDS-PAGE.

2.19.12. Elastase treatmemt.

The [^{35}S]Cys labelled immunoprecipitated TGFα precursor was resuspended in 100 μl elastase buffer (250 mM Tris HCl, pH 8.8, 10 mM β–

mercaptoethanol, 20 mM EDTA). 2 μg elastase was added to a 50 μl aliquot and the reaction incubated at 37°C for 1 h at which time, 10 μl of 5 x SDS sample buffer was added and the reaction mix analysed by SDS-PAGE.

CHAPTER THREE.

STRUCTURE-FUNCTION ANALYSIS OF EGF.

3.1. INTRODUCTION.

3.1.1. The EGF family of growth factors.

The peptide growth factor EGF has been purified from several species. The highly conserved nature of its primary structure, indicates that the molecule folds into a strict three-dimensional conformation that must be rigorously adopted if receptor binding is to occur (see Figure 3.1). 70% homology exists between murine (Cohen, 1959), human (Starkey et al., 1975) and guinea pig EGF, the latter species lacking the carboxyl-terminal Arg residue (Rubin and Bradshaw, 1984). The rat form of EGF, displaying greater than 80% homology with human and murine EGF, has five residues missing from the carboxyl terminus (Simpson et al., 1985). In addition, it does not contain any Trp residues, indicating that replacement of the corresponding Trp residues in the other forms of EGF does not affect receptor binding or mitogenic potency (Schaudies and Savage, 1986). The strict disulphide pairings of the six Cys residues in EGF folds the molecule into a stable, compact structure. The pairing of Cys^6 with Cys^{20}, 14 with 31 and 33 with 42 (positions refer to murine EGF) define three looped regions A, B and C (Savage et al., 1973).

While searching protein sequence databases for EGF-like homologies, Blomquist et al. (1984) identified a sequence in the Vaccinia virus 19 kD early protein (see Figure 3.2). This polypeptide was isolated from the culture supernatants of Vaccinia virus-infected cells, and was termed Vaccinia virus growth factor (VVGF) after it was established that
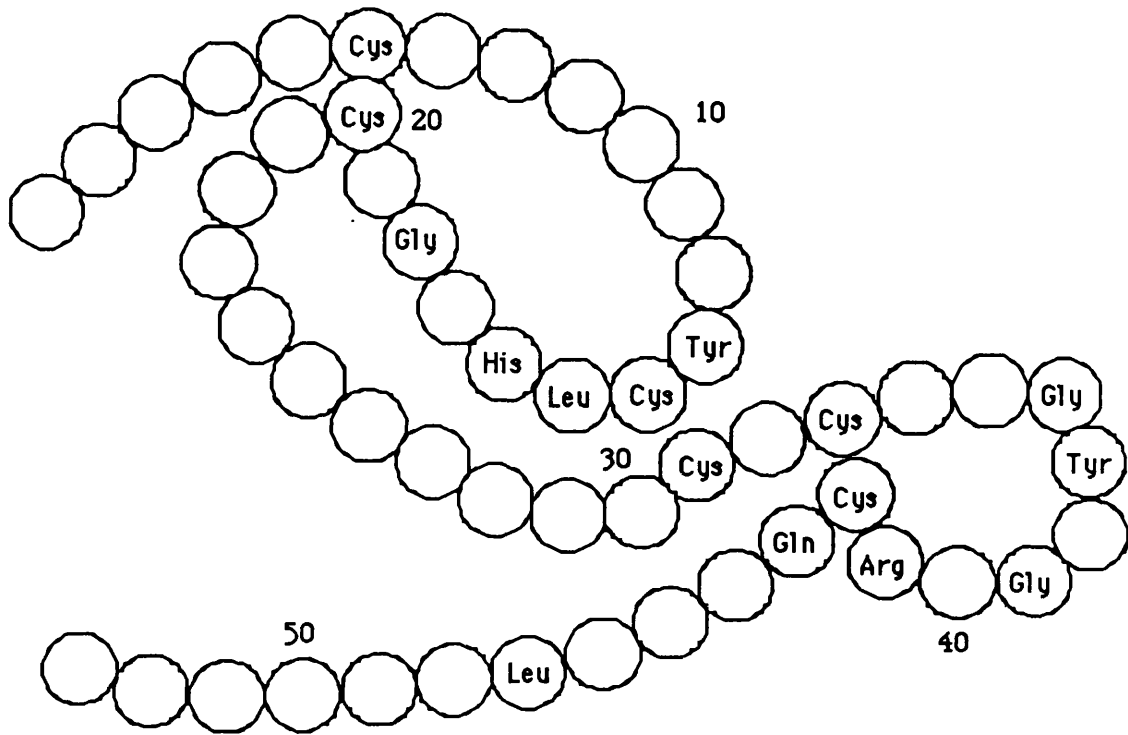
Figure 3.1. The amino acid sequence of human EGF indicating the residues that are conserved or conservatively changed in the EGF-like family of growth factors.

```
hEGF    --NSDSECPLSHDGYCLHDGVCMYIEA---LDKYACNCVVGYIGERCQYRDLKWWELR

mEGF    --NSYPGCPSSYDGYCLNGGVCMHIES---LDSYTCNCVIGYSGDRCQTRDLRWWELR

rEGF    --NSNTGCPPSYDGYCLNGGVCMYVES---VDRYVCNCVIGYIGERCQHRDLR

gpEGF   --QDAPGCPPSHDGYCLHGGVCMHIES---LNTYACNCVIGYVGERCEHQDLDWE

hTGFα   VVSHFNDCPDSHTQFCFH-GTCRFLVQ---EDKPACVCHSGYVGARCEHADLLA

rTGFα   VVSHFNKCPDSHTQYCFH-GTCRFLVQ---EEKPACVCHSGYVGVRCEHADLLA

VVP     DIPAIRLCGPEGDGYCLH-GDCIHARD---IDGMYCRCSHGYTGIRCQHVVLLVDYQRS

SFVP    IVKHVKVCNHDYENYCLNNGTCFTIALDNVSITPFCVCRINYEGSRCQFINLVTY

MVP     IIKRIKLCNDDYKNYCLNNGTCFTVALNNVSLNPFCACHINYVGSRCQFINLITIK

AR           NPCNAEFQNFCIH-GECKYIEH---LEKVTCNCQQEYFGERCEK
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Figure 3.2. The aligned sequences of the EGF family of growth factors showing the conserved residues in bold type. The sequences are, in order, human EGF, mouse EGF, rat EGF, guinea pig EGF, human TGFα, rat TGFα, the vaccinia protein (C-terminal region), the Shope fibroma virus protein (C-terminal region), the myoxoma virus protein (C-terminal region) and amphiregulin.

it stimulated the anchorage-independent growth of fibroblasts in the presence of TGFβ (Twardzik, 1985). The region of the molecule between residues 40 and 91 was shown to display 36% sequence identity with both human and murine EGF, including the alignment of all six Cys residues (Reisner, 1985). The secreted 77 amino acid form, residues 29 - 96 of the primary translation product, was purified and found to stimulate DNA synthesis in quiescent Swiss 3T3 cells and to compete with EGF for receptor binding, indicating that VVGF was able to bind to the EGF receptor (Stroobant et al., 1985).

Shope fibroma virus (SFV) induces benign fibromas in adult rabbits and encodes an 80 amino acid VVGF-related gene product (SVGF) possessing significant homology with EGF and TGFα within its carboxyl-terminal portion (Chang et al., 1987; Ye et al., 1988). A 55 residue peptide, corresponding to the EGF-homologous region, was synthesised and it was determined that the disulphide bond pattern was identical to those found in EGF. The peptide illicited similar biological responses to EGF, including precocious eyelid opening and tooth eruption, but possessed 10% of the activity of EGF in receptor binding and soft agar colony-formation assays (Ye et al., 1988).

Myxoma virus, which causes myxomatosis in rabbits, encodes a 85 amino acid EGF-like peptide displaying 80% homology with the EGF-like domain of SVGF (Upton et al., 1987). Interestingly, malignant rabbit fibroma virus (MRV) which arises as a consequence of recombination between the SFV and Myxoma virus genomes, induces malignant fibromas (Chang et al., 1987). The recombination event inserts the SVGF coding sequence into the Myxoma genome, indicating that this gene product potentially mediates the tumourigenicity of both MRV and SFV.

Infection with SFV or MRV inhibited the proliferation of EGF-responsive cell lines, but not of a nonresponsive cell line, indicating that these viruses directed the synthesis of an EGF antagonist (Strayer and Leibowitz, 1987). Indeed, pretreatment of the cells with EGF prevented these antiproliferative effects. These observations indicated that, in addition to SVGF, a MRV peptide competed with EGF for binding to the EGF receptor. The expression of biologically active EGF-like sequences by the members of the poxvirus family suggested that these peptides may play an important role in the viral life cycle, potentially by providing growth stimulation and proliferation signals for their host epidermal cells.

Amphiregulin (AR), a single chain 78 amino acid peptide of molecular weight 9060 daltons was isolated from the culture medium of a human breast carcinoma cell line treated with PMA (Shoyab et al., 1988). AR is able to inhibit the growth of A431 and other human tumour cell lines and stimulate the proliferation of NRK fibroblasts, monkey kidney cells and human pituitary tumour cells. The amino acid sequence of AR was used to screen a database and it was shown that AR shared extensive sequence homology with the EGF family of growth factors (Shoyab et al., 1989). The alignment of the AR sequence with those of the other EGF family members reveals that AR contains all of the six Cys residues and maintains the strict pattern of Cys residue spacing. In addition, AR possesses several of the other conserved residues. Furthermore, it was demonstrated that AR was able to bind to the EGF receptor albeit with reduced affinity.

The observation that AR and the viral growth factors are able to bind to the EGF receptor, led to the suggestion that they may adopt similar tertiary structures to EGF and TGFα. In addition to the EGF family

of growth factors, domains more distantly related to EGF have been identified in many other proteins, although these EGF-like motifs do not appear to bind to the EGF receptor. The group of polypeptides possessing EGF-like domains and their potential function within these molecules is discussed in Chapter 4. Alignment of the amino acid sequences of the EGF family of growth factors and analysis of the EGF-like domains within proteins, enables the determination of the highly conserved residues. It can be postulated that these residues play an important role in the determination of the three dimensional structure of the EGF family of growth factors.

3.1.2. ^{1}H NMR analysis of the tertiary structure of EGF.

Initial investigations into the tertiary structure of murine EGF, employing the technique of far UV circular dichroism (CD) spectroscopy, suggested the presence of a high proportion of β structure (Taylor et al., 1970). Subsequent CD analysis predicted a 22% β structure with no indication of any α helicity (Holaday et al., 1976). In the last few years, the application of ^{1}H NMR techniques has greatly facilitated investigations into the solution structure of EGF, thereby extending these initial observations. Mayo et al. (1986) proposed the existence of a large β-sheet in murine EGF involving residues 29 - 37 and 46 - 53 as the antiparallel strands. More complete ^{1}H NMR data did not confirm the presence of this large β-sheet, instead, a structure involving two distinct antiparallel β-sheets was proposed (Montelione et al., 1986). A double hairpin backbone conformation was suggested for the highly conserved region Cys^{37} - Leu^{47}, with segment Cys^{33} - Cys^{42} interacting with segment Gln^{43} - Asp^{46}. The presence of an amino terminal β-sheet was indicated involving Gly^{19} - Ile^{23} and Ser^{28} - Cys^{32} as the two antiparallel strands, with the segment Cys^6 - Cys^{14} adopting a multiple-

bend or short irregular helical structure. The β-bend at Ser^{25}/Leu^{26} was classified as type I and that at Ile^{35}/Gly^{36} as type II, the segment Gly^{39} - Gln^{43} adopting a mutiple-bend configuration. Furthermore, it was proposed that the segment Ser^2 - Gly^5 was loosely attached as a third antiparallel strand (Montelione et al., 1987). Kohda et al. (1988) generated ^{1}H NMR data which led them to propose a slightly different tertiary structure for murine EGF, where the amino and carboxyl-terminal domains were more proximal, thereby generating a more globular structure.

Concurrently, similar techniques were being employed to determine the tertiary structure of human EGF (Carver et al., 1986). It was predicted that the segment Gly^{18} - Ile^{23} paired with segment Glu^{28} - Val^{34} in an antiparallel β-sheet. Subsequent investigations revealed the presence of a smaller antiparallel β-sheet involving Tyr^{37}/Ile^{38} and Tyr^{44}/Arg^{45} (Cooke et al., 1987). The most recent model for the tertiary structure of human EGF proposed that the molecule comprised two domains, the N-terminal domain representing residues 1 - 32 and the C-terminal domain residues 33 - 53 (Campbell et al. 1989). According to this model, the N-terminal domain contains a double stranded antiparallel β-sheet linking segment 19 - 23 with segment 28 - 32, with residues 8 - 12 forming an irregular helical loop. The two strands of the main sheet are linked by a turn involving residues 24 - 27 and the second strand leads into the C-terminal domain commencing at about Cys^{33}. The segment 34 - 37 forms a type II β-turn, and a small stretch of antiparallel β-sheet pairs residues 37 and 38 with 44 and 45. A turn involving residues 39 - 43 allows the third disulphide bond to form and the minor β-sheet to close (see Figure 3.3). Following residue 46, the chain is less well defined, although
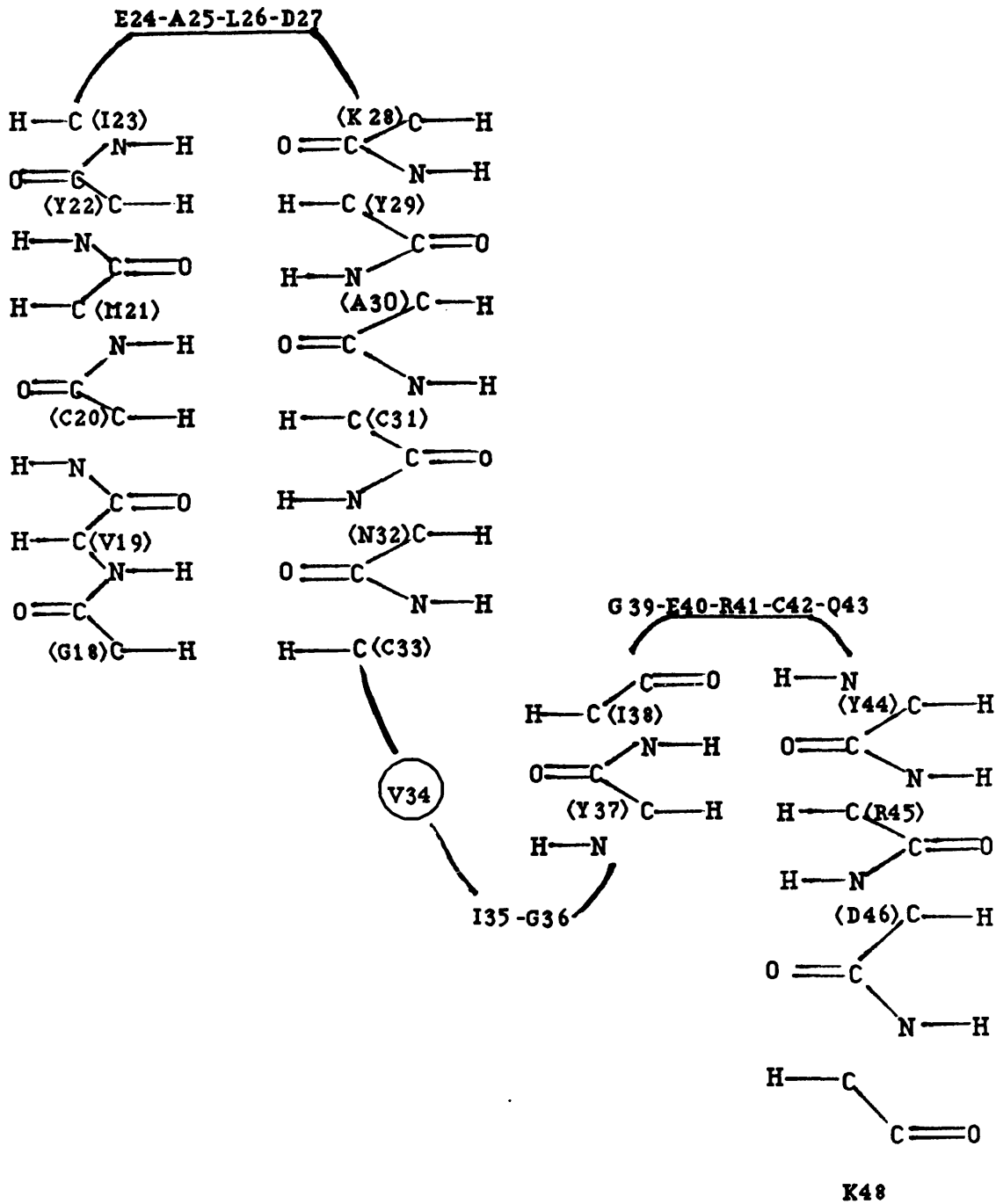
Figure 3.3. Secondary structures in human EGF, planar representation of the antiparallel β sheets.

interactions between 49-50 and 35-37 have been detected.

Recent ^{1}H NMR analysis on the rat form of EGF has determined the presence of two regions with antiparallel β-sheet structure (Mayo et al., 1989). Val^{19} - Val^{23} and Arg^{28} - Asn^{32} form the two antiparallel strands of the first β-sheet with the chain reversal occurring in the segment Glu^{24} - Asp^{27}. A β-turn at Val^{34} - Tyr^{37} leads into the first strand of the short β-sheet. A turn provided by the segment Gly^{39} - Gln^{43} folds the second strand, His^{44} - Arg^{45} antiparallel to the first. The amino terminal 17 residues seem to exist as a multiple-bend conformation folded onto the amino terminal β-sheet.

3.1.3. A model for the tertiary structure of EGF.

Thus ^{1}H NMR analyses of human, murine and rat EGF have led to the proposal of a very similar model for the three dimensional structure of all three EGF species. This model predicts that the backbone of the molecule is arranged in two antiparallel β-sheets with chain reversals occurring at tight β-turns thereby folding the molecule into a compact globular bilobed structure. It has been suggested that the large β-sheet (residues 19 - 32) does not contribute directly to the receptor recognition site, but acts as a scaffold to maintain the binding site in its precise conformation (Campbell et al., 1989). The observation that the β-sheet is extended by ten residues in VVGF without loss of binding capacity correlates with this view (Chang et al., 1987). The demonstration that Met^{21} in EGF can be mutated to a Leu residue without seriously affecting binding, indicates that residue 21 is unlikely to be critical for binding and is more likely to perform a structural role (Sumi et al., 1985). The involvement of residue 21 in the maintenance of

the three-dimensional conformation was demonstrated by the loss of stability (Holaday et al., 1976) and activity of EGF (Anzano et al., 1982) following CNBr cleavage.

This three-dimensional model can be used to predict features of the molecule which are responsible for binding to and activating the EGF receptor. However, more meaningful predictions of the active conformation of EGF can be made when information as to the positions of the highly conserved residues is employed in combination with the three dimensional model (Figure 3.4). It can be assumed that residues involved in receptor binding would be conserved or conservatively changed throughout the EGF-family of growth factors. A total of sixteen such residues have been identified that may be required either to form a suitable conformation (structural) or for direct participation in the recognition of the receptor (functional). It is therefore necessary to distinguish between structural and functional residues, although it must be recognised that the alteration of a structural residue may also effect the maintenance of the receptor-binding face of the molecule or directly interfere with binding. Cys and Gly residues are often structural, their involvement in the establishment of a precise conformation dictating that their substitution would render the molecule inactive. The three-dimensional model of human EGF implicates the Gly residues at positions 18, 36 and 39 in β-turns in the polypeptide backbone (Campbell et al., 1989). Thus, the six Cys and three Gly residues fulfil a structural role. The only other residue that is conserved or conservatively changed throughout the entire family of EGF-like growth factors and EGF-like domains is Tyr^{37}, suggesting a possible structural role rather than an involvement in receptor binding. However, a functional role can also be envisaged for Tyr^{37}

```
hEGF      NSDSECPLSHDGYCLHDGVCMYIEA...LDKYACNCVVGYIGERCQYRDLKWWELR
mEGF      NSYPGCPSSYDGYCLNGGVCMHIES...LDSYTCNCVIGYSGDRCQTRDLRWWELR
rEGF      NSNTGCPPSYDGYCLNGGVCMYVES...VDRYVCNCVIGYIGERCQHRDLR
gEGF      QDAPGCPPSHDGYCLHGGVCMHIES...LNTYACNCVIGYVGERCEHQDLDWE
hTGFαVVSHFNDCPDSHTQFCFH.GTCRFLVQ...EDKPACVCHSGYVGARCEHADLLA
rTGFαVVSHFNKCPDSHTQYCFH.GTCRFLVQ...EEKPACVCHSGYVGVRCEHADLLA

VVP   DIPAIRLCGPEGDGYCLH.GDCIHARD    IDGMYCRCSHGYTGIRCQHVVLLVDYQR
SFVP  IVKHVKVCNHDYENYCLNNGTCFTIALDNVSITPFCVCRINYEGSRCQFINLVTY
MVP   IIKRIKLCNDDYKNYCLNNGTCFTVALNNVSLNPFCACHINYVGSRCQFINLITIK
```

```
hFIX    DGDQCESNPCLNGGSCKDDI..NSYECWCPFGFEGKNCEL
hFX     DGDQCETSPCQNQGKCKDGL..GEYTCTCLEGFEGKNCEL
hFVII   DGDQCASSPCQNGGSCKDQL..QSYICFCLPAFEGRNCET
Notch   DIDECQSNPCLNDGTCHDKI..NGFKCSCALGFTGARCQI
uEGFI   NIDECASAPCQNGGICIDGI..NGYTCSCPLGFSGDNCEN
hFXII   ASQACRTNPCLHGGRCLEVE..GHRLCHCPVGYTGPFCDV      ·
hTPA    PVKSCSEPRCFNGGTCQQALYFSDFVCQCPEGFAGKCCEI
```

```
hLDLR   DIDEC.QDPDT.C.SQL.CVNLE.GGYKCQCEEGFQLDPHTKACKA
hPS     DVDECSLKP.SIC.GTAVCKNIL.GDFECECPEGYRYNLKSKSCED
hTMD    DVDDCILEP.SPC.PQR.CVNTQ.GGFECHCYPNYDLV.DGE.CVE
hPC     SFLNCSLDNGG.C.THY.CLEEV.GWRRCSCAPGYKLGDDLLQCHP
mEGFP   GADPCLYRNGG.C.EHI.CQESL.GTARCLCPEGFVKAWDGKMCLP
hFIX    LDVTCNIKNGR.C.EQF.CKNSADNKVVCSCTEGYRLAENQKSCEP
```

Figure 3.4. (A) Aligned sequences of the EGF-like family of growth factors showing the sixteen conserved amino acids. The sequences are, in order, human EGF, murine EGF, rat EGF, guinea pig EGF, human TGFα, rat TGFα, the vaccinia virus protein (C-terminal region), the Shope fibroma virus protein (C-terminal region) and the myoxoma virus protein (C-terminal region).
(B). Alignment of the EGF-like units of some extracellular multi-domain proteins. The sequences are, in order, human Factor IX (47-84), Factor X (46-83), Factor VII (46-83), Drosophlia *notch* (526-563), sea urchin uEGF-1 (12-49), human Factor XII (155-192), human TPA (82-121), human LDL receptor (333-364), human Protein S (160-202), human thrombomodulin (325-364), human Protein C (94-135), murine preproEGF (748-788) and human Factor IX (84-126).

since ^{1}H NMR analysis indicates it is solvent-accessible (Montelione et al., 1987; Cooke et al., 1987).

The model for the tertiary structure of human EGF predicts that the remaining highly conserved residues, Tyr^{13}, Leu^{15}, His^{16}, Arg^{41}, Gln^{43} and Leu^{47}, lie on the same face of the molecule, consistent with a potential functional role in the establishment of the receptor-recognition site (Campbell et al., 1989). The putative involvement of residues from several regions of the primary sequence in the formation of the receptor binding site is supported by the inability of any synthetic fragments to bind to the receptor or illicit a mitogenic response at physiological concentrations (Komariya et al., 1984; Heath and Merrifield, 1986). Residues 13, 15, 41 and 47 are conserved or conservatively changed in only the EGF family of growth factors and not in the EGF-like domains, consistent with a potential functional role. The conservation of His/Gln^{16} and Glu/Gln^{43} in several of the non-growth factor sequences suggests a structural role for these residues. Indeed, they are located at the interface between the two domains and may be involved in determining their correct relative orientation. The involvement of any of the structurally or functionally important residues can be tested by their mutagenesis to several different residues. Not only can the spatial positioning of important residues be determined, but also the relevance of the nature of their side chains.

Thus, a model for the tertiary solution structure of EGF has been proposed based on the positioning of highly conserved residues and information generated by ^{1}H NMR analysis. This information has been relatively slow to emerge as a result of the difficulty in purifying sufficient quantities of material to allow detailed structural

characterisations. Ultimately, the most precise three-dimensional structure information will be generated by diffraction methods employing crystals of EGF. Attempts to crystallise EGF to date have repeatedly failed, most likely as a consequence of the inability to purify large amounts of biologically active material. EGF is produced at very low concentrations in body fluids, approximately 1 mg can be purified from 1000 litres of urine (Cohen and Carpenter, 1975) and therefore, it has proved necessary to devise methods for the production of recombinant material.

Several systems have been designed to express EGF in *E. coli*, exploiting various signal sequences to ensure secretion of the peptide into the periplasmic space (Oka et al., 1985; Engler et al., 1988; Ray et al., 1988). However, such systems resulted in a low yield of biologically active EGF which was difficult to purify from the cells. In order to circumvent the need to purify EGF from a cell pellet, several groups have developed expression systems in yeast (Urdea et al., 1983; Brake et al., 1984). Again, although the EGF is secreted into the medium, the problem associated with yeast expression systems is one of low yield. In addition, yeast cells release carboxypeptidases into the medium which remove varying numbers of residues from the carboxyl terminus, thereby generating a heterogeneous population of EGF molecules (George-Nascimento et al., 1988). The presence of several forms of EGF within a sample poses problems for biophysical characterisation and potential pharmaceutical applications. There is therefore a real requirement for an expression system which would generate a single, biochemically defined EGF species that could be produced in large quantities and readily purified from the extracellular medium.

3.1.4. Aims of this study.

Only through continued ^{1}H NMR analysis, in association with crystallisation and subsequent diffractional analyses will progress be made towards the complete elucidation of the three-dimensional structure of EGF and the conformation of its receptor binding site. These biophysical techniques require large quantities of pure material. 20 mg is required per ^{1}H NMR analysis and at least 100 mg is necessary to ensure the successful growth of crystals. The generation of such large amounts of EGF has previously been a stumbling block. Furthermore, the production of large amounts of EGF would allow the continuation of CD and spectroscopic analyses on the ligand-EGF receptor complex, thereby investigating the potential conformational changes in the receptor associated with ligand binding (Greenfield et al., 1989). The predicted involvement of key residues in binding to and activating the EGF receptor can be evaluated by the generation of specific mutant peptides. ^{1}NMR analysis of the mutant peptides will discriminate between residues involved directly in ligand-receptor interactions and those responsible for establishing the three-dimensional structure of the binding site. Mutation of residues involved in maintaining the three-dimensional receptor binding conformation will result in gross alteration of the three-dimensional structure of the peptide, leading to the dramatic perturbation of the ^{1}NMR spectrum. In contrast, mutation of residues that interact directly with the receptor will generate only minor conformational changes involving the side chains of the residues proximal to the site of mutation. The aim of this study was therefore to establish a system for the large scale expression of pure native EGF and mutant peptides which will provide the starting material for further biophysical investigations. This system utilises the

insect cell line, Spodoptera frugiperda (Sf9), infected with a recombinant baculovirus carrying a synthetic coding sequence for human EGF.

3.2. RESULTS.

3.2.1. Expression of EGF in insect cells.

The full length cDNA for EGF encodes a 1200 amino acid precursor protein (Bell et al., 1986) from which the mature 53 amino acid EGF peptide is released by proteolytic processing (Taylor et al., 1970) (Figure 3.5). It would be impractical to express the entire 1200 residue precursor protein in the baculovirus system especially since the processing pathway for the generation of the mature EGF peptide would most likely not operate in the insect cells (Frey et al., 1979). It was therefore decided to create the coding sequence for only the mature growth factor. In addition, the coding sequence would need to encode a signal peptide for the efficient export of the EGF molecule into the medium so as to aid the purification of the growth factor. The cDNA sequence for the EGF precursor is 4.87 kb in length with only 159 bases encoding the mature 53 residue peptide (Bell et al., 1986). The intervening DNA sequence between the putative codon for the carboxyl terminus of the signal peptide at Ala^{22} and the codon for the amino terminus of the mature EGF peptide at Asn^{971} comprises 2844 nucleotides. It was anticipated that the process of looping out these intervening nucleotides by *in vitro* mutagenesis would be very inefficient and therefore, a synthetic gene was constructed from oligonucleotides.

Four forms of EGF have been purified from rat submaxillary glands, ranging in length from 44 to 48 residues. These forms of rat EGF
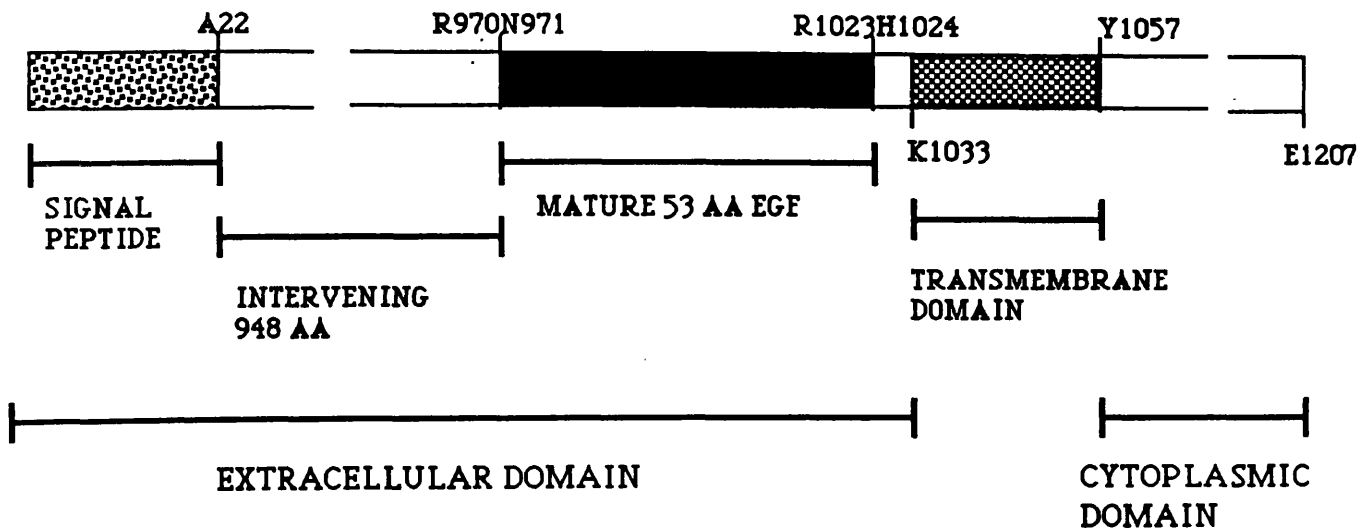
Figure 3.5. The structure of preproEGF highlighting the processing sites for the removal of the signal peptide at A22 and the generation of the mature 53 aa EGF at R970-N971 and R1023-H1024.

lack 5 residues from the carboxyl terminus and up to three from the amino terminus and all bind to the receptor with equal affinity and are equipotent in illiciting a mitogenic response in Swiss 3T3 cells (Simpson et al., 1985). Thus, the shortest EGF molecule retaining full biological activity includes residues 4 to 48 of the 53 amino acid peptide. A sequence encoding the truncated molecule was therefore designed.

The exact site at which the signal peptide is removed from the rest of preproEGF is unknown although it is postulated to occur after Ala^{22}. It was therefore decided to utilise the sequence of a signal peptide which is efficiently removed in the insect cells. By employing such a signal sequence, the problem that the EGF signal peptide may not be removed thereby preventing the secretion of EGF, or that extra amino acids at the amino terminus may somehow prevent EGF interacting with its receptor will be avoided. In insect cells, the IL-2 signal sequence is efficiently recognised allowing the secretion of the biologically active IL-2 molecule into the extracellular medium (Smith et al., 1985). The signal peptide is 23 amino acids in length and therefore, a synthetic DNA sequence encoding a hybrid 76 amino acid peptide was designed from oligonucleotides of varying length. In addition, two stop codons and the restriction sites required for subsequent subcloning into a suitable expression vector were introduced.

3.2.2. Construction of the synthetic gene for EGF.

The assembly of the double-stranded coding sequence employed ten oligonucleotides of between 24 and 51 bases in length (Figure 3.6). Each strand was assembled from five oligonucleotides, each one complementary to the two overlapping oligonucleotides on the opposite

322	323	255	256	257

BamHI ▭▭▭▭ ▬▬▬▬▬ Kpnl

324	325	308	260	261

IL-2
SIGNAL SEQUENCE   HUMAN EGF (4-48)

BamHI ▬▬▬▬▬▬▬▬▬▬▬▬▬ Kpnl

CHIMERIC EGF MOLECULE
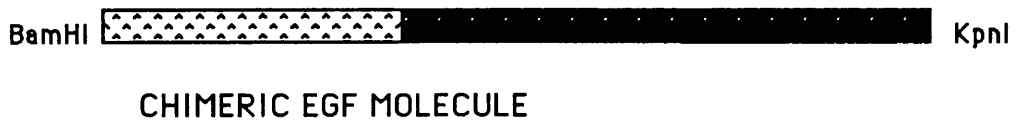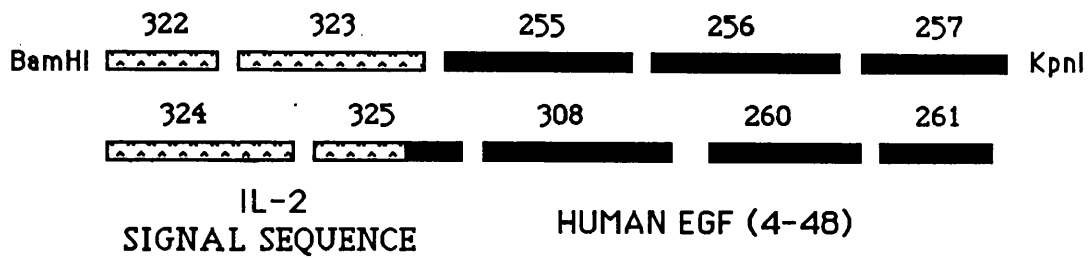
Figure 3.6. The scheme for the construction of the human EGF (4-48)
IL-2 chimeric molecule. The numbers of the oligonucleotides refer to
the text.

▨ IL-2 signal sequence

■ human EGF (4-48)

strand over at least 50% of their length. The baculovirus vector employed was pAC373 which comprises the 7 kb EcoRI fragment of the AcNPV genome, containing the polyhedrin gene, cloned into pUC8 (Summers and Smith, 1983) (Figure 3.7). As a result of Bal31 digestion and the addition of BamHI linkers, the sequence between -8 (8 nucleotides upstream from the ATG and 40 bases downstream from the transcription start site) and the natural BamHI site at +171 is deleted. The EGF coding sequence was introduced into pAC373 using the BamHI restriction site at the 5' end of the sequence and the KpnI site at the 3' end. A 5' BamH1 single-stranded overhang and a 3' Kpn1 single-stranded overhang were therefore created.

The following oligonucleotides were synthesised:

322   29mer

GATCCATGTACAGGATGCAACTCCTGTCT

323   45mer

TGCATTGCACTAAGTCTTGCACTTGTCACAAACAGTGCACCTACT

324   45mer

ATCGTGGGACAGGGGACATTCAGAAGTAGGTGCACTGTTTGTGAC

325   49mer

AAGTGCAAGACTTAGTGCAATGCAAGACAGGAGTTGCATCCTGTACATG

255   51mer

TCTGAATGTCCCCTGTCCCACGATGGGTACTGCCTCCATGATGGTGTGTGC

117

256 51mer

ATGTATTGAAGCATTGGACAAGTATGCATGCAACTGTGTTGTTGGCTAC

257 44mer

ATCGGGGAGCGATGTCAGTACCGAGACCTGAAGTGATGAGGTAC

308 51mer

CTTGTCCAATGCTTCAATATACATGCACACACCATCATGGAGGCAGTACCC

260 42mer

ACATCGCTCCCCGATGTAGCCAACAACACAGTTGCATGCATA

261 25mer

CTCATCACTTCAGGTCTCGGTACTG

The oligonucleotides were assembled into the double-stranded molecule according to the scheme outlined in Figure 3.6.

Approximately 20 µg of each oligonucleotide was applied to a 15% (w/v) polyacrylamide gel and visualised by UV shadowing against a thin-layer chromatography plate. The bands containing the oligonucleotides were excised from the gel and the DNA recovered from the acrylamide. 100 ng of each oligonucleotide was end-labelled using T_4 kinase and 100 µCi [γ-^{32}P] ATP (10mCi/ml, Amersham) and analysed on a 15% (w/v) polyacrylamide gel in order to ascertain their relative size and purity (Figure 3.8). The oligonucleotides are synthesised without a 5' phosphate group and in order to act as a substrate for T_4 DNA ligase, a 5' phosphate group must be added by T_4 kinase. The only oligonucleotides that require a 5' phosphate group are the internal

Figure 3.7. Restriction endonuclease map of the transfer vector pAc373 (Smith et al., 1985). The plasmid was derived from a plasmid which comprised a 7 kb EcoRI fragment containing the AcNPV polyhedrin gene cloned into the EcoRI-HindIII fragment of pUC8. As a result of Bal31 mutagenesis and the addition of BamHI linkers, pAC373 contains a deletion of the sequence between -8 (8 bases upstream from the polyhedrin ATG and approximately 40 bases downstream from the polyhedrin transcription start site) and the natural BamHI site at nucleotide +171. The plasmid also contains the polyhedrin polyadenylation signal and the ampicillin resistance gene and origin of replication for selection and propagation in *E. coli*.

pAc373
9.65 kb

HindIII
BstEII
PvuII
XhoI
SalI
SalI
EcoRV
BamHI
HindIII
KpnI
polyhedrin
promoter
HindIII
HindIII
PvuII
EcoRI
Amp
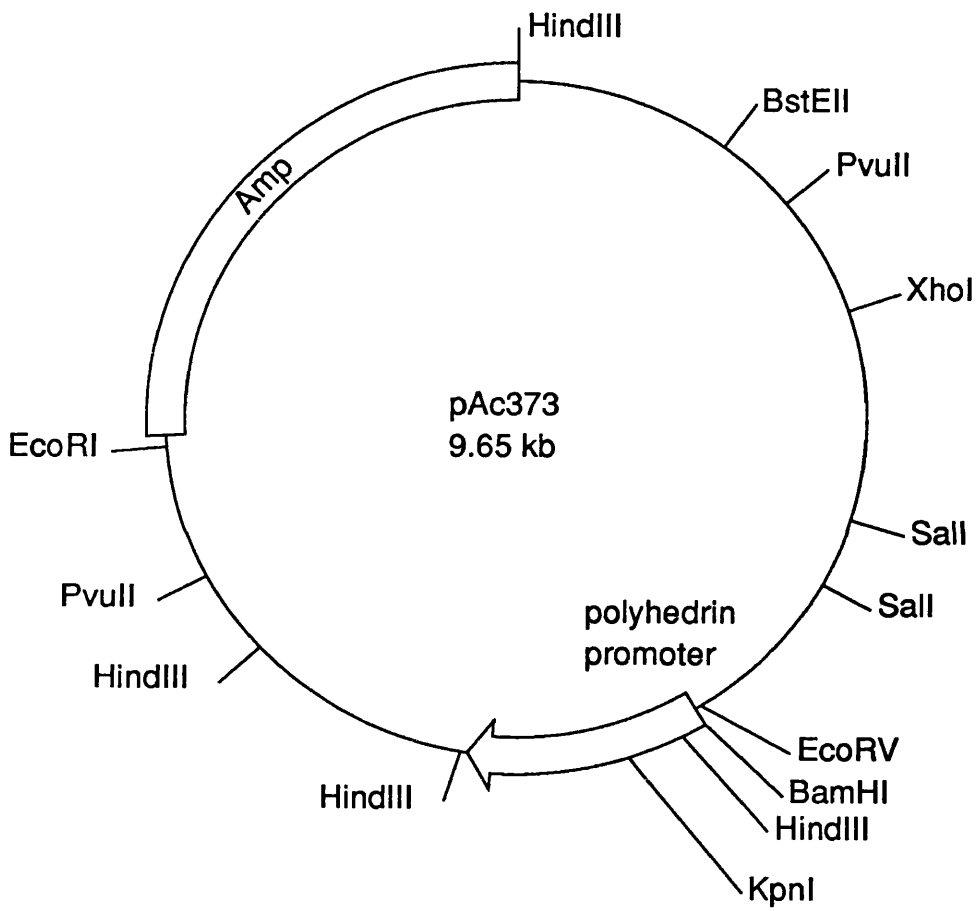
Figure 3.8.   100 ng of each of the oligonucleotides were 5' end labelled by the incorporation of [γ-^{32}P] ATP (specific activity 10 mCi/ml, Amersham) by the action of T$_4$ kinase and analysed by 15% (w/v) PAGE.   The gel was exposed to Hyperfilm (Amersham) for 10 min at room temperature.

Lane 1.   255   51 mer.

Lane 2.   256   51 mer.

Lane 3.   257   44 mer.

Lane 4.   258   24 mer.

Lane 5.   259 (308)   51 mer.

Lane 6.   260   42 mer.

Lane 7.   261   25 mer.
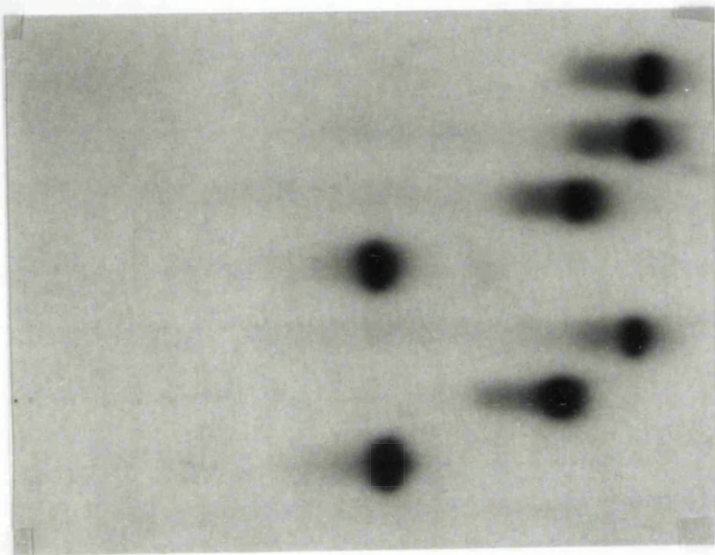
+ 255
+ 256
+ 257
+ 258
+ 259
+ 260
+ 261

oligonucleotides that is, 323, 255, 256 and 257 on the coding strand and 260, 308, 324 and 325 on the non-coding strand. The 5' phosphate groups required for ligating the synthetic gene with the vector will be supplied by the vector and therefore, the oligonucleotides 322 and 261 do not need to be kinased.

100 pM of 322 and 261 were each resuspended in 20 μl of water. 100 pM of each of the other eight oligonucleotides were kinased individually by resuspending the DNA in 7 μl of water and adding the following reagents:

10 mM ATP	4 μl
0.5 M Tris-HCl (pH7.6)	2 μl
0.1 M MgCl$_2$	2 μl
1 mM Spermidine	2 μl
50 mM DTT	2 μl
T$_4$ Kinase	1 μl

The reaction mix was incubated at 37°C for 30 min and then at 75°C for 20 min to inactivate the enzyme. 8 μl aliquots of each of a pair of overlapping oligonucleotides (322 + 324, 323 + 325, 255 + 308, 256 + 260 and 257 + 261) were annealled by incubation at 90°C for 5 min followed by slow cooling back to room temperature. 5 μl aliquots of each of the annealled pairs of oligonucleotides were mixed, incubated at 50°C for 5min and then slow cooled back to room remperature. The oligonucleotides were ligated together by the addition of 3.5 μl of 10 x Ligation buffer (0.25 M Tris-HCl pH7.6, 0.2 M DTT, 50 mM MgCl$_2$), 3.5 μl of

10 mM ATP and 2 µl of T4 DNA ligase (1u/µl) and incubation at 16°C overnight.

3.2.3. Generation of SK.EGF.

The synthetic EGF gene was initially subcloned into the Bluescript vector SK$^+$ (Stratagene). The Bluescript vectors have large polylinkers containing the restriction sites for several common enzymes, thereby facilitating the subcloning of restriction fragments. In addition, the polylinker contains many of the restriction sites present in the M13 vectors so restriction fragments can be readily moved between phage and plasmid vectors. The polylinker is present in the N-terminal portion of a LacZ gene fragment and therefore bacterial strains, such as XL-1 blue transformed with Bluescript plasmids will be able to metabolise X-gal. In the presence of IPTG, these transformants will grow as blue colonies. However, if the LacZ gene is interrupted by the presence of a cloned DNA fragment, the resultant plasmids will direct the growth of white colonies. The blue/white colour selection therefore greatly facilitates the identification of recombinants. 5 µg of SK$^+$ DNA was digested with KpnI for 60 min at which time, 0.1 x volume sample was analysed on an agarose gel to check that the KpnI digestion had gone to completion. The salt concentration was then adjusted for digestion with BamHI. The vector fragment was electroeluted and the DNA phenol: chloroform extracted, ethanol precipitated and resuspended in 20 µl of TE. 0.5 µl of the purified SK$^+$ DNA was mixed with 15 µl of the synthetic gene and ligated overnight in a ligation volume of 30 µl. The ligation mix was used to transform an aliquot of frozen competent XL-1 blue, X-gal and IPTG added and the transformation mix plated onto a nitrocellulose filter on a L-agar plate supplemented with ampicillin. All the resultant colonies were blue indicating that the Lac

Z gene had not been inactivated by the subcloning since the 220 bp EGF sequence had inserted inframe and allowed read through into the Lac Z gene. It was therefore necessary to screen the colonies with a radioactively labelled EGF probe.

3.2.4. Radioactively screening colonies.

A replica of the master filter was taken and incubated at $37^\circ C$ for 4 h until the colonies were visible (the master filter was also regenerated). The colonies on the replica filter were lysed by NaOH treatment and the DNA irreversibly bound to the filter. The temperature at which hybridisation was performed (Td) was determined from the base composition of the oligonucleotide and by applying the formula:

$$Td = 4^\circ C \text{ per GC pair } + 2^\circ C \text{ per AT pair}$$

where the number of GC and AT pairs is 13 and 12 respectively. The filter was therefore prehybridised in 6 x SSC, 10 x Denhardt's at $76^\circ C$ for at least 60 min, and hybridised overnight with the end-labelled 261 oligonucleotide. The filter was washed to remove non-specifically bound probe in 6 x SSC, 0.1% (w/v) SDS at room temperature for 10 min followed by a 2 min wash at a temperature $5\text{-}10^\circ C$ below the Td. The filter was exposed to Hyperfilm (Amersham) and the hybridising colonies identified on the master filter (Figure 3.9). All of the colonies on the master hybridised to the EGF-specific probe, suggesting that the subcloning reaction had been extremely successful. Fifteen colonies were picked and plasmid DNA prepared according to the minipreperation method. An aliquot of the DNA was digested with KpnI and then BamHI, treated with RNase 1 "A" and the samples analysed on

123

Figure 3.9. XL-1 blue transformed with the SK.EGF ligation mix were plated onto a nitrocellulose filter on a L-agar plate supplemented with ampicillin. The colonies were allowed to develop by incubation at 37°C overnight and a replica filter generated. The replica and master filters were incubated at 37°C for 4 h at which time, the colonies on the replica filter were lysed, the DNA denatured by NaOH treatment and irreversibly bound to the filter by baking, under vacuum, at 80°C for 2h. The filter was prehybridised in 6 x SSC, 10 x Denhardt's solution for 4 h at a hybridisation temperature (T_d) of 76°C. The oligonucleotide 261 was 5'-end labelled by the incorporation of [γ-^{32}P] ATP (specific activity 10 mCi/ml, Amersham) by the action of T_4 kinase and unincorporated radionucleotide removed by centrifugation through a Sephadex G25 column. Hybridisation was performed at 76°C overnight, at which time, the filter was washed in 6 x SSC, 0.1% (w/v) SDS for 10 min at room temperature and then for 2 min at 66°C. The filter was then exposed to Hyperfilm (Amersham) for 30 min at -80°C.
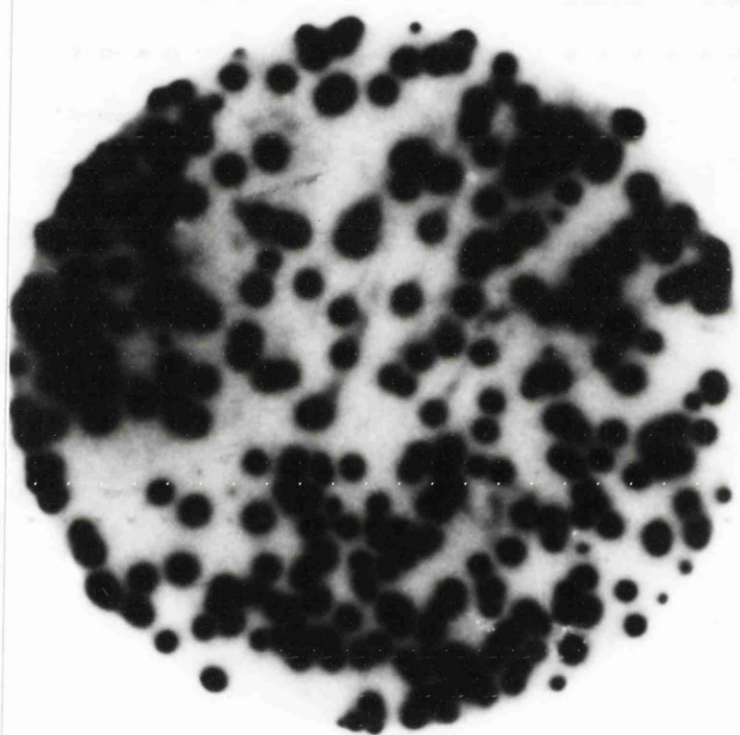
an agarose gel to determine the presence of the 220 bp EGF insert. Ten of the 15 samples contained the EGF coding sequence and the DNA from one, designated SK.EGF, was prepared according to the plasmid maxipreperation method. The EGF coding sequence was 'shotgun' cloned following BamHI and KpnI digestion into Mp19 in order to ascertain that the sequence was correct throughout its entire length. Twenty-four plaques were picked, single-stranded DNA prepared and sequenced initially by performing the technique of 'T-tracking'. In this technique, which is a quick and accurate way of determining an alteration in the DNA sequence, the synthesised DNA strand is labelled, extended and terminated in only the T reaction sequence. A potential subclone, designated Mp19.EGF, was then fully sequenced and the EGF coding sequence determined to be correct (Figure 3.10).

3.2.5. Generation of pAC.EGF.

5 μg of pAC373 DNA was digested with KpnI and then BamHI and then treated with calf intestinal phosphatase (CIP) to remove the 5' phosphate groups. T4 DNA ligase requires a 5' phosphate group to join DNA molecules and will therefore be unable to utilise the 5' dephosphorylated vector. Thus, the phosphatasing reaction reduces the background of recircularised plasmid, generated as a result of incomplete digestion, in the subsequent ligation reaction. The vector fragment was purified by electroelution, phenol: chloroform extracted, ethanol precipitated and resuspended in 20 μl of TE. 5 μg of SK.EGF was digested and purified in the same way as the vector DNA and resuspended in 20 μl of TE. 5 μl of the EGF insert was ligated overnight with 0.5 μl of the purified plasmid, 0.25 x volume of the ligation mix used to transform an aliquot of frozen competent XL-1 blue, and the

Figure 3.10. M13 dideoxy sequencing of Mp18 and Mp19.EGF. The first set of four lanes represents Mp18.EGF labelled in G, A, T and C respectively, showing the sequences of 322, 323, 255 and 256. The second set of four lanes represents Mp19.EGF labelled in C, T, A and G respectively, showing the sequence of 257.
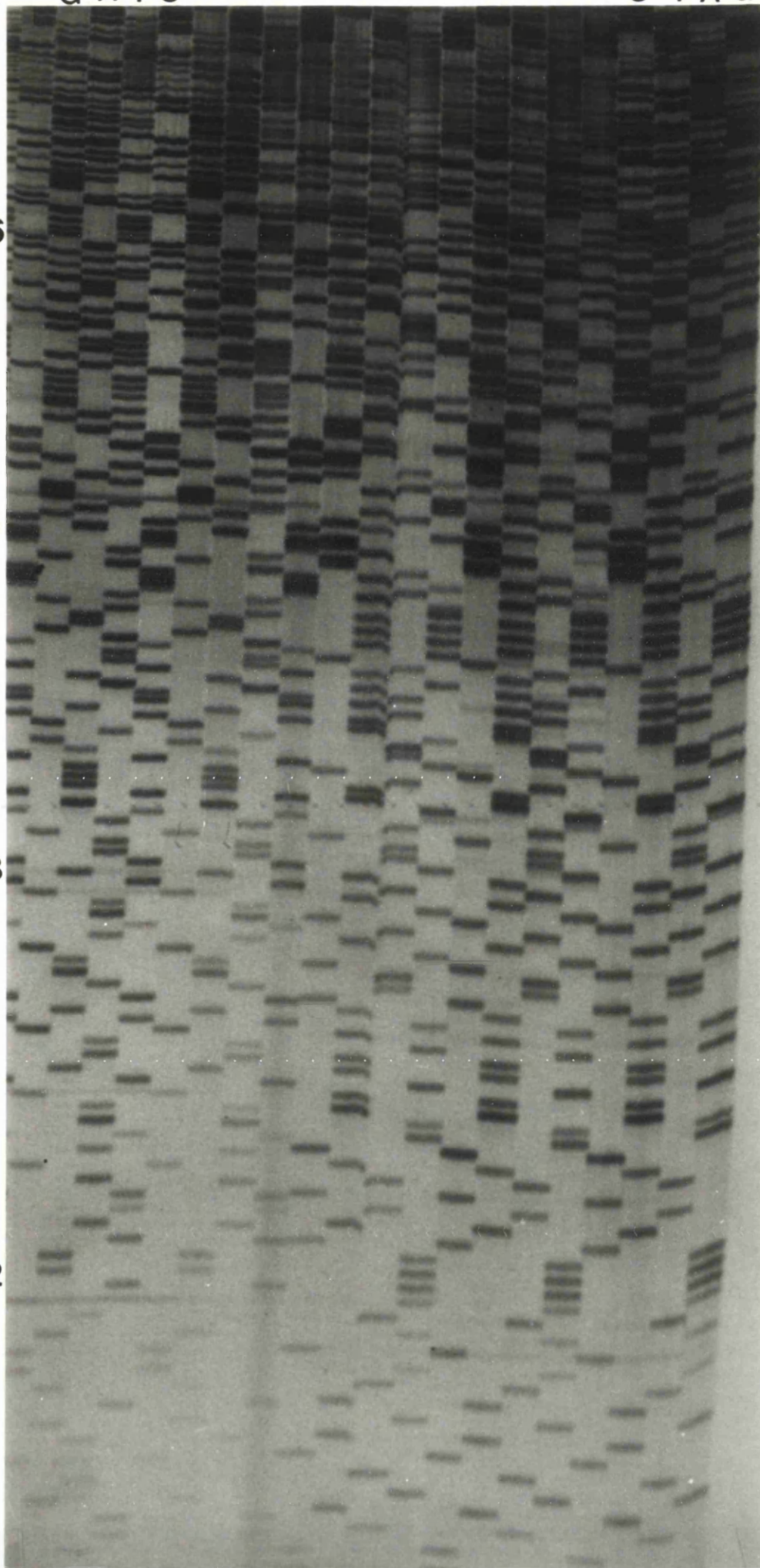
transformation mix plated to a L-agar plate supplemented with ampicillin. Plasmid DNA was prepared according to the minipreperation method and an aliquot digested with KpnI and then BamHI. Following RNase 1 "A" treatment, the samples were analysed by 0.8% (w/v) agarose gel electrophoresis. Six of the plasmid preperations contained the 220 bp EGF insert and the DNA of one designated pAC.EGF, was prepared by the plasmid maxipreperation method.

3.3. Transfection of Sf9 cells with pAC.EGF.

The pAC.EGF DNA was cotransfected into Sf9 cells with AcNPV DNA. After 4-6 days, the cultures were examined for positive signs of infection including the appearence of polyhedra, an increase in cell size and cell lysis. Extracellular virus particles were harvested and plated on fresh monolayers and the recombinant virus identified by plaque hybridisation. The filters were hybridised with the synthetic EGF gene labelled to a high specific activity by random priming. The filters were washed to a stringency of 0.1 x SSC, 0.1% (w/v) SDS at 65°C, to remove the nonspecifically bound probe and exposed to Hyperfilm (Amersham) (Figure 3.11). Areas of positive hybridisation on the film were determined and the corresponding areas of agar identified. Four positives were picked from each plate and two utilised for the subsequent round of screening. After three rounds of plaque hybridisation, the percentage of recombinant virus relative to wildtype was high enough to allow visual screening for occlusion negative plaques. Several potential recombinant viruses were picked and a further round of visual screening performed so as to validate the observation and to ensure no wild type virus was present.

Figure 3.11. Plaque hybridisation of recombinant EGF virus particles. The viral particles were lysed and the denatured DNA irreversibly bound to the filter by NaOH treatment followed by baking, under vacuum, at 80°C for 2 h. The filters were prehybridised in prehybridisation buffer (50% (v/v) formamide, 5 x SSC, 5 x Denhardt's solution, 5 mM $NaPO_4$ pH 6.8, 1 mM sodium pyrophosphate, 0.1 mM ATP, 0.1% (w/v) SDS, 0.1 mg/ml sonicated salmon sperm DNA) at 42°C for 4 h. The synthetic EGF gene was labelled with [$\alpha-^{32}P$] dCTP (specific activity 10 mCi/ml, Amersham) by the action of DNA Polymerase 1. Unincorporated radionucleotide was removed by centrifugation through a Sephadex G-50 column. The filters were hybridised at 42°C overnight at which time, non-specifically bound probe was removed by washing at 65°C to a stringency of 0.1 x SSC, 0.1% (w/v) SDS. The filters were then exposed to Hyperfilm (Amersham) overnight at- 80°C.
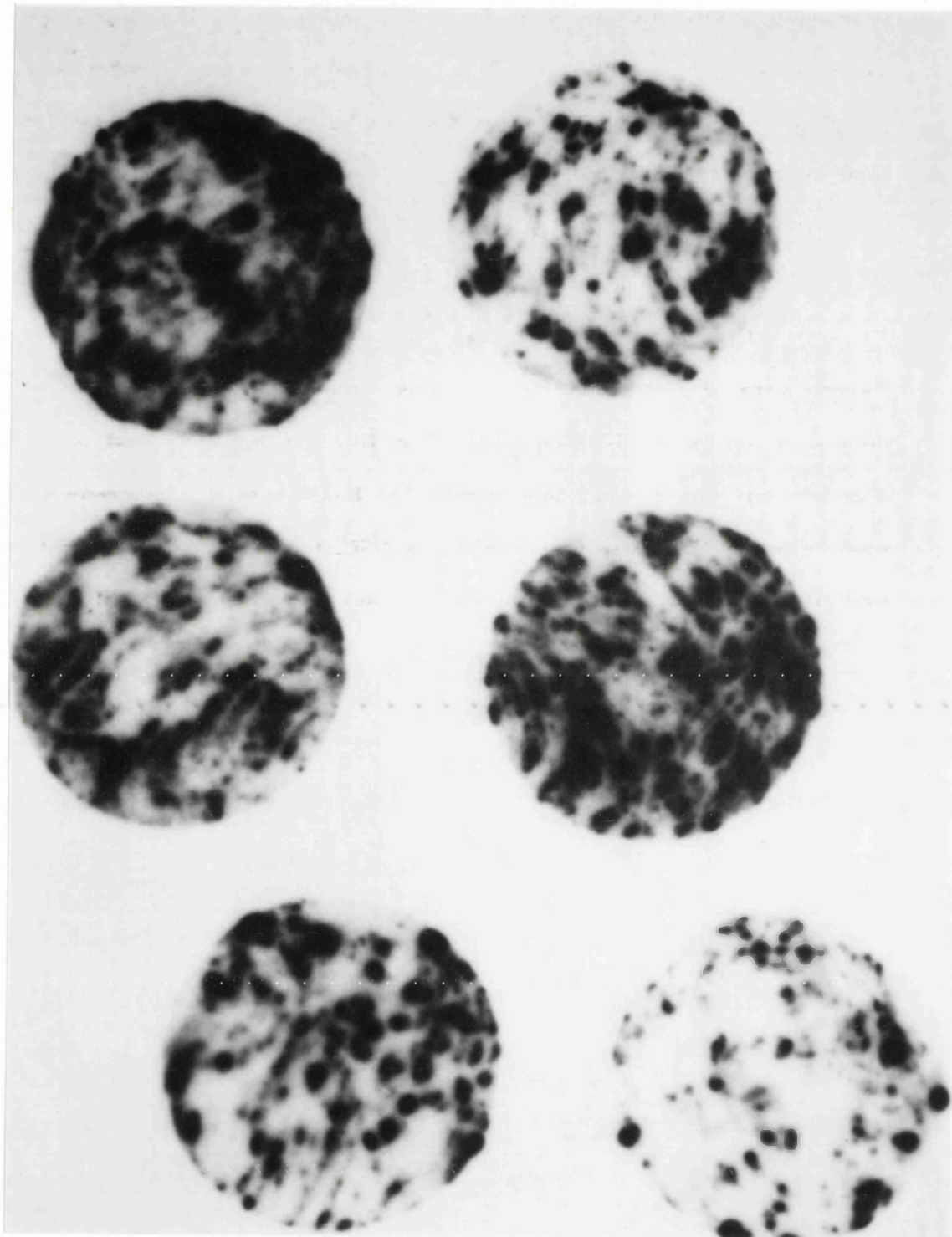
3.4. The expression of EGF (EGF$_i$) in insect cells.

Not all recombination events are equivalent and therefore, different recombinants can express the product at different levels (Summers and Smith, 1987). For this reason, several potential recombinants were initially assessed for their ability to direct the synthesis of EGF$_i$.

2.5×10^6 Sf9 cells were infected with each of the recombinant viruses and after 72 h, the cells were assessed for EGF$_i$ expression by [^{35}S]Cys labelling. The lysates and medium were immunoprecipitated with an anti-EGF monoclonal antibody (Oncogene Sci Inc) and analysed by 15% (w/v) SDS-PAGE. The gel was fixed in destain for 20 min followed by 20 min in Amplify (Amersham), to intensify the ^{35}S signal upon autoradiography. Figure 3.12 indicates that all the recombinants express a small (< 6 kD) peptide that is specifically recognised by an anti-EGF monoclonal antibody. No such peptide can be immunoprecipitated from wild type infected cells. The cells are able to secrete the EGF$_i$ into the medium, indicating that the IL-2 signal peptide has been correctly recognised and processed. Furthermore, the peptide can be immunoprecipitated from the cell lysates indicating that not all of the EGF is exported from the cells. From these results, it is difficult to determine the exact molecular weight of the EGF$_i$ since low molecular weight peptides migrate as diffuse bands on analysis by high percentage SDS-PAGE. It is also difficult to predict how much EGF$_i$ is expressed since low molecular weight peptides readily diffuse out of the gel upon fixing and amplification.

The production of EGF$_i$ was monitored during the post-infection period from 48-96 h. Aliquots of 2.5×10^6 Sf9 cells were infected with

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Figure 3.12. 2.5×10^6 Sf9 cells were infected with different EGF_i viruses and labelled with $[^{35}S]Cys$ (specific activity 1300 mCi/ml, Amersham) at 72 h post infection. The medium and lysates were immunoprecipitated with the anti-EGF monoclonal antibody (Oncogene Sci Inc) and analysed by 15% (w/v) SDS-PAGE. The gel was fixed, 'amplified' (Amersham), dried and exposed to Hyperfilm (Amersham) for 3 days at $-80^{\circ}C$.

Lane 1.   wild type infected lysate.

Lane 2. EGF_i1 infected medium.

Lane 3. EGF_i1 infected lysate.

Lane 4. EGF_i2 infected medium.

Lane 5. EGF_i2 infected lysate.

Lane 6. EGF_i3 infected medium.

Lane 7. EGF_i3 infected lysate.

Lane 8. EGF_i4 infected medium.

Lane 9. EGF_i4 infected lysate.

Lane 10. EGF_i5 infected medium.

Lane 11. EGF_i5 infected lysate.
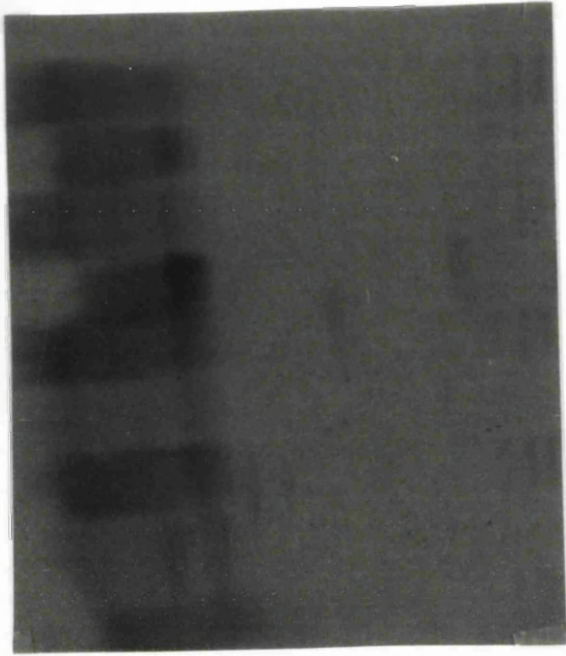
+ WILD TYPE

+ EGF 1 MEDIUM

+ EGF 1 LYSATE

+ EGF 2 MEDIUM

+ EGF 2 LYSATE

+ EGF 3 MEDIUM

+ EGF 3 LYSATE

+ EGF 4 MEDIUM

+ EGF 4 LYSATE

+ EGF 5 MEDIUM

+ EGF 5 LYSATE

EGF

the EGF$_i$ virus and [^{35}S]Cys labelled at 48, 72 and 96 h post infection. The lysates and medium were immunoprecipitated with the anti-EGF monoclonal antibody and analysed by Tricine-SDS-PAGE according to Schagger and von Jagow (1987) (Data not shown). The results indicated that EGF$_i$, with a molecular weight of approximately 5 kD, was apparent in the lysates by 48 h post infection and that it could be immunoprecipitated from the medium by 72 h post infection.

3.5. The Receptor binding activity of EGF$_i$.

Although it has proved possible to immunoprecipitate EGF$_i$ from the lysates and medium of cells infected with the EGF$_i$ virus, it is necessary to determine whether the EGF$_i$ has been folded into its active three-dimensional conformation. The ability of EGF$_i$ to bind to the EGF receptor was assessed in an [^{125}I]EGF competition assay, where a constant concentration of [^{125}I]EGF was present and increasing amounts of EGF$_i$-conditioned medium added. The parameters of ligand binding are most frequently derived by transforming data into the form of a Scatchard plot (Scatchard, 1949). The analysis of high affinity binding sites in cells that overexpress the EGF receptor is compromised by the fact that at low concentrations of EGF where high affinity interactions are favoured, the amount of bound EGF is greater than 50% of the total EGF. This violates the conditions of Scatchard analysis and means that the calculation of the concentration of free ligand is subject to considerable error. To avoid this problem, the NR6+ fibroblast line, which has been transfected with the full length human EGF receptor, (DiFiore et al., 1987a) and express approximately 5×10^5 receptors per cell was used in preference to A431 cells which express approximately 2.5×10^6 receptors per cell (Haigler et al., 1978). 1.0×10^7 Sf9 cells were

infected with the EGF_i virus under serum-free conditions, and the medium harvested 72 h post infection. The extracellular virus particles were removed by ultracentrifugation at 100,000 g for 30 min, and the pH adjusted to 7.4. The EGF standards were prepared in duplicate in IPL41 medium, 0.1% (w/v) BSA (adjusted to pH 7.4), as were the serial dilutions of the EGF_i-conditioned medium. The results in Figure 3.13 indicate that the EGF_i-conditioned medium can compete with $[^{125}I]EGF$ for binding to the EGF receptor expressed on NR6+ fibroblasts. Since all samples were prepared in IPL41 medium, the effect of the EGF_i present in the conditioned medium is in excess of the medium alone. In addition, since the insect cells were grown in the absence of serum, the receptor binding capacity cannot be attributed to the growth factors present in the serum. By comparison with the EGF standard curve, it can be determined that EGF_i is produced at approximately 100 ng/ml of medium. These results directly confirm those of Simpson et al. (1985) in that the deletion of 3 and 5 residues from the amino and carboxyl termini respectively does not render the molecule unable to bind to the EGF receptor.

In an attempt to further characterise the EGF_i, 500 ml of serum-free medium containing EGF_i was prepared and partially purified. 2.0×10^7 Sf9 cells were seeded into 50 ml of serum-free IPL41 in each of ten 500 ml flasks and infected with EGF_i virus. After 72 h, the cells were removed by centifugation at 7600g for 20 min, (the medium was frozen in 100 ml aliquots prior to further purification). The pH of the medium was adjusted to pH 1.5 by the addition of concentrated HCl and ultracentrifuged at 100,000g for 30 min to remove any particulate matter. 50 ml of the acidifed medium was applied to a C_{18} Sephak cartridge (Waters) according to the manufacturer's instructions, the

Figure 3.13.   1.0 x 10^7 Sf9 cells were infected with the EGF$_i$ virus under serum-free conditions and harvested 72 h post infection.   The extracellular virus particles were removed by ultracentrifugation at 100,000g for 30 min, the pH adjusted to 7.4 and BSA added to 0.1% (w/v). 2.0 x 10^4 NR6+ fibroblasts were plated in DMEM, 10% (v/v) FCS to 24 well plates and grown for 2-3 days until just subconfluent.   5 x 10^4 cpm [^{125}I] EGF (specific activity of 100 μCi/μg, Amersham) was added to each well and EGF standards, prepared in PBS, 0.1% (w/v) BSA, or serial dilutions of EGF$_i$ medium dispensed in duplicate.   The cells were incubated at 4°C for 4 h and then lysed in 0.5 M NaOH, 0.1% (w/v) SDS.   The lysates were transferred to scintillation vials and counted on the γ channel of a scintillation counter for 1 min.   The mean of duplicate values for [^{125}I]EGF bound was plotted against nM EGF or serial dilution.

Panel A.   EGF standard curve using known concentrations of a human EGF standard.

Column 1.   0.2 nM EGF.

Column 2.   1 nM EGF.

Column 3.   2 nM EGF.

Column 4.   5 nM EGF.

Column 5.   10 nM EGF.

Panel B.   Serial dilutions of the EGF$_i$-conditioned medium.

Column 1.   undiluted.

Column 2.   serial dilution 1.

Column 3.   serial dilution 2.

Column 4.   serual dilution 3.

Column 5.   serial dilution 4.

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EGF standard curve

cpm bound

10000
8000
6000
4000
2000
0

0.2   1   2   5   10

nM EGF

EGF medium

cpm bound

8000
6000
4000
2000
0

1   1:2   1:2   1:2   1:2

serial dilution
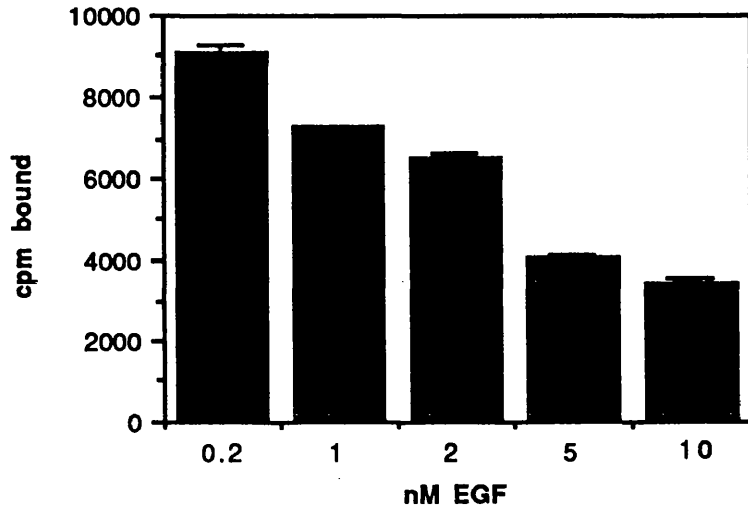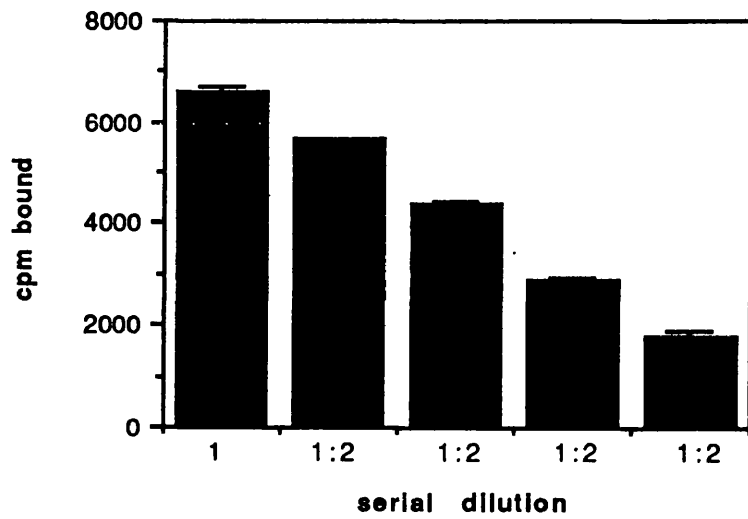
eluate applied to a second cartridge incase the capacity of the first had been exhausted. The EGF_i was eluted with 60% acetonitrile and lyophilised. An EGF_i stock solution was prepared in 100 mM HCl at a concentration of approximately 100 ng/μl. The $[^{125}I]EGF$ competition assay was repeated with varying concentrations of EGF_i against a more extensive standard curve. Figure 3.14 indicates that the concentrated EGF_i-conditioned medium competes with $[^{125}I]EGF$ for binding to the EGF receptor in a concentration-dependent manner.

3.6. The mitogenic capacity of EGF_i.

The ability of the EGF_i to bind to the EGF receptor has been indicated by the results of the $[^{125}I]EGF$ competition assay. However, the biological activity of the EGF_i must be assessed by its ability to stimulate DNA synthesis in quiescent cells, as measured by the uptake of $[^3H]$Thymidine. The NR6+ fibroblast cell line was used inconjunction with the control line of NR6 fibroblasts, which do not express the EGF receptor (Pruss and Herschman, 1977). The analysis was performed in duplicate, with EGF standards at concentrations of 0.5 nM and 100 nM and 1 μl samples of undiluted and 1/10 and 1/100 dilutions of the concentrated EGF. The validity of the assay was determined by the maximal response of the cells to 10% (v/v) FCS and the nonresponsiveness of cells grown in the absence of EGF. The mitogenic capacity of concentrated medium, conditioned by uninfected Sf9 cells, was also measured. In order to ascertain whether the Sf9 cells produced any growth factor-like activities that would act synergistically with the EGF_i, 5 nM standard EGF and an aliquot of the unconditioned medium were analysed together. The results in Figure 3.15 indicate that EGF_i is able to induce a mitogenic response in NR6+ fibroblasts. This response

Figure 3.14. 2.0×10^7 Sf9 cells were seeded in 50 ml of serum-free IPL41 into each of ten 500 ml flasks and infected with the EGF_i virus. At 72 h post infection, the cells were removed by centrifugation at 7600g for 20 min. The pH was adjusted to 1.5 and the particulate matter removed by ultracentrifugation at 100,000g for 30 min. 50 ml of the acidified medium was applied to a C_{18} Sephak cartidge (Waters) and the EGF eluted with 60% (v/v) acetonitrile. The EGF was lyophilised and a stock solution prepared in 100 mM HCl at a concentration of approximately 100 ng/μl. The $[^{125}I]EGF$ competition assay was repeated with known concentrations of the human EGF standard and various concentrations of the concentrated EGF_i. The mean of duplicate values for $[^{125}I]EGF$ bound was plotted against nM standard EGF and nM EGF_i.

Panel A. EGF standard curve.

Column 1.   0 nM EGF.

Column 2.   0.2 nM EGF.

Column 3.   1 nM EGF.

Column 4.   2 nM EGF.

Column 5.   5 nM EGF.

Column 6.   10 nM EGF.

Panel B. EGF_i.

Column 1.   1 nM EGF_i.

Column 2.   2 nM EGF_i.

Column 3.   5 nM EGF_i.

Column 4.   10 nM EGF_i.

Column 5.   20 nM EGF_i.

EGF standard curve

Concentrated EGF

Figure 3.15. 2 x 10^4 NR6 or NR6+ fibroblasts were plated to 24 well plates in DMEM, 10% (v/v) FCS and incubated for 2-3 days until confluent. The cells were then made quiescent by incubation for 5 days at 37°C in starving medium (DMEM supplemented with 10% (v/v) original medium). The EGF standards and test samples were added in duplicate and the cells incubated for a further 18 h at which time, 1 μCi [^{3}H]Thymidine (specific activity 5 Ci/mmol, Amersham) was added to each well. The incubation was extended for a further 4 h, the cells lysed in 0.5 M NaOH and the DNA precipitated by the addition of 2 x volume 10% (w/v) TCA and incubation at 4°C overnight. The DNA was immobilised on glass scintered filters and counted on the [^{3}H] programme of the scintillation counter. The mean of the values for [^{3}H]Thymidine incorporation was plotted against the test sample as shown.

Panel A.   NR6+ fibroblasts.

Column 1.   0 nM EGF standard.

Column 2.   0.5 nM EGF standard.

Column 3.   100 nM EGF standard.

Column 4.   10% (v/v) FCS.

Column 5.   1 μl concentrated uninfected medium.

Column 6.   1 μl concentrated uninfected medium plus 5 nM EGF.

Column 7.   1 μl undiluted concentrated EGF$_i$.

Column 8.   1 μl 1/10 dilution concentrated EGF$_i$.

Column 9.   1 μl 1/100 dilution concentrated EGF$_i$.

Column 10.   1 μl undiluted concentrated TGFα_i.

Column 11.   1 μl 1/10 dliution concentrated TGFα_i.

Column 12.   1 μl 1/100 dilution concentrated TGFα_i.

Panel B. As above but with NR6 fibroblasts.

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NR6+ THYMIDINE INCORPORATION

NR6 THYMIDINE INCORPORATION

is due to EGF_i binding to and activating the EGF receptor as indicated by the lack of response to EGF of the non-receptor expressing NR6 line. Furthermore, the observation that the concentrated medium conditioned by uninfected cells was unable to induce [^{3}H]Thymidine uptake indicates that the positive mitogenic response to the EGF_i conditioned medium was due to the presence of EGF and not some component of the medium. The inability of the conditioned medium from uninfected cells to enhance the effect of the EGF standard sample demonstrates that the effect seen with EGF_i is due to EGF alone and not due to any cooperativity with any growth factor-like activity associated with uninfected Sf9 cells.

3.7. EGF_i activation of the EGF receptor protein-tyrosine kinase.

3.7.1. *In vitro* kinase assay with A431 membranes.

The ability of EGF_i to bind to the EGF receptor and induce mitogenesis in fibroblasts has been indicated by the results of the previous experiments. The affect of EGF_i on the protein-tyrosine kinase activity of the EGF receptor was assessed in a number of ways. Aliquots of A431 membranes were preincubated at 4^oC for 10 min with 1 µg of standard EGF or with 1 µl of EGF_i, in a final volume of 50 µl kinase buffer. 0.5 µCi of [γ-^{32}P] ATP (specific activity 10 mCi/ml, Amersham) was added and the reaction mix incubated at room temperature for 10min. The products of the reaction were analysed by 7.5% (w/v) SDS-PAGE and, as can be seen from Figure 3.16B, both the standard EGF and the EGF_i stimulate the EGF receptor protein-tyrosine kinase activity as indicated by EGF receptor autophosphorylation.

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Figure 3.16A. 2.5 x 10^6 Sf9 cells were infected with the EGFR$_i$ virus and the cells harvested at 24, 48, 72 and 96 h post infection. The cells were resuspended in 1 ml PBS, with or without 1 μg/ml EGF and incubated at 27°C for 10 min, at which time the cells were lysed with hot sample buffer containing 500 μM sodium orthovanadate. The samples were analysed by 7.5% (w/v) SDS-PAGE, immunoblotted and probed with the phosphotyrosine-specific antibody.

Lane 1. wild type infected - EGF.

Lane 2. wild type infected + EGF.

Lane 3. 24 h - EGF.

Lane 4. 24 h + EGF.

Lane 5. 48 h - EGF.

Lane 6. 48 h + EGF.

Lane 7. 72 h - EGF.

Lane 8. 72 h + EGF.

Lane 9. 96 h - EGF.

Lane 10. 96 h + EGF.

Lane 11. A431.

Figure 3.16B. Aliquots of A431 membranes were preincubated at 4°C for 10 min with 1 μg EGF or 1 μl EGF$_i$ in a final volume of 50 μl kinase buffer. 0.5 μCi of [γ-^{32}P] ATP (specific activity 10 mCi/ml, Amersham) was added and the reaction incubated at room temperature for 10 min. The products of the reaction were analysed by 7.5% (w/v) SDS-PAGE and the gel exposed to Hyperfilm (Amersham) for 30 min at -80°C.

Lane 1. + 1 μg standard EGF.

Lane 2. - standard EGF.

Lane 3. + 1 μl EGF$_i$ concentrated medium.

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A
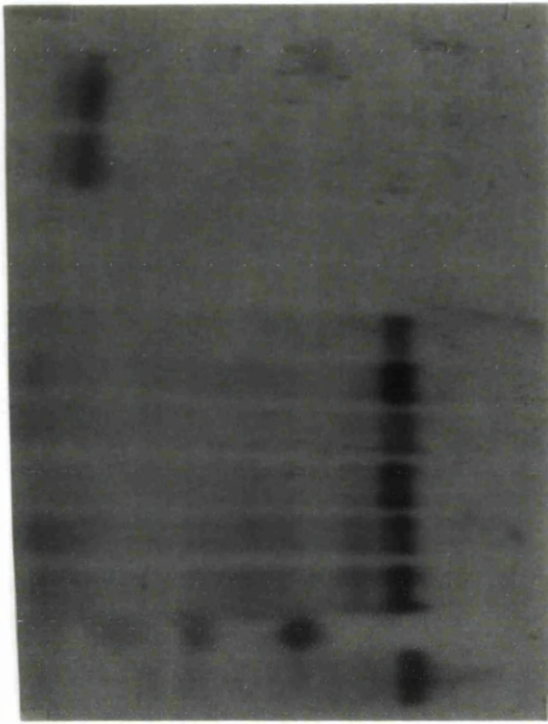
WILD TYPE - EGF
WILD TYPE + EGF
24H - EGF
24H + EGF
48H - EGF
48H + EGF
72H - EGF
72H + EGF
96H - EGF
96H + EGF

A 4 3 1

EGFR

B
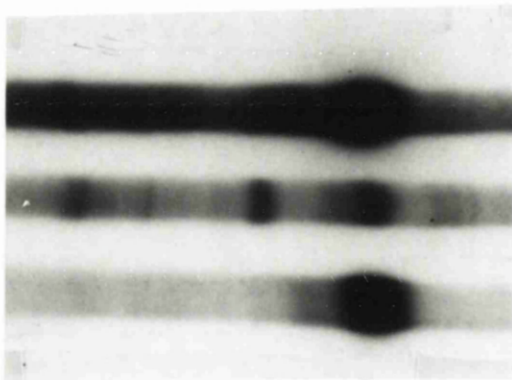
+ EGF STANDARD

- EGF STANDARD

EGF CONCENTRATE

3.7.2. The abiliity of EGF to activate the EGFRᵢ protein-tyrosine kinase.

The abili ty of EGFᵢ to activate the protein-tyrosine kinase of the EGF receptor (EGFRᵢ) expressed in insect cells was also assessed. It has previously been determined that the protein-tyrosine kinase activity of the EGFRᵢ was EGF-inducible (Greenfield et al., 1987). However, some controversy exists as to whether the protein-tyrosine kinase activity of the EGFRᵢ becomes EGF-independent with time following infection. In order to assess the EGF-dependency of the protein-tyrosine kinase activity of the EGFRᵢ, a time course of EGFRᵢ infection was performed. 2.5×10^6 Sf9 cells were infected with EGFRᵢ virus and the cells harvested at 24, 48, 72 and 96 h post infection. The cells were resuspended in 1 ml PBS, with or without 1 µg/ml EGF and incubated at 27°C for 10 min, at which time, the cells were lysed in hot sample buffer containing 500 µM sodium orthovanadate. The samples were analysed by 7.5% (w/v) SDS-PAGE, immunoblotted and probed with a phosphotyrosine-specific polyclonal antibody. As can be seen from Figure 3.16A, the EGFRᵢ is specifically recognised by the anti-phosphotyrosine antibody, indicating the presence of phosphorylation on Tyr residues. At 48 h post infection, the level of phosphotyrosine in the EGFRᵢ is increased on EGF treatment. However, at 72 h and 96 h post infection, the EGFRᵢ basal autophosphorylation state is higher, the addition of EGF having only a minimal effect. By comparison with the EGF receptor present in A431 cells (Lane 11), the molecular weight of EGFRᵢ is lower than 170 kD. These results are in agreement with those of Greenfield et al., (1987) who determined the molecular weight of EGFRᵢ to be 155 kD, attributing the size difference to the inability of insect cells to completely glycosylate the receptor by the addition of high mannose carbohydrate.

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3.7.3. *In vitro* kinase assay of EGFR$_i$.

The ability of the EGF$_i$ to stimulate the EGFR$_i$ protein-tyrosine kinase activity was determined by *in vitro* kinase assay at 48 h post infection when the kinase activity of EGFR$_i$ is still subject to EGF stimulation. 2.5×10^6 Sf9 cells were infected with the EGFR$_i$ virus and the cells harvested at 48 h post infection. The cells were resuspended in either 5 ml of EGF$_i$-conditioned medium or PBS, containing 1 μg/ml of standard EGF or 1 μl of concentrated EGF$_i$, and incubated at 27°C for 10min. The cells were lysed and the EGFR$_i$ immunoprecipitated by reaction with an anti-EGF receptor monoclonal antibody ICR9. An *in vitro* kinase assay was performed and the products of the reaction analysed by 7.5% (w/v) SDS-PAGE. As can be seen from Figure 3.17A, at 48 h post infection, the EGFR$_i$ protein-tyrosine kinase activity was stimulated by treatment with both the EGF standard and EGF$_i$-conditioned medium before and after concentration. Therefore, EGF$_i$ is able to activate the EGFR$_i$ protein-tyrosine kinase.

3.7.4. Phosphotyrosine analysis of EGFR$_i$.

The ability of EGF$_i$ to activate the EGFR$_i$ protein-tyrosine kinase was confirmed by phosphotyrosine immunoblot analysis. At 48 h post infection, EGFR$_i$ infected cells were treated with EGF standard or EGF$_i$ as before. The cells were lysed in hot sample buffer containing 500 μM sodium orthovanadate and analysed by 7.5% (w/v) SDS-PAGE. The gel was immunoblotted and phosphotyrosine containing proteins detected with the anti-phosphotyrosine antibody. The results in Figure 3.17B. indicate that the exposure of the EGFR$_i$ at 48 h post infection to EGF$_i$ causes receptor autophosphorylation.

Figure 3.17A. 2.5×10^6 Sf9 cells were infected with the EGFR$_i$ virus and the cells harvested at 48 h post infection. The cells were resuspended in either 5 ml of EGF$_i$ of TGFα_i-conditioned medium or PBS containing 1 µg/ml EGF or 1 µl concentrated EGF$_i$ or TGFα_i or mixed with 2.5×10^6 Sf9 cells, (harvested at 48 h post infection) expressing preproTGFα, and incubated at 27°C for 10 min. The cells were lysed and immunoprecipitated with the anti-EGF receptor monoclonal antibody ICR9. The immunoprecipitates were resuspended in 50 µl kinase buffer, 0.5 µCi [γ-^{32}P] ATP added and incubated at room temperature for 10 min. The products of the reaction were analysed by 7.5% (w/v) SDS-PAGE and the gel was exposed to Hyperfilm (Amersham) for 30 min at -80°C.

Lane 1.   + 1 µg EGF standard.
Lane 2.   - EGF.
Lane 3.   + 1 µl EGF$_i$ concentrated medium.
Lane 4.   + 5 ml EGF$_i$ conditioned medium.
Lane 5.   + 1 µl TGFα_i concentrated medium.
Lane 6.   + 5 ml TGFα_i conditioned medium.
Lane 7.   + 2.5×10^6 Sf9 cells expressing preproTGFα.

Figure 3.17B. 2.5×10^6 Sf9 cells were infected with the EGFR$_i$ virus and harvested at 48 h post infection. The cells were treated as above and lysed in hot sample buffer containing 500 µM sodium orthovanadate. The cell lysates were analysed by 7.5 % (w/v) SDS-PAGE, the gel immunoblotted and probed with the anti-phosphotyrosine antibody. The filter was exposed to Hyperfilm (Amersham) at -80°C for 3 days.

Lane 1.   ─ 1 µg EGF standard.
Lane 2.   + EGF.
Lane 3.   + 1 µl EGF$_i$ concentrated medium.
Lane 4.   + 5 ml EGF$_i$ conditioned medium.
Lane 5.   + 1 µl TGFα_i concentrated medium.
Lane 6.   + 5 ml TGFα_i conditioned medium.
Lane 7.   + 2.5×10^6 Sf9 cells expressing preproTGFα.

A

WILD TYPE

+ EGF STANDARD

- EGF STANDARD

+ EGF CONCENTRATE

+ EGF MEDIUM

+ TGFα CONCENTRATE

+ TGFα MEDIUM
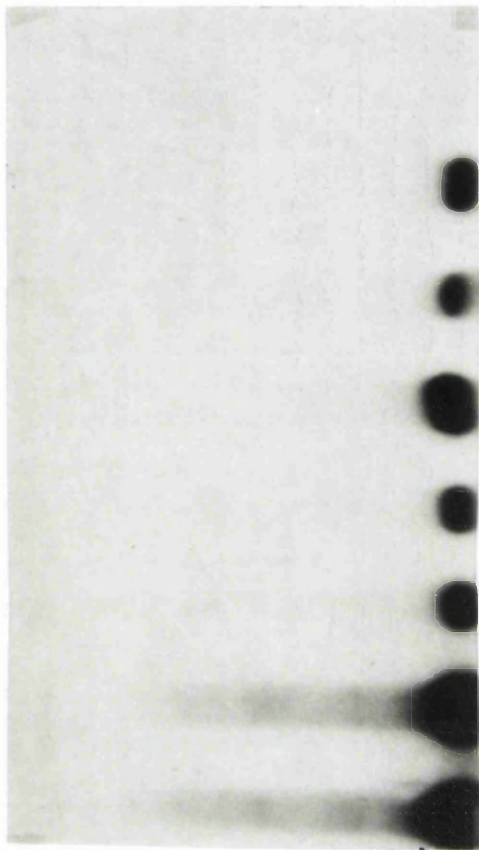
+ TGFα PRECURSOR CELLS

170 kD ↕ EGFR

B

WILD TYPE

-EGF STANDARD

+EGF STANDARD

EGF CONCENTRATE

EGF MEDIUM

TGFα CONCENTRATE

TGFα MEDIUM
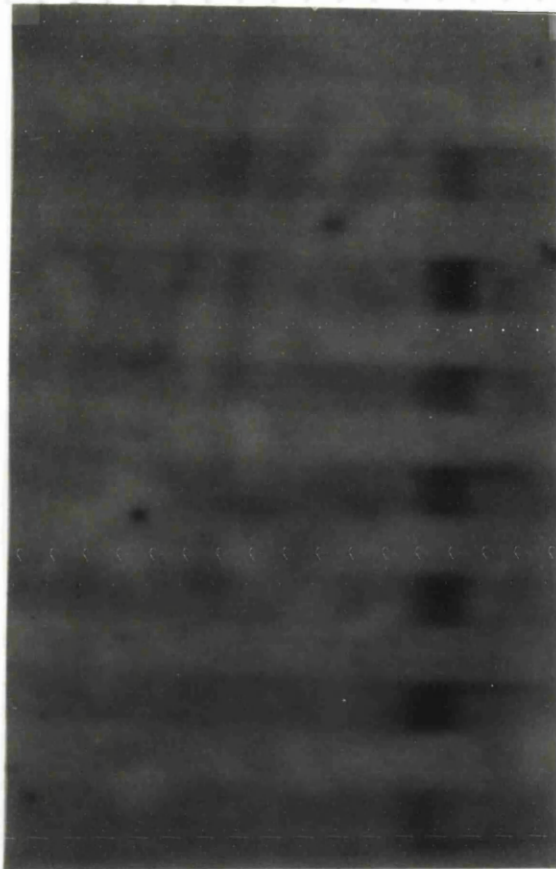
TGFα PRECURSOR CELLS

3.8. HPLC analysis of EGF$_i$

In an attempt to determine the amount of EGF$_i$ secreted into the medium, 100 ml of the conditioned medium was analysed by HPLC. The medium was acidified and applied to the C_{18} Sephak cartridge as before. The eluate was lyophilised and resuspended in 0.1% (v/v) TFA. A sample of the standard EGF was applied to a C_{18} HPLC column and the EGF eluted with a continuous gradient of 0-65% (v/v) acetonitrile. The elution of EGF was followed by monitoring the absorbance by scanning over the range 200-400 nm. The height of the peak of absorbance at 214nm, associated with the amide bond, was taken as an estimation of the amount of EGF present. The absorbance at 280 nm associated with aromatic residues was not measured as a consequence of the absence of the two carboxyl terminal Trp residues in EGF$_i$. The EGF$_i$ was analysed under the conditions established for the standard EGF. However, a contaminant that co-eluted from the Sephak cartridge eluted throughout the entire acetonitrile gradient. The contaminant strongly absorbed over the entire UV range monitored and therefore masked the OD_{214} absorbance signal associated with the EGF.

IPL41 medium contains amino acids, vitamins, inorganic salts, organic acids, sugars, yeast extract, calcium chloride and sodium bicarbonate. In addition, cod liver oil and a lipid emulsion are added to protect the cells from the shearing effects created by the stirring of suspension cultures (Table 2.1). It is possible that it is the cod liver oil and lipids in the emulsion that are co-eluting with the EGF from the Sephak cartridge. The removal of the lipids and other medium components smaller than 1000 daltons was combined with a concentration procedure. Large volumes of conditioned medium can be

effectively concentrated and buffer-exchanged employing a Minitan S (Millipore) to pump the medium under pressure across a 1000 dalton filter (Millipore). The medium continuously recirculates causing the removal of molecules of molecular weight less than 1000 daltons. The lipids would essentially be trapped in the membrane, thereby removing them from the sample. 100 ml of the EGF_i-conditioned medium was filtered and concentrated to a volume of 25 ml using the Minitan S (Millipore) apparatus. The medium was then dialysed extensively against PBS and a 5 ml aliquot applied to the C_{18} cartridge as before. The sample was lyophilised, resuspended in 0.1% (v/v) TFA and applied to the C_{18} HPLC column. However, although it was estimated that 95% of the contaminant had been removed by the filtration procedure, the 214nm EGF-specific absorbance peak was still obscured by the absorbance of the other components of the sample. Therefore, the concentration of the EGF_i could not be measured by HPLC analysis at this stage of purification.

5.9. DISCUSSION.

A synthetic gene encoding the truncated (4-48) version of human EGF linked to the signal sequence of IL-2 was constructed from ten overlapping oligonucleotides (3.2). DNA sequencing confirmed that the sequence of the double stranded molecule was correct (3.2.4). The coding sequence was integrated into the AcNPV genome and a recombinant virus directing the expression of human EGF (EGF_i) isolated (3.3). The expression of EGF_i, with approximate molecular weight of 5 kD, is apparent by 48 h post infection, consistent with the transcriptional activation of the late polyhedrin promoter (For review see, Summers and Smith, 1987). The EGF_i expression level reaches a maximum by 72 h post infection and this level is maintained for a

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further 24 h until the cells begin to lyse. EGF_i is secreted into the extracellular medium indicating that the Sf9 cells recognise and efficiently remove the IL-2 signal peptide when it is linked to a heterologous peptide. The removal of the signal peptide from the rest of the peptide ensures that the receptor binding capacity of the growth factor is not impaired by the presence of an amino terminal extension. Furthermore, the secretion of EGF_i will greatly facilitate subsequent purification procedures. The successful recognition of the IL-2 signal sequence by the Sf9 cells means that it could be employed to direct the secretion of other heterologous proteins for which the extent of the signal sequence is unknown. Indeed, this system could be used to direct the export of intracellular proteins or the cytoplasmic domains of transmembrane proteins.

Conditioned medium was harvested at 72 h post infection in order to maximise the amount of EGF_i synthesised prior to cell lysis which may result in the liberation of degrative enzymes into the medium. In addition, cell lysis may lead to the accumulation of cellular components which could interfere with the subsequent purification procedure. Preliminary results indicate that EGF_i is secreted into the medium at a concentration of approximately 100 ng/ml. This estimated concentration was calculated from the relative competing concentration of EGF_i in the $[^{125}I]EGF$ competition assay and therefore represents the concentration of EGF_i able to bind to the EGF receptor. It was observed that a large amount of the EGF_i remains inside the cell, possibly due to the high expression levels of EGF_i exceeding the capacity of the export system. EGF is extremely acid stable (Cohen and Carpenter, 1975) and therefore, the EGF_i remaining in the cell pellet can be

relatively easily extracted with acid, thereby increasing the yield of EGF_i significantly.

It is unlikely that the yield of EGF_i could be improved at the level of transcription or translation since only the 5' untranslated sequence of the polyhedrin gene was present in the construct. The inclusion of the 5' untranslated sequence of foreign coding sequences has the potential to impair transcription or translation if it is excessively long or contains any regions of potential secondary structure (Lucklow and Summers, 1988). Conversley, the polyhedrin 5' untranslated sequence should allow transcription and translation to occur at maximal efficiency. It is possible that the translation efficiency could be improved by biasing the codon usage to that operating in invertebrates, although there is no evidence to suggest that such a bias would have a positive effect.

The value for the concentration of EGF_i was estimated by the ability of aliquots of conditioned medium to compete with $[^{125}I]EGF$ for receptor binding. This conditioned medium was prepared by culturing 1.0×10^7 cells in 10 ml of medium in a 250 ml tissue culture flask and does not represent the maximal cell density that can be achieved. Sf9 cells can grow either as monolayer cultures or in suspension in Techne stirring vessels (Summers and Smith, 1987). Thus, the potential exists for increasing the yield of EGF_i substantially by increasing the cell density relative to the culture volume. The maximum cell density that can be achieved without reducing the viability of the cells is 5.0×10^6 cells/ml in a culture volume of 400 ml in a 1 litre Techne stirring vessel (Annette Waugh, personal communication). Using this approach, a yield of 2 mg/ litre of the purified extracellular domain of the EGF receptor has been achieved (C Greenfield, personal communication).

Several systems have been designed to express EGF in *E. coli*, exploiting various signal sequences to ensure secretion of the peptide into the periplasmic space, thereby facilitating purification. The alkaline phosphatase (phoA) signal sequence, fused to a synthetic gene for human EGF directed the secretion of biologically active EGF into the periplasmic space (Oka et al., 1985; Engler et al., 1988). Similar results were obtained utilising the signal sequence of the outer membrane protein A (ompA) fused to a synthetic murine EGF gene (Ray et al., 1988). Although yield ranging from 100-400 μg/litre have been reported, a high percentage of foreign proteins expressed in *E. coli* accumulate in insoluble refractile bodies. Subsequent purification of the protein from these bodies causes the disulphide bonds to form incorrectly and the protein becomes aggregated, insoluble and inactive. A redox buffer such as glutathione, can be used to reshuffle the disulphide bonds, but a large proportion of the molecules remain inactive. The insect cell EGF expression system possesses many advantages over the *E. coli* system in that the peptide can be purified relatively easily from the extracellular medium with the correct pattern of disulphide bonds, thereby generating biologically active growth factor at potentially far superior yields.

In order to circumvent the need to purify EGF from a cell pellet, a system to direct the secretion of EGF into the culture medium has been developed in Bacillus subtilis, utilising a synthetic gene for EGF fused to the signal peptide of MWP, one of the major cell wall proteins, resulting in a yield of 240 μg/litre of biologically active EGF (Yamagata et al., 1989). A more universal solution to this problem has been the development of yeast expression systems. Purified human EGF synthesised as a result of the expression of a synthetic gene under the

control of the glutaraldehyde dehydrogenase (GAPDH) promoter was biologically active *in vitro* in receptor binding assays, and *in vivo* in causing precocious eyelid opening in newborn mice (Urdea et al., 1983). However, this expression system resulted in a yield of secreted EGF at only 30 μg/ litre. In an alternative strategy, a synthetic gene for human EGF was fused to the leader region of the peptide mating pheremone α factor, the product was processed correctly and biologically active EGF was efficiently secreted at a concentration of 5μg/ml (Brake et al., 1984). When this latter strategy was employed, it was observed that four forms of EGF, A, B, C and D, could be isolated from the conditioned medium (George-Nascimento et al., 1988). All four forms were equivalent in promoting DNA synthesis in fibroblasts and competed equally for receptor binding. It was determined that the carboxyl-terminal Arg residue was missing in all four forms and that the A and B forms contained only 4 of the 5 Leu residues. These observations indicated that the yeast cells were removing Leu^{52} and Arg^{53} by carboxyl-terminal processing. Furthermore, D and C forms were converted into B and A respectively by the action of a carboxypeptidase in the medium, D converting to C by oxidation at Met^{21}. Thus, although the yeast system produces a relatively high yield of EGF, the presence of a heterogeneous population of molecules in a sample poses problems for biophysical characterisation and potential pharmaceutical applications.

The advantage of a bacterial or yeast expression system over a eukaryotic system is that the latter most often requires expensive culture medium supplemented with serum. Furthermore, eukaryotic cells adapt poorly to large-scale culturing procedures. However, eukaryotic expression systems perform all the protein modification,

processing and transport systems that may be necessary to produce the product in a biologically active form. Insect cells employ many of the post-translational modifications occuring in higher eukaryotes and therefore present an ideal system for the generation of fully biologically active recombinant proteins. The advantage of the insect expression system over other eukaryotic systems is that insect cells will grow well in inexpensive synthetic media. Furthermore, insect cells can be cultured in the absence of serum thereby facilitating purification procedures and dramatically reducing expenditure.

EGF$_i$ is able to compete with [^{125}I]EGF for binding to the EGF receptor expressed on NR6+ fibroblasts (DiFiore et al., 1987a) (3.5). The ability of this truncated (4-48) form of human EGF to bind to the EGF receptor indicates that both amino and carboxyl terminal truncations of the ligand can be tolerated without loss of receptor binding capacity. The apparent lack of a functional requirement for the amino and carboxyl termini of EGF is reflected by the presence of several native forms of biologically active EGF in body fluids from a variety of sources. Two forms of biologically active human EGF have been isolated from urine, the full length molecule and a shorter version, γ-urogastrone which lacks the amino terminal Arg residue (Cohen and Carpenter, 1975). Four forms of EGF, all capable of receptor binding, have been identified in human breast milk (Petrides et al., 1985). Indeed, four forms of EGF have been purified from rat submaxillary glands, ranging in length from 44 to 48 residues (Simpson et al., 1985). These forms of rat EGF lack 5 residues from the carboxyl terminus and up to three from the amino terminus and all bind to the receptor with equal affinity and are equipotent in illiciting a mitogenic response in Swiss 3T3 cells.

The observation that five residues can be deleted from the amino terminus of human EGF without loss of receptor binding capacity is in direct agreement with the findings of Burgess et al. (1988). Two forms of murine EGF were isolated following trypsinisation of the full length form, the 1 - 48 form bound to the receptor and was mitogenic, whereas the 1 - 45 form was unable to bind to the receptor.

EGF_i stimulates DNA synthesis in quiescent NR6+ fibroblasts, but not in NR6 fibroblasts, as measured by the incorporation of $[^3H]$Thymidine (3.6). This mitogenic effect was due to the EGF_i secreted into the medium and not the medium alone. Furthermore, the medium conditioned by uninfected Sf9 cells was tested for the presence of 'competence factors' which enable cells to become responsive to the subsequent addition of EGF (Stiles et al., 1979). The observation that the addition of an aliquot of this concentrated medium to 5 nM standard EGF did not augment the EGF-effect suggests that growth medium of uninfected Sf9 cells does not contain any competence factors.

The ability of EGF_i to activate the EGF receptor protein-tyrosine kinase was assessed by *in vitro* kinase assay on A431 membranes (3.7.1). In addition, the EGF_i was also able to stimulate $EGFR_i$ autophosphorylation as shown by *in vitro* kinase assay (3.7.3) and phosphotyrosine immunoblot analysis (3.7.4). Thus, EGF_i is able to bind to the EGF receptor and activate the intrinsic protein-tyrosine kinase activity, thereby illiciting a mitogenic response in fibroblasts.

The question of the EGF-dependency of the protein-tyrosine kinase activity of the $EGFR_i$ has been addressed (3.6.2), indicating that the basal level of the protein-tyrosine kinase activity of the receptor increases over the post infection period. At 48 h post infection, the

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protein-tyrosine kinase activity of the $EGFR_i$ is stimulated by the addition of EGF. At 72 h and 96 h post infection, the basal protein-tyrosine kinase activity is higher, the addition of EGF having only a minimal effect. A possible explanation for the observed constitutive protein-tyrosine kinase activity of the $EGFR_i$ by 72 h post infection, is that by this time, expression is at a maximum level and the concentration of the EGF receptor at the membrane is very high. Similar results were obtained for the expression of the PDGF receptor in insect cells, although it was determined that the protein-tyrosine kinase activity of the receptor became ligand-independent as early as 48 h post infection (Morrison et al., 1989). The constitutive activation of the PDGF receptor at 48 h post infection, when the EGF receptor protein-tyrosine kinase activity is still dependent on EGF, could be attributed to a higher expression level achieved in their system. The PDGF receptor concentration at the membrane would therefore be higher than that of the EGF receptor at an earlier stage in the infection. Alternatively, there could be a discrepancy in the actual time post infection when the cell were harvested, if the cells were harvested only a few hours later, a significantly higher level of product would have accumulated. An oligomerisation model for signal transduction has been proposed (Yarden and Schlessinger, 1987), in which EGF binding causes receptor dimerisation with associated activation of the protein-tyrosine kinase. In insect cell membranes at 72 h post infection, the concentration of the EGF receptor may be sufficiently high to promote dimerisation in the absence of EGF, thereby stimulating the protein-tyrosine kinase activity. However, at 48 h post infection, the concentration of the EGF receptor is such that the activation of the protein-tyrosine kinase is dependent on receptor dimerisation.

HPLC analysis was performed in an attempt to quantitate the yield of EGF_i. However, any EGF-specific absorbance was masked by the presence of a contaminant which co-eluted with EGF_i from the C_{18} Sephak cartridge. Therefore, the EGF_i sample would have to be more completely purified before an accurate figure for the concentration could be determined by HPLC analysis. IPL41 medium contains amino acids, vitamins, inorganic salts, organic acids, sugars, yeast extract, calcium chloride and sodium bicarbonate. In addition, cod liver oil and a lipid emulsion is added to protect the cells from the shearing effects created by the stirring of suspension cultures (Table 2.1). It is possible that it is the cod liver oil and the lipids in the emulsion that are co-eluting with the EGF from the Sephak cartridge. In an attempt to remove these medium components, the medium was filtered through a 1000 dalton filter (Millipore) using a Minitan S (Millipore) apparatus and then applied to the C_{18} Sephak cartridge. However, upon HPLC analysis, the 214 nm EGF absorbance peak was still obscured. These results indicate that not all of the cod liver oil and lipids have been removed by the filtration step. These remaining components could be removed by gel permeation chromatography or partition with a non-polar organic solvent prior to the Sephak step. It is anticipated that the background absorbance would thus be reduced significantly allowing detection of the EGF-specific absorbance at 214 nm.

From the existing ^{1}H NMR data and information as to the positioning of the highly conserved residues, predictions can be made as to the residues which are responsible for binding to and activating the receptor. These predictions can be evaluated by mutational analysis so that, not only can the spatial positioning of these residues be determined but also the involvement of their specific sidechains.

Preliminary mutational analysis has suggested the intimate involvement of Leu^{47} in receptor binding (Ray et al., 1988; Burgess et al., 1988). The importance of Leu^{47} was initially postulated by the observation that its removal by carboxypeptidase treatment led to a marked reduction in receptor binding and mitogenic potency (Burgess et al., 1988). Furthermore, the low affinity of amphiregulin (AR) for the EGF receptor may reflect the fact that its carboxyl-terminal residue corresponds to residue 46 of EGF and consequently, AR lacks the equivalent of Leu^{47} (Shoyab et al., 1988). Mutation of Leu^{47} in EGF to either Val or Ser resulted in loss of receptor binding capacity, although mutation to the hydrophilic Ser residue had a more dramatic effect than to the similar hydrophobic Val (Ray et al., 1988). These results were confirmed and extended by Engler et al. (1988) who mutated Leu^{47} to His resulting in an almost complete loss of receptor binding. Information obtained from the ^{1}H NMR analysis of murine EGF indicated that the defined structure stops after Leu^{47} (Montelione et al., 1987). More recent ^{1}H NMR analysis of a mutant peptide, in which Leu^{47} was replaced with a Ser residue, indicated that only minor conformational changes were associated with the replacement of Leu^{47} (Moy et al., 1989). These changes involved the side chains of only those residues spatially adjacent to Leu^{47} and did not involve any other regions of the molecule. These observations therefore provide evidence that Leu^{47} is involved directly in the interaction of EGF with its receptor and not indirectly by determining the conformation of the receptor binding site. Mutational analysis of the corresponding residue in human TGFα (Leu^{48}) yielding an inactive molecule has confirmed the proposed importance of Leu^{47}. Interestingly, mutation of the adjacent residue Asp^{47}, that is conserved between EGF and TGFα, indicated that neither the negative charge nor the polarity of the Asp residue was essential

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for receptor binding (Lazar et al., 1988). ^{1}H NMR analysis of murine EGF has indicated that, although the adjacent residues, Asp[46] and Leu[47] are both solvent accessible, their sidechains point in different directions in the β-sheet, suggesting potentially very different involvements in the structure and function of EGF (Montelione et al., 1987). In addition, several other residues have been mutated (Ray et al., 1988). Pro[7] was mutated to Thr and retained greater than 50% of the binding affinity of the wild type peptide, indicating an apparent lack of requirement for this highly conserved and structurally distinctive residue in the correct folding of the molecule. The peptide in which Tyr[29] was replaced with Gly retained only a low level of activity. This observation supports the proposal that the clustering of the aromatic residues, Pro[7], His[10], Tyr[13], Tyr[22] and Tyr[29] (positions refer to human EGF) is thermodynamically favourable and may be partly responsible for the folding and stability of the molecule.

The isolation of a recombinant baculovirus directing the expression of biologically active human EGF paves the way foward for large scale production exploiting the on site 6 and 60 litre bioreactor facilities. The availability of large quantities of biologically active EGF for further ^{1}H NMR analysis and ultimately, diffractional analysis of EGF crystals, means that the complete determination of the three-dimensional structure of EGF is within sight. Furthermore, the generation of large amounts of EGF will allow the continuation of CD and spectroscopic analyses of the conformational changes occurring in the receptor upon ligand binding (Greenfield et al., 1989).

Mutational analysis of the other conserved residues, 13, 15, 16, 41 and 43 and any other residues postulated to play a role in the generation of the receptor binding site can be evaluated using the baculovirus

system. ^{1}H NMR analysis of these mutant peptides will discriminate between those residues involved directly in ligand-receptor interactions and those responsible for establishing the three-dimensional structure of the binding site. The generation of mutant peptides which bind to the receptor without illiciting a biological response will discriminate between those residues involved in binding to the receptor and those involved in receptor activation. The ability to dissociate receptor binding from receptor activation will lead to the development of potential EGF antagonists which will have important clinical implications in the treatment of certain malignancies which are known to display an elevated level of EGF receptors.

CHAPTER FOUR.

THE EXPRESSION OF PREPROTGF IN INSECT CELLS.

4.1. INTRODUCTION.

4.1.1. The structure of preproTGFα.

The molecular cloning of the cDNAs for human and rat TGFα indicated that the growth factor is synthesised as part of a larger precursor, preproTGFα (Derynck et al., 1984; Lee et al., 1985). Human preproTGFα comprises 160 amino acids, one residue longer than the rat form (all subsequent amino acid assignments refer to the human sequence). The initiator Met (residue 1) is followed by a region of approximately twenty-two uncharged and predominantely hydrophobic amino acids, a feature characteristic of the signal peptides of secretory and membrane-associated proteins (Perlman and Halvorson, 1983). There is a potential site for N-linked glycosylation at Asn^{25} (Figure 4.1).

The mature 50 amino acid TGFα species is released from the rest of the preprotein by the action of a protease with elastase-like specificity (Derynck et al., 1984). The cleavage site, with the recognition sequence Ala - Val - Val, is present at both the amino and carboxyl termini of the mature growth factor, with cleavage occurring between the Ala residue and the Val dipeptide at Ala^{39} - Val^{40} and Ala^{89} - Val^{90}. This type of processing is distinct from that of most polypeptide hormones where cleavage occurs at single or paired basic residues.

The mature TGFα sequence is followed by an extremely hydrophobic core, consisting almost exclusively of Ile, Leu and Val residues, extending between residues 98 and 121 and flanked by paired basic residues (Lys^{96} - Lys^{97} and Arg^{122} - Lys^{123}). The latter feature is characteristic of the 'stop-transfer' transmembrane domain of integral

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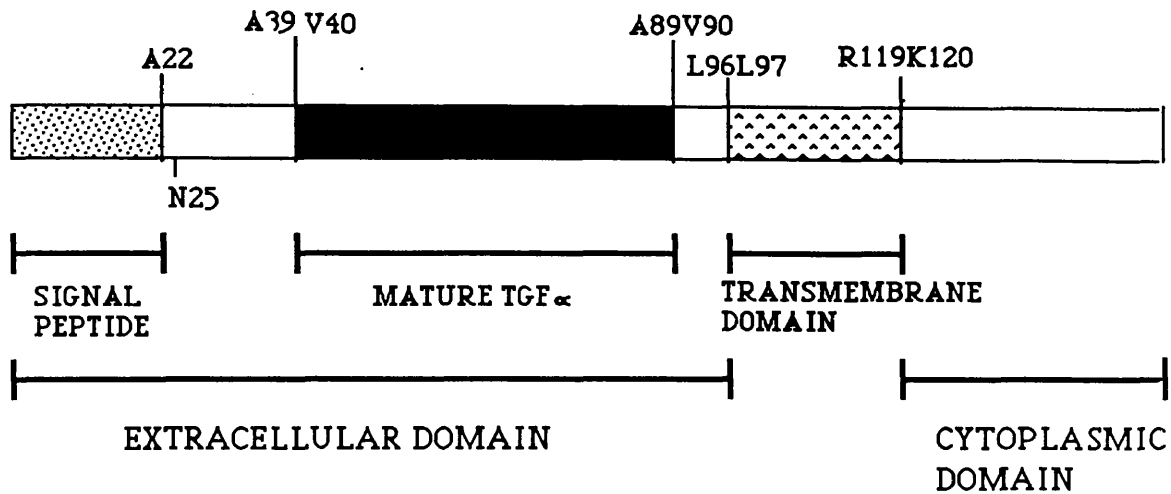
Figure 4.1. The structure of preproTGFα showing the processing sites for the removal of the signal peptide at A22 and the site for cleavage by the trypsin-like activity at L96-L97. Mature TGF α is generated by the action of the specific protease at A39-V40 and A89-V90. A potential glycosylation site is at N25.

membrane proteins (Blobel, 1980) and is absent from the sequence of secretory proteins, including all known prohormones which as a result, are completely translocated across the endoplasmic reticulum membrane. These observations suggested that preproTGFα was synthesised as an integral membrane glycoprotein with the 50 amino acid TGFα sequence exposed to the extracellular phase where it could be released from the rest of the preprotein by specific proteolytic processing. If the preprotein is indeed anchored in the membrane, the carboxyl terminal domain would face the cytoplasmic side of the endoplasmic reticulum. It is interesting to note that although the entire sequence of human and rat preproTGFα are very homologous (93%), the carboxyl terminus is the most highly conserved region of the molecule (Derynck et al., 1984). This Cys-rich region has only a single conservative Val to Ile change at position 99 and is therefore even more highly conserved than the 50 amino acid mature TGFα which has four differences.

Why is TGFα, a secreted growth factor, synthesised as a large membrane-associated glycoprotein? Indeed, other growth factors including EGF (Scott et al., 1983a), CSF-1 (Rettenmier et al., 1987) and TNF (Kriegler et al., 1988) are all released by proteolytic processing of large integral membrane precursors. Have these precursors evolved from purely mechanistic considerations, functioning only to anchor the nascent growth factors in the membrane thereby ensuring their correct three-dimensional conformation? An alternative role for the TGFα precursor can be postulated in the light of the extensive conservation of the entire precursor and when the sequences of both preproTGFα and preproEGF are examined.

4.1.2. The structure of preproEGF.

EGF is synthesised as a 1200 amino acid membrane-bound glycoprotein which is over twenty times larger than the mature growth factor. The extracellular domain of preproEGF contains nine Cys-rich regions of approximately forty residues with EGF-like homology (Bell et al., 1986) (Figure 4.2). The mature EGF peptide, corresponding to the Cys-rich region closest to the transmembrane domain (1033 - 1057), is flanked at the amino and carboxyl termini by the dipeptides Arg^{970} - Asn^{971} and Arg^{1023} - His^{1024} respectively. In the submaxillary glands, pancreas, small intestine and mammary gland, the growth factor is released from the rest of the precursor by the action of an Arg-specific protease cleaving at these flanking sequences (Taylor et al., 1970). Neither dipeptide junction occurs at equivalent positions around the other eight homologous segments and therefore, none of these sequences are released as biologically active factors by the action of this protease. Although none of the other EGF-like segments have been isolated, it cannot be ruled out that one or more of them could be released as biologically active entites by the action of alternative proteases. Interestingly, preproEGF is produced by, but not processed, in the cells of the distal tubule of the kidney where it accumulates as a membrane-associated glycoprotein. This observation suggests a potential function for EGF in its membrane-associated preproEGF form (Rall et al., 1985).

4.1.3. Is the precursor a receptor?

It has previously been determined that the extracellular domain of the EGF receptor contains several copies of the EGF-like sequence, four of approximately 40 amino acids and two of half that size (Pfeffer and Ullrich, 1985). Such structural similarities between secreted
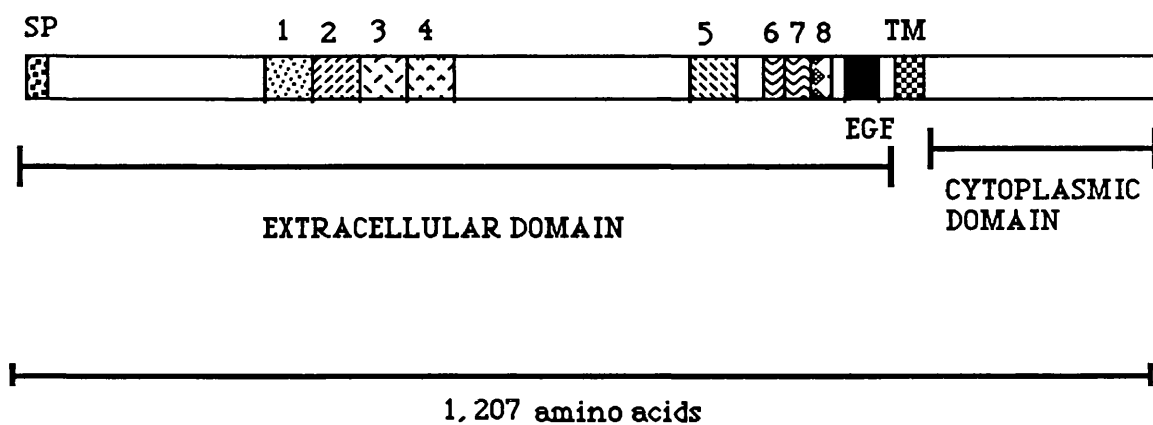
Figure 4.2. The structure of preproEGF showing the eight EGF-like repeat units and the position of mature EGF.

▨ signal peptide

▨ transmembrane domain

molecules and their membrane receptors have been previously reported, the receptor for the trans-epithelial transport of two Immunoglobulins; IgA and IgM, displays structural homologies with its ligands (Mostov et al., 1984). These findings have lead to the suggestion that the precursor for EGF may be a multifunctional protein serving as a receptor for an unknown ligand in addition to acting as a precursor to one or more biologically active peptides (Pfeffer and Ullrich, 1985).

4.1.4. Potential functions of the EGF-like repeat.

In addition to the members of the EGF-like family of growth factors, TGFα, VVGF (Blomquist et al., 1984), SVGF (Chang et al., 1987) and MRV (Strayer et al., 1987), single or multiple copies of EGF-like sequences have been found in many different proteins. These observations suggest that these domains share some common functional features (Figure 4.3).

The low density lipoprotein (LDL) receptor mediates the cellular uptake of LDL, a cholesterol-carrying plasma lipoprotein, thereby supplying the cell with cholesterol. The extracellular domain of the LDL receptor between residues 457-595, contains three EGF-like repeats with 40% homology to the region between residues 565-701 in preproEGF (Russell et al., 1984). The homology decreases in the regions around the core but remains significant at approximately 30% for several hundred more amino acids. Analysis of the structure of the LDL receptor gene in subjects with familial hypercholesterolemia has shown that deletion of exons 7 and 8, encoding the first two copies of the EGF-like repeat, strongly interferes with the binding capacity and specificity of the receptor (Yamamoto et al., 1986).

The transferrin receptor, an integral membrane glycoprotein composed of two identical subunits, mediates iron uptake via

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hEGF    NSDSECPLSHDGYCLHDGVCMYIEA...LDKYACNCVVGYIGERCQYRDLKWWELR

mEGF    NSYPGCPSSYDGYCLNGGVCMHIES...LDSYTCNCVIGYSGDRCQTRDLRWWELR

rEGF    NSNTGCPPSYDGYCLNGGVCMYVES...VDRYVCNCVIGYIGERCQHRDLR

gEGF    QDAPGCPPSHDGYCLHGGVCMHIES...LNTYACNCVIGYVGERCEHQDLDWE

hTGFαVVSHFNDCPDSHTQFCFH.GTCRFLVQ...EDKPACVCHSGYVGARCEHADLLA

rTGFαVVSHFNKCPDSHTQYCFH.GTCRFLVQ...EEKPACVCHSGYVGVRCEHADLLA

VVP  DIPAIRLCGPEGDGYCLH.GDCIHARD    IDGMYCRCSHGYTGIRCQHVVLLVDYQR

SFVP  IVKHVKVCNHDYENYCLNNGTCFTIALDNVSITPFCVCRINYEGSRCQFINLVTY

MVP   IIKRIKLCNDDYKNYCLNNGTCFTVALNNVSLNPFCACHINYVGSRCQFINLITIK
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hFIX    DGDQCESNPCLNGGSCKDDI..NSYECWCPFGFEGKNCEL

hFX     DGDQCETSPCQNQGKCKDGL..GEYTCTCLEGFEGKNCEL

hFVII   DGDQCASSPCQNGGSCKDQL..QSYICFCLPAFEGRNCET

Notch   DIDECQSNPCLNDGTCHDKI..NGFKCSCALGFTGARCQI

uEGFI   NIDECASAPCQNGGICIDGI..NGYTCSCPLGFSGDNCEN

hFXII   ASQACRTNPCLHGGRCLEVE..GHRLCHCPVGYTGPFCDV

hTPA    PVKSCSEPRCFNGGTCQQALYFSDFVCQCPEGFAGKCCEI
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hLDLR   DIDEC.QDPDT.C.SQL.CVNLE.GGYKCQCEEGFQLDPHTKACKA

hPS     DVDECSLKP.SIC.GTAVCKNIL.GDFECECPEGYRYNLKSKSCED

hTMD    DVDDCILEP.SPC.PQR.CVNTQ.GGFECHCYPNYDLV.DGE.CVE

hPC     SFLNCSLDNGG.C.THY.CLEEV.GWRRCSCAPGYKLGDDLLQCHP

mEGFP   GADPCLYRNGG.C.EHI.CQESL.GTARCLCPEGFVKAWDGKMCLP

hFIX    LDVTCNIKNGR.C.EQF.CKNSADNKVVCSCTEGYRLAENQKSCEP
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Figure 4.3. (A) Aligned sequences of the EGF-like family of growth factors showing the sixteen conserved amino acids. The sequences are, in order, human EGF, murine EGF, rat EGF, guinea pig EGF, human TGFα, rat TGFα, the vaccinia virus protein (C-terminal region), the Shope fibroma virus protein (C-terminal region) and the myoxoma virus protein (C-terminus).

(B). Alignment of the EGF-like units of some extracellular multi-domain proteins. The sequences are, in order, human Factor IX (47-84), Factor X (46-83), Factor VII (46-83), Drosophlia *notch* (526-563), sea urchin uEGF-1 (12-49), human Factor XII (155-192), human TPA (82-121), human LDL receptor (333-364), human Protein S (160-202), human thrombomodulin (325-364), human Protein C (94-135), murine preproEGF (748-788) and human Factor IX (84-126).

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internalisation and recycling of the iron-carrying serum protein transferrin (reviewed by Newman et al., 1982). An eleven amino acid segment of each subunit shares seven residues in common with preproEGF with an overall homology of 21% over a stretch of 240 amino acids (McCelland et al., 1984; Schneider et al., 1984). This area of tentative homology overlaps with the region of preproEGF that is related to the LDL receptor, occurring in a similar segment of both of the receptors a short distance from their transmembrane domains.

It has been postulated that the EGF-like repeats in extracellular matrix glycoproteins and in the extracellular portions of some membrane proteins function as signals for cellular growth and differentiation (Appella et al., 1988; Engel, 1989). The EGF-like domains form parts of large molecules and often of supramolecular assemblies which are well suited to stimulate neighbouring cells in a specific and vectorial manner. Components of the extracellular matrix with growth factor-like activity, such as tenascin (Pearson et al., 1988) with an uninterrupted array of thirteen EGF-like repeats in each of its six-arms, may stimulate target cells in a much more selective way than diffusible growth factors.

Laminin, another component of the extracellular matrix, associates with components of the basement membrane (Panayotou et al., 1989). Laminin is a large, multidomain glycoprotein involved in the promotion of cell attachment, locomotion, proliferation and differentiation, each of the different domains of the molecule implicated in different cellular functions (reviewed by Timpl, 1982). Laminin is the first matrix protein to be detected at early stages of embryogenesis, where its expression and deposition is intimately associated with the formation of epithelial cell layers (Dziadek and Timpl, 1985). The mitogenically active domain 1 may be accessible to

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cells only during the early stages of tissue development, before laminin is assembled into an intact basement membrane, and potentially becomes re-exposed after damage to the membrane caused by injury. Under these conditions, laminin may exert its growth factor-like activity on neighbouring cells and via an autocrine mechanism, on cells that produce it. It is of great interest to note that the short arm of the mitogenically active domain I contains a large number of EGF-like Cys-rich motifs.

Another protein displaying the EGF-like repeat is urokinase (uPA) which catalyses the proteolytic activation of inactive plasminogen to the broad spectrum serine protease, plasmin (Moonen et al., 1982). This reaction is crucial in regulating extracellular proteolysis and hence a variety of phenomena that require degradation of the extracellular matrix and the basement membrane. Some normal and neoplastic cells possess a specific uPA receptor which may serve to focus the regulatory proteolytic activity of uPA, and it is this receptor-binding specificity which resides within the EGF homologous region of the molecule (Stoppelli et al., 1986).

Thrombomodulin is an endothelial cell surface protein which forms a 1:1 complex with thrombin, thereby activating protein C (Jackman et al., 1986). Thrombomodulin therefore plays a role as a physiologically important anticoagulant on the endothelial cell surface. The extracellular domain of thrombomodulin which contains six EGF-like repeats, is responsible for both the binding of thrombin and the activation of protein C (Zushi et al., 1989). It was predicted that each EGF-like structure in thrombomodulin has a similar secondary structure to human EGF. Indeed, the pertubation of this secondary structure by denaturation or deletion led to the loss of protein C activation, suggesting that the EGF-like repeats control thrombin binding and

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cofactor activation (Zushi et al., 1989). One to three copies of the EGF-like motif have been found in several other proteins including the serine proteases involved in the formation (factor VII, IX, X, XII and protein C, S and Z) and breakdown of fibrin clots (tPA) (reviewed by Appella et al., 1988).

All of the proteins containing the EGF-like motif are known to, or may be expected to participate in protein-protein or protein-cell interactions. They are growth factors, receptors or receptor-like proteins, or proteins of the coagulation and fibrinolytic pathways. The function of these proteins suggests that EGF-like motifs are involved in ligand-receptor interactions, either in the determination of the receptor binding site of the ligand or the ligand acceptor site of the receptor.

The postulated involvement of the EGF-like repeat in cell-cell signalling is supported by the discovery of several invertebrate proteins containing repeating units with extensive homology to the EGF-like repeat. The function of the neurogenic genes of Drosophila are required for the normal pattern of committment of neural and epidermal progenitor cells (Lehmann et al., 1983). The seperation of neuroblasts from the ectoderm into the inner part of the embryo is one of the first steps of CNS development in insects. In Drosophila, this process is under the constant control of the neurogenic genes. Indeed, loss of function mutations in any of these genes results in the development of all cells of the neurogenic ectoderm into neuroblasts (Lehmann et al., 1983). Once an ectodermal cell becomes a neuroblast, it inhibits its neighbours from following the same pathway suggesting that cell-cell signalling plays an important role in the committment of cell fate. One of the members of the neurogenic locus encodes the integral membrane protein, *notch* (Kidd et al., 1986). The extracellular

domain of *notch* contains 39 Cys-rich repeating units, the first 36 sharing extensive homology with the EGF-like repeat (Wharton et al., 1985). It has been suggested that the *notch* protein functions as a dimeric or multimeric assembly formed as a consequence of interaction between the EGF-like repeats, with at least one of the repeats involved in interactions with the product of another neurogenic gene, *E(spl)* (Kelley et al., 1987). Another Drosophila protein displaying the EGF-like repeating motif is the product of the *slit* gene (Rothberg et al., 1988). The *slit* protein, containing seven EGF-like repeats, plays a role in the development of the embryonic nerve cord, lending support to the proposed important function that invertebrate proteins, containing tandem arrays of EGF-like repeats, have throughout development.

Developmentally important proteins containing EGF-like repeats have been discovered for other invertebrate species. The protein product of the C. elegans *Lin-12* gene controls certain binary decisions during development and contains eleven EGF-like repeats (Greenwald, 1985). Partial sequence information generated for the sea urchin gene product *uEGF-I*, implicated as having a function in early development when cell fates are assigned, shows that it contains at least eighteen EGF-like repeats (Hursh et al., 1987). In the light of the obvious importance of the EGF-like repeat in such a wide range of both vertebrate and invertebrate proteins intimately involved in cell-cell signalling, it can be postulated that preproEGF and preproTGFα may mediate physiological cell-cell recognition events, in addition to serving as vehicles to ensure the correct transport and secretion of the growth factors.

4.1.5. Aims of this study.

It would be extremely useful to express the preproTGFα in the baculovirus expression system in order to study its processing. Will

insect cells produce the specific protease required to cleave the precursor to the mature 50 amino acid TGFα species, or will the precursor remain intact and membrane-associated? The isolation of a recombinant baculovirus directing the expression of preproTGFα, in association with the EGF receptor recombinant virus (Greenfield et al., 1987) would enable investigation into potential interactions between preproTGFα and the EGF receptor. If preproTGFα is biologically active in insect cells, it should stimulate the intrinsic protein-tyrosine kinase activity of the EGF receptor. The system will stand as a useful model to investigate the interaction of growth factors with their receptors and will allow the determination of the significance of this type of pathway for growth factor secretion. It has been proposed that preproEGF and preproTGFα may function as receptors for unknown ligands (Pfeffer and Ullrich, 1985). The observation that the cytoplasmic domain of preproTGFα is by far the most highly conserved region of the molecule (Derynck et al., 1984) suggests an important physiological role for this region of preproTGFα, potentially through intracellular signalling. Such receptor-like activity of preproTGFα could be evaluated in the insect cells by co-expression with implicated cellular components. In addition, the large scale expression and purification of preproTGFα should facilitate the isolation of the specific protease responsible for the release of the mature growth factor from the conditioned media of transformed cells.

4.2. RESULTS.

4.2. CONSTRUCTION OF PAC.252.

4.2.1. Cloning strategy for the generation of pAC.252.

The actual sequence of the TGFα cDNA (Derynck et al., 1984) was different from the published sequence around the translation start site, in so much that the ATG was not present.

PUBLISHED SEQUENCE

5' GAATTCCCCGCCCGTAAAATGGTCCCC 3'

ACTUAL SEQUENCE

5' GAATTCATGCGTTCTTACAGGGTCCC 3'

It was therefore necessary to recreate the translational initiation site (ATG). In addition, a Kozak consensus sequence for translation was introduced in order to try to maximise translational efficiency (Kozak, 1984, 1987). Furthermore, the upstream ATG was mutated so as to prevent premature initiation. The 5' untranslated region was kept to a minimum, since it has been determined that potential regions of secondary structure can reduce the level of expression significantly (Lucklow and Summers, 1988). These changes were introduced by *in vitro* mutagenesis employing an oligonucleotide of 30 bases with the sequence:

5' ATCGAATTCAAGCGTTCTACCATGGTCCCC 3'

The mutated coding sequence was introduced into the baculovirus expression vector pAC373.

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4.2.2. The generation of SK.TGFα.

The TGFα cDNA was supplied in the plasmid Sp64 denoted SpTGFα (Derynck et al., 1984) and was subcloned into the Bluescript vector, SK$^+$ (Stratagene) in order to facilitate subsequent subcloning procedures. 5μg of SpTGFα DNA was digested in a double digest, with EcoR1 and Sau3A liberating the TGFα coding sequence as a 650 bp fragment. The 650 bp insert was isolated by electroelution, purified by phenol:chloroform extraction, ethanol precipitated and resuspended in 20 μl of TE. 5 μg of SK$^+$ was digested with EcoR1 and BamH1 (Sau3A and BamH1 create identical 5' and 3' overhangs). The digested plasmid DNA was then treated with CIP and the phosphatased DNA purified as before and resuspended in 20 μl of TE. 5 μl of the purified TGFα insert was ligated with 0.5 μl of the phosphatased SK$^+$ DNA and transformed into competent XL-1 blue. The transformation culture was plated onto an L-agar plate supplemented with ampicillin and DNA prepared from twenty four white colonies by the plasmid minipreparation method. An aliquot of the DNA was digested with EcoR1 and Sau3A and treated with RNase 1 'A' prior to analysis by agarose gel electrophoresis. A large amount of plasmid DNA was then prepared from a successful subclone by the maxipreparation method.

4.2.3. *In vitro* mutagenesis of the TGFα coding sequence.

The TGFα DNA was 'shot gun' cloned following EcoRI and XbaI digestion into Mp19, in order to perform the mutagenesis reaction (the BamHI site on the M13 polylinker is lost on the stuffer fragment). Single-stranded DNA was prepared from several plaques, according to the M13 minipreparation method, and 'T-tracked'. A candidate sample was then fully sequenced to ascertain the presence of the valid TGFα

sequence and designated Mp19.TGFα. It is of paramount importance to ensure that the oligonucleotide utilised in the *in vitro* mutagenesis reaction has the sequence which is complementary to the single-stranded DNA used as the template in the reaction. The strand synthesised in the sequencing reaction, using Mp19.TGFα as the template, was the coding strand in the 5'->3' direction. It can therefore be concluded, that the single-stranded DNA template (the cloned strand) is the non-coding strand in the 5'->3' direction and thus, the oligonucleotide must be synthesised 5'->3' as the coding strand. The oligonucleotide was synthesised, approximately 20 μg purified by 15% (w/v) PAGE, and a stock solution prepared at 5 OD$_{260}$ units/ml. The mutagenesis reaction was performed using an *in vitro* mutagenesis kit (Amersham) with one complete minipreparation of Mp19.TGFα single-stranded DNA as template and the purified oligonucleotide stock. The resultant mutated DNA was used to transform an aliquot of competent XL-1 blue and the transformation reaction plated onto an L-agar plate, supplemented with ampicillin. Single-stranded M13 DNA was prepared, 'T-tracked' and a potential mutant, designated Mp19.252, sequenced to determine the presence of the entire mutant oligonucleotide. The insert was subcloned into Mp18 so as to generate Mp18.252. The entire TGFα sequence was then sequenced, on both strands from the universal primer in Mp18 and Mp19, in order to be confident that no unwanted base changes had been created during the mutagenesis reaction.

The TGFα 252 coding sequence was 'shot gun' cloned into the Bluescript vector, SK$^+$ following EcoRI and HindIII digestion. DNA was prepared from twelve white colonies by the plasmid minipreparation method and an aliquot digested with EcoRI and HindIII. All of the twelve minipreparations contained 252 (Figure 4.4) and the DNA of one clone,

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Figure 4.4. One Mp19.252 double stranded (RF) minprepqration was digested at 37°C for 1 h with EcoRI and HindIII in a final volume of 30 μl. The enzymes were inactivated by incubation at 75°C for 10 min. 1μg of SK+ was also digested with EcoRI and HindIII in a final volume of 30 μl and the enzymes heat-inactivated as before. 0.1 x volume of the digests were combined and ligated overnight at 16°C in a 20 μl ligation volume. 0.25 and 0.1 x volumes of the ligation mix were used to transform an aliquot of SK+ competent cells, X-gal and IPTG added and the transformation plated to a L-agar plate supplemented with ampicillin. Twelve white colonies were picked from the plate on which the colonies were well sepcrated, and plasmid DNA prepared according to the plasmid miniprepcration method. 5 μl of the DNA was digested with EcoRI and HindIII in a final volume of 25 μl and the digests treated with 1 μl of 1 μg/ml RNase 1 'A' for 15 min at 37°C prior to analysis by 0/8% (w/v) agarose gel electrophoresis against 1 kb molecular weight markers (BRL).

Lane 1.   1 kb molecular weight size markers.

Lane 2.   EcoRI/HindIII digestion of SK.252 1.

Lane 3.   EcoRI/HindIII digestion of SK.252 2.

Lane 4.   EcoRI/HindIII digestion of SK.252 3.

Lane 5.   EcoRI/HindIII digestion of SK.252 4.

Lane 6.   EcoRI/HindIII digestion of SK.252 5.

Lane 7.   EcoRI/HindIII digestion of SK.252 6.

Lane 8.   EcoRI/HindIII digestion of SK.252 7.

Lane 9.   EcoRI/HindIII digestion of SK.252 8.

Lane 10.   EcoRI/HindIII digestion of SK.252 9.

Lane 11.   EcoRI/HindIII digestion of SK.252 10.

Lane 12   EcoRI/HindIII digestion of SK.252 11.

Lane 13   EcoRI/HindIII digestion of SK.252 12.
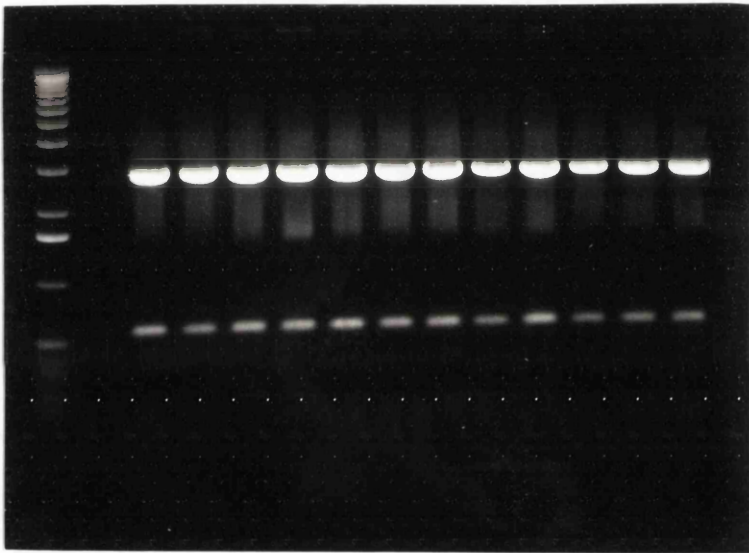
designated SK.252, was prepared by the plasmid maxipreperation method.

4.2.4. The generation of pAC.252.

The 252 coding sequence was subcloned into the plasmid pAC373 in order to generate the recombinant baculovirus required to produce the TGFα precursor in Sf9 cells. 5 μg of pAC373 DNA was digested with KpnI and after 1 h, a sample was removed for analysis by 0.8% (w/v) agarose gel electrophoresis to determine whether digestion by KpnI had gone to completion. The salt concentration was then raised for digestion with BamHI. The DNA was phosphatased, electroeluted, phenol:chloroform extracted, ethanol precipitated and resuspended in 20 μl of TE. 5 μg of the SK.252 DNA was digested with BamHI and KpnI and the insert DNA purified using the same procedure as for pAC373. 0.5μl of the phosphatased vector and 5 μl of the insert were ligated and competent XL-1 blue transformed. The transformation reaction was plated onto a L-agar plate, supplemented with ampicillin and plasmid DNA prepared by the minipreperation method. An aliquot of the DNA was digested with KpnI and then BamHI, treated with RNase 1 "A" and analysed by 0.8% (w/v) agarose gel electrophoresis to determine the presence of the insert. Twenty-two of the 24 samples contained the 252 sequence and a large scale highly pure preperation of the DNA of a correct clone, designated pAC252, was performed by the plasmid maxipreperation method.

4.3. Transfection of Sf9 cells with pAC.252.

The pAC.252 DNA was cotransfected into Sf9 cells with AcNPV DNA and after 4-6 days, the cultures were examined for positive signs of

infection. The extracellular virus particles were harvested and plated on fresh monolayers and the recombinant virus identified by plaque hybridisation. The filters were hybridised with the TGFα cDNA 1.3kb EcoR1 fragment, washed at a stringency of 0.1 x SSC, 0.1% (w/v) SDS at 65°C and exposed to Hyperfilm (Amersham). Four rounds of plaque hybridisation were performed followed by two rounds of visual screening.

4.4. The expression of preproTGFα.

Several potential recombinants were assessed for their ability to direct the synthesis of preproTGFα. 2.5 x 10⁶ Sf9 cells were infected with each of the potential recombinant viruses and the cells harvested at 72 h post infection. The cells were lysed and the medium immunoprecipitated with an anti-TGFα monoclonal antibody (Oncogene Sci Inc). The lysates and immunoprecipitated medium was analysed by 15% (w/v) SDS-PAGE and immunoblotted with the anti-TGFα antibody. As can be seen from Figure 4.5, all recombinants expressed two TGFα specific peptides of approximate molecular weights 18 and 25 kD. No such peptides were recognised in the cells or medium of wild type infected cells. The mature 6 kD form of TGFα could not be immunoprecipitated from the medium nor identified in the cell lysates, suggesting that the processing pathway for the generation of mature TGFα is not operating in insect cells. The apparent molecular weights of the two TGFα species suggest that they represent the entire TGFα precursor in a non-glycosylated and a glycosylated form.

Analysis of CHO cells transfected with the TGFα cDNA detected a diffuse 18-22 kD population of soluble species reduced to 13.5-15 kD on treatment with endoglycosidase F (Bringman et al., 1987). During pulse-

Figure 4.5. 2.5 x 10^6 Sf9 cells were infected with each of four different preproTGFα recombinant viruses. At 72 h post infection, the cells were lysed in Triton x 100 lysis buffer and the medium immunoprecipitated with the anti-TGFα antibody (Oncogene Sci Inc). The lysates and immunoprecipitates were analysed by 15% (w/v) SDS-PAGE and immunoblotted with the anti-TGFα antibody. The blot was exposed to Hyperfilm (Amersham) overnight at -80°C.

Panel A.   Cell lysates.

Lane 1.   wild type infected lysates.

Lane 2.   virus 1 infected lysates.

Lane 3.   virus 2 infected lysates.

Lane 4.   virus 3 infected lysates.

Lane 5.   virus 4 infected lysates.

Panel B.   Immunoprecipitated medium.

Lane 1.   wild type infected medium.

Lane 2.   virus 1 infected medium.

Lane 3.   virus 2 infected medium.

Lane 4.   virus 3 infected medium.

Lane 5.   virus 4 infected medium.

A
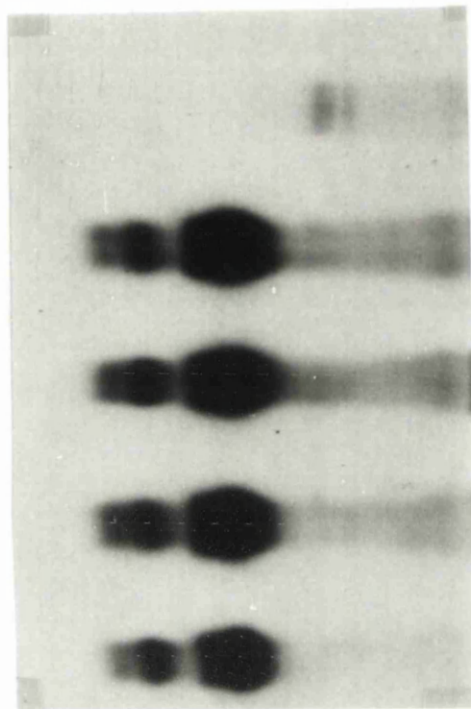
WILD TYPE LYSATE

1 LYSATE

2 LYSATE

3 LYSATE

4 LYSATE

18 kD   25 kD
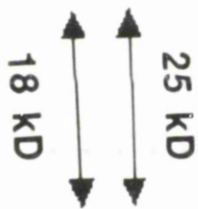
B

WILD TYPE MEDIUM

1 MEDIUM

2 MEDIUM

3 MEDIUM

4 MEDIUM

chase analysis, the first species to arise was a short-lived membrane associated 25 kD species assumed to be the entire glycosylated precursor. These and other results indicated that heterogeneous glycosylation of TGFα forms, termed meso-TGFα released by variable proteolytic processing of the transmembrane precursor, account for the population of soluble TGFα. The observation that the TGFα species can be immunoprecipitated from the medium (see figure 4.5B) suggests that similar processing may be occurring in insect cells, although why the cellular and extracellular forms have the same apparent molecular weights is surprising. Alternatively, the TGFα species could be accumulating in the medium as a consequence of cell lysis.

The problem as to whether partially processed forms of TGFα are actually secreted into the medium was addressed by monitoring TGFα expression in the post infection period. Aliquots of 2.5×10^6 Sf9 cells were infected with the preproTGFα virus and lysed at 24, 48 and 72 h post infection. The medium was immunoprecipitated with the anti-TGFα monoclonal antibody and the lysates and medium analysed by 15% (w/v) SDS-PAGE and immunoblotting with the anti-TGFα monoclonal antibody. As can be seen from Figure 4.6A, the two forms of TGFα are already visible in the cell lysates at 24 h post infection and continue to accumulate over 48-72 h post infection. However, the TGFα species appear in the medium 48 h post infection when the cells are still viable, indicating their appearence is unlikely to be due to cell lysis. An alternative explanation for the presence of these TGFα forms in the medium could be due to the accumulation of extracellular virus particles (ECV) which bud out of the membrane and therefore display any membrane-associated proteins. ECV begin to appear in the medium by 48 h post infection which would be consistent with the results of the

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Figure 4.6A. 2.5 x 10^6 Sf9 cells were infected with the preproTGFα virus and harvested at 24, 48 and 72 h post infection. The cells were lysed with Triton x 100 lysis buffer and the medium immunoprecipitated with the anti-TGFα antibody. The lysates and immunoprecipitates were analysed by 15% (w/v) SDS-PAGE and immunoblotted with the anti-TGFα antibody.

Lane 1.   wild type medium.

Lane 2.   medium 24 h post infection.

Lane 3.   lysate 24 h post infection.

Lane 4.   medium 48 h post infection.

Lane 5.   lysate 48 h post infection.

Lane 6.   medium 72 h post infection.

Lane 7.   lysate 72 h post infection.

Figure 4.6B.   The time course of infection was repeated but the medium was cleared of extracellular virus particles by centrifugation at 100,000g for 30 min prior to immunoprecipitation.

Lane 1.   wild type medium.

Lane 2.   medium 24 h post infection.

Lane 3.   medium 48 h post infection.

Lane 4.   medium 72 h post infection.

Lane 5.   wild type lysate.

Lane 6.   lysate 24 h post infection.

Lane 7.   lysate 48 h post infection.

Lane 8.   lysate 72 h post infection.

A
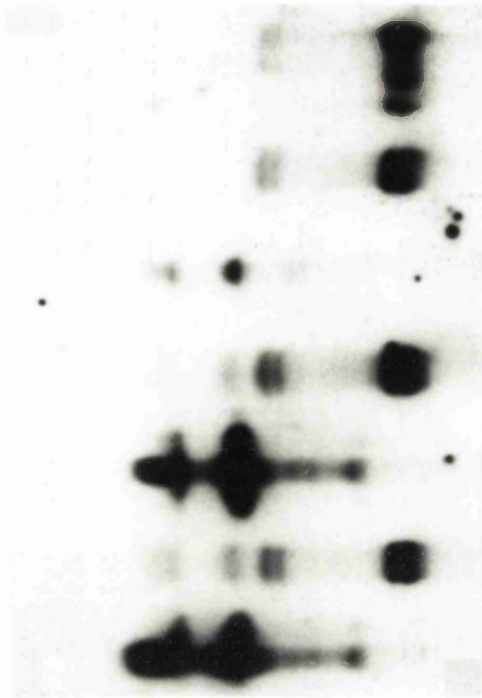
+ WILD TYPE
+ 24H MEDIUM
+ 24H LYSATE
+ 48H MEDIUM
+ 48H LYSATE
+ 72H MEDIUM
+ 72H LYSATE

18 KD   25 KD
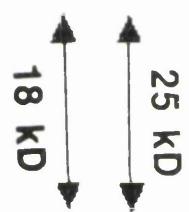
B
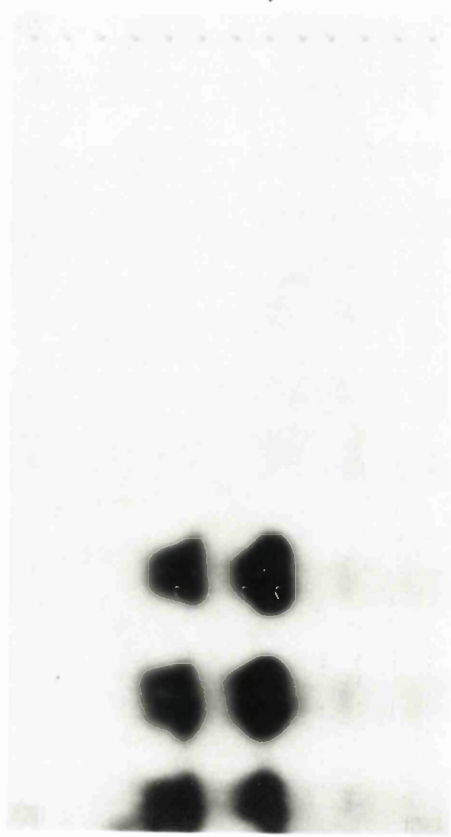
+ WILD TYPE MEDIUM
+ 24H MEDIUM
+ 48H MEDIUM
+ 72H MEDIUM
+ WILD TYPE LYSATE
+ 24H LYSATE
+ 48H LYSATE
+ 72H LYSATE

time course. The time course of expression was repeated but the medium was cleared of the ECV particles by ultracentrifugation at 100,000g for 30 min prior to immunoprecipitation. As can be seen from Figure 4.6B, the two TGFα forms could not be detected in the medium cleared of ECV. These results strongly suggest that the TGFα species are not secreted into the medium as a consequence of partial proteolytic processing but remain membrane-associated. The two forms may therefore represent nonglycosylated and glycosylated versions of the full length preproTGFα.

4.5. Assessment of the glycosylation state of preproTGFα.

To determine whether the large molecular weight polypeptide represents the glycosylated version of preproTGFα, the glycosylation state was assessed by *in vivo* tunicamycin or by *in vitro* endoglycosidase F treatment. 2.5×10^6 Sf9 cells were infected with the preproTGFα virus and labelled with 100 μCi/ml [^{35}S]Cys (specific activity 1300 mCi/ml. Amersham) at 48 h post infection. Tunicamycin treated cells were pretreated for 2 h and then labelled and chased in the presence of 5μg/ml tunicamycin. Cells were then lysed and immunoprecipitated with the anti-TGFα monoclonal antibody. For analysis by endoglycosidase F, the immunoprecipitated preproTGFα was treated at the stated enzyme concentration for 24 h at 37°C. The products of the reactions were analysed by 12.5% (w/v) SDS-PAGE, the gel fixed, 'amplified' (Amersham) and exposed to Hyperfilm (Amersham). As can be seen from Figure 4.7, following tunicamycin or endoglycosidase F treatment the 25 kD form of TGFα is not apparent but the 18 kD form remains. These results indicate that the 25 kD peptide represents the glycosylated full length TGFα precursor. The 18 kD peptide therefore

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Figure 4.7. 2.5 x 10^6 Sf9 cells were infected with the preproTGFα virus and labelled with 100 μCi/ml [^{35}S]Cys (specific activity 1300 mCi/ml, Amersham) at 48 h post infection. Tunicamycin treated cells were pretreated for 2 h and then labelled and chased in the presence of 5 μg/ml tunicamycin. The cells were lysed and immunprecipitated with the anti-TGFα antibody. For analysis by endoglycosidase F, the immunoprecipitate was resuspended in endo F buffer and digested with 10 mU endoglycosidase F for 24 h at 37°C. For digestion with elastase, the immunoprecipitate was resuspended in elastase buffer and treated with 1 or 2 μg of elastase for 1 h at 37°C. The reaction products were analysed by 12.5% (w/v) SDS-PAGE. The gel was fixed, 'amplified' (Amersham), dried and exposed to Hyperfilm (Amersham) overnight at -80°C.

Lane 1.   wild type lysate.

Lane 2.   - tunicamycin.

Lane 3.   + 5 μg/ml tunicamycin.

Lane 4.   + endoglycosidase F.

Lane 5.   + 1 μg elastase.

Lane 6.   + 2 μg elastase.
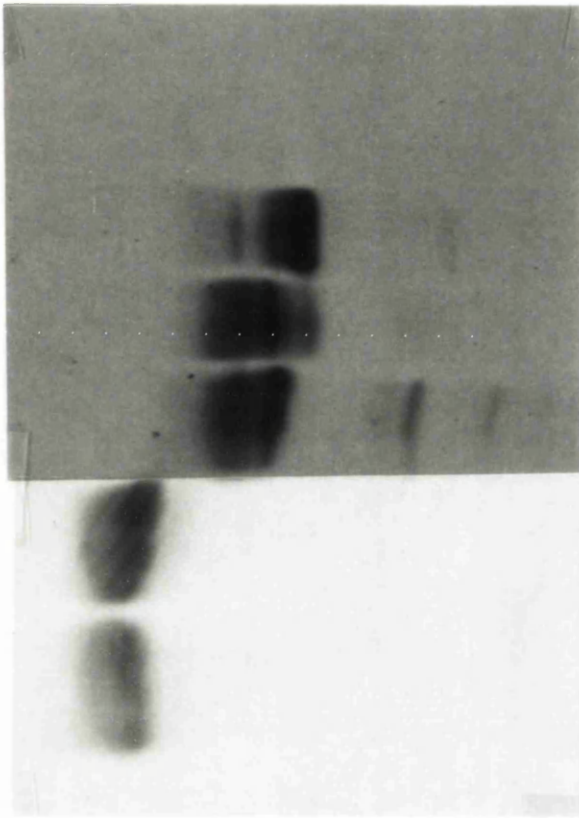
WILD TYPE

- TUNICAMYCIN

+ TUNICAMYCIN

+ ENDOGLYCOSIDASE F

+ 1μg ELASTASE

+ 2μg ELASTASE

25 kD
18 kD

6 kD

TGFα

most likely represents the unglycosylated version of the precursor and is detected potentially as a result of the high expression level exhausting the glycosylation machinery of the cell.

4.6. Elastase treatment of preproTGFα.

PreproTGFα is processed to release the mature 50 amino acid species by the action of a protease with elastase-like specificity (Derynck et al., 1984). The observation that the 6 kD mature form of TGFα could not be immunoprecipitated from the conditioned medium suggested that the specific proteolytic processing pathway does not operate in the insect cells. From sequence analysis of the pAC.252 coding sequence, it can be determined that the recognition sites have not been inadvertently mutated (data not shown). The ability of proteolytic processing to occur at these sites was evaluated by *in vitro* elastase treatment. 2.5×10^6 Sf9 cells were infected with the preproTGFα virus and [^{35}S]Cys labelled at 48 h post infection. The cells were lysed and immunoprecipitated with the anti-TGFα monoclonal antibody. The immunoprecipitates were resuspended in elastase buffer (250 mM Tris-HCl, pH 8.8, 10 mM β-mercaptoethanol, 20 mM EDTA) and digested with 1 or 2 μg elastase at 37°C for 1 h. The reaction products were analysed by 12.5% (w/v) SDS-PAGE and the gel fixed, 'amplified' (Amersham) and exposed to Hyperfilm (Amersham). As can be seen from Figure 4.7, elastase treatment cleaved both the 18 and 25 kD TGFα species into the mature 6 kD form. These results indicate that insect cells do not express the specific protease responsible for the generation of the mature 6 kD TGFα species from its precursor.

4.7. The ability of preproTGFα to stimulate the protein-tyrosine kinase activity of the EGF receptor.

4.7.1. *In vitro* kinase assay.

If the mature TGFα species is correctly folded into its biologically active three-dimensional conformation within the preprotein, it should retain its ability to bind to and activate the EGF receptor. The ability of preproTGFα to stimulate the $EGFR_i$ protein-tyrosine kinase activity was evaluated by *in vitro* kinase assay. Aliquots of 2.5 x 10^6 Sf9 cells were infected with either the preproTGFα or the $EGFR_i$ virus. It had previously been determined that the protein-tyrosine kinase activity of the $EGFR_i$ was EGF stimulatable at 48 h post infection but became EGF-independent by 72 h post infection (Figure 3.16B). The infected cells were harvested at 48 h post infection, combined and resuspended in 5 ml of PBS and incubated at 27^oC for 10 min. The cells were then lysed and immunoprecipitated with the anti-EGF receptor monoclonal antibody, ICR9. An *in vitro* kinase assay was performed on the immunoprecipitates and the reaction products analysed by 7.5% (w/v) SDS-PAGE. As can be seen from Figure 3.17A, membrane-associated preproTGFα is able to stimulate the EGF receptor protein-tyrosine kinase activity. By comparison with the activation achieved with 1 μg/ml EGF, it seems that preproTGFα stimulates the protein-tyrosine kinase activity to a greater extent. This elevated level could be due to the higher relative concentration of ligand in the preproTGFα sample. Since it is not possible to determine the amount of preproTGFα expressed in the insect cells, it is difficult to quantitatively determine the amount present in the sample relative to the EGF standard. In order to clarify this point, it would be necessary to mix varying numbers of preproTGFα

expressing cells with those expressing the EGFR₁ or to completly purify the preproTGFα polypeptide and prepare a sample of known concentration.

4.7.2. Phosphotyrosine immunoblot analysis.

The *in vivo* activation of the EGF receptor protein-tyrosine kinase by preproTGFα treatment was determined by phosphotyrosine immunoblotting. The cells were treated as above, although they were lysed in hot sample buffer containing 500 μM sodium orthovanadate. The lysates were subjected to 7.5% (w/v) SDS-PAGE analysis and the gel immunoblotted with an anti-phosphotyrosine polyclonal antibody. The results in Figure 3.17B indicate that treatment with preproTGFα stimulates the EGFR₁ protein-tyrosine kinase as shown by autophosphorylation on Tyr residues. The results of these two experiments suggest that the membrane-associated TGFα precursor is able to bind to the EGF receptor thereby activating the intrinsic protein-tyrosine kinase activity. The inability of the insect cells to process the precursor to the 6 kD form, coupled with the fact that the preproTGFα cells and not the conditioned medium were mixed with the EGFR₁ suggests that it is the membrane-associated form which possesses the receptor binding capacity.

4.8. DISCUSSION.

The baculovirus expression system has been used successfully to isolate a recombinant virus directing the expression of the TGFα precursor in insect cells (4.4). An anti-TGFα monoclonal antibody specifically recognises two polypeptides of approximate molecular weights 18 and 25 kD, their membrane localisation indicating that the TGFα signal sequence has been recognised by the insect cells (4.4).

These polypeptides most likely represent the nonglycosylated and glycosylated forms of the full length TGFα precursor, the nonglycosylated version accumulating due to expression levels exceeding the capacity of the glycosylation machinery. Tunicamycin and endoglycosidase F treatment converted the 25 kD form into the 18 kD form, thereby confirming this suggestion (4.5).

The suggestion that the 18 kD form of preproTGFα represents the full-length unglycosylated version of preproTGFα is supported by the results of *in vitro* translation experiments employing the rat cDNA sequence (Lueheke et al., 1988). It was demonstrated that a 20 kD membrane-associated species was synthesised when the cDNA was translated in the presence of microsomes, suggesting potential N-linked glycosylation most likely occurring at Asn^{25}. The observation that the 25 kD form of preproTGFα, expressed in the insect cells, is sensitive to treatment with tunicamycin or endoglycosidase F supports the proposal that the full-length preproTGFα is glycosylated. The discrepancy between the molecular weights of the glycosylated preproTGFα species most likely reflects the different systems used to analyse the polypeptide. Indeed, pulse-chase analysis of CHO cells transfected with the human TGFα cDNA estimated the molecular weight of the entire glycosylated precursor to be 25 kD (Bringman et al., 1987).

It has been well documented that transformed cells accumulate substantial amounts of a range of bioactive TGFα species of approximately 20 kD. These forms are of great interest because they constitute the most abundant forms of TGFα found in the medium of various human tumour-derived cell lines (Teixido and Massague, 1988). A heterogeneous population of TGFα species is also produced in retrovirally transformed cells where a soluble, glycosylated 17-19 kD

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species, thought to be a secreted form of the entire precursor, but not the mature 6 kD form, is detected (Ignotz et al., 1986). In contrast JM1 cells, a rat hepatocellular carcinoma cell line, process a 18-21 kD population to the 6 kD growth factor (Lueheke et al., 1988). Analysis of CHO cells transfected with the TGFα cDNA detected a diffuse 18-22 kD population of soluble species reduced to 13.5-15 kD on treatment with endoglycosidase F (Bringman et al., 1987). These and other results indicated that heterogeneous glycosylation of TGFα forms, termed meso-TGFα released by variable proteolytic processing of the transmembrane precursor, account for the population of soluble TGFα. The range in sizes of the reported meso TGFα species may reflect variable processing at the carboxyl terminus of the mature TGFα as a consequence of the action of the specific protease at Ala^{89} and a protease with trypsin-like activity at the dibasic sequence Lys^{96} - Lys^{97} (Derynck et al., 1984). The variable processing will almost certainly depend on the particular cell type or environment studied.

The insect cells do not appear to process the preproTGFα polypeptide by heterologous carboxyl-terminal processing to yield meso TGFα species, nor do they express the specific protease responsible for the release of the mature 50 amino acid TGFα form. It had been postulated that since an unusual protease was involved in the processing of preproTGFα, proteolysis was a regulatory event in the generation of the mature growth factor. The inability of the insect cells to release any of these TGFα species into the medium supports the proposal that the processing arising in tumour-derived cells or transformed cells may be a direct consequence of the expression of a tumour-specific protease or the deregulation of the TGFα processing pathway. However, BHK fibroblasts transfected with the cDNA

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generated a membrane associated 13-17 kD population which was processed to yield the soluble 6 kD form (Gentry et al., 1987). The ability of non-transformed BHK fibroblasts to completely process the precursor indicates that these cells express the protease. It is therefore unlikely that preproTGFα processing is mediated by a tumour-specific protease, although the level of the protease could be elevated in transformed cells, the expression of the protease regulated by the expression of TGFα itself.

It can be seen that transformed cells and cells transfected with the TGFα cDNA variably process the preproTGFα polypeptide. Furthermore, a wide range of high molecular weight TGFα species have been detected as the predominant activity in certain normal and transformed cells, platelets (24 kD), human rhabdomyosarcoma (18 kD, 15-48 kD), melanoma (22.5 kD), mammary carcinoma (30 and 68 kD) and the urine of some cancer patients (30 kD) (Sherwin et al., 1983). The unusually high molecular weight of these TGFα forms may be due to heavy glycosylation or association with other proteins. The availability of a system for the expression of preproTGFα will enable the potential functions of preproTGFα to be assessed and the relevance of the high molecular weight species existing *in vivo* to be determined.

The membrane-associated preproTGFα was able to stimulate the protein-tyrosine kinase activity of the EGF receptor expressed on adjacent cells, suggesting that the release of the mature growth factor into the medium is not required for receptor activation (4.7). These results are in agreement with those of Wong et al. (1989) who transfected BHK fibroblasts with a preproTGFα construct in which all the cleavage sites were mutated. When co-incubated with A431 cells, these cells which express only the membrane-associated preproTGFα

species, stimulated EGF receptor autophosphorylation. Similar results were observed when CHO cells were transfected with a comparable mutated preproTGFα coding sequence (Brachmann et al., 1989). Furthermore, NIH 3T3 cells transfected with the full length EGF cDNA display membrane-associated preproEGF which binds to and activates the EGF receptor expressed on adjacent A431 cells (Mroczkowski et al., 1988, 1989). It has recently been demonstrated that the membrane-associated precursor form of amphiregulin is able to stimulate tyrosine phosphorylation of the putative receptor protein-tyrosine kinase, c-erbB-3 (Plowman et al., submitted).

Thus, the transmembrane form of TGFα can functionally interact with the EGF receptor on adjacent cells. Such interactions between cells could play an important role in various *in vivo* systems known to display elevated levels of preproTGFα such as in solid tumours, where many normal cells are interdispersed with transformed cells, and in proliferating skin keratinocytes. It is therefore possible that TGFα producing cells transfer a mitogenic signal to normal fibroblasts not only by releasing the growth factor, but also via cell-cell contact through preproTGFα and the EGF receptor. The signal generated by the membrane-bound TGFα may serve to prolong the mitogenic signal by preventing the internalisation and down-regulation of the EGF receptor. Furthermore, the action of a membrane-associated growth factor could stimulate target cells in a far more selective way than its diffusible counterpart.

The postulated involvement of preproTGFα in mediating physiological cell-cell recognition events is supported by the discovery of EGF-like repeats in a wide range of both vertebrate and invertebrate proteins intimately involved in cell-cell interactions. The EGF-like

repeats in extracellular matrix glycoproteins and the extracellular domains of some membrane proteins form parts of large molecules or supramolecular assemblies which are ideal for relaying growth and differentiation signals to adjacent cells in a specific and vectorial manner. The existence of the EGF-like repeat in several proteins of the Drosophila neurogenic locus have implicated them in the cellular communication involved in the control of cell fate. The involvement of EGF-like repeats in other developmentally important invertebrate proteins has further demonstrated their role in cell-cell signalling.

The presence of several copies of the EGF-like repeat in the extracellular domain of the EGF receptor suggested that it may function as a receptor for an unknown ligand (Pfeffer and Ullrich, 1985), the size and membrane-localisation of preproTGFα implying a similar role for preproTGFα. Furthermore, the extremely high degree of homology existing in the cytoplasmic domain predicts an important role for preproTGFα potentially in intracellular signalling.

Thus, preproTGFα may play a number of physiologically relevant roles as well as ensuring the correct transport and secretion of the growth factor. The expression of preproTGFα in the baculovirus system will enable further investigation into these alternative roles for preproTGFα. In addition, the large scale expression and purification of preproTGFα could prove instrumental in the isolation of the TGFα-specific protease from the conditioned medium of transformed cells.

CHAPTER FIVE.

STRUCTURE-FUNCTION ANALYSIS OF TGFα.

5.1. INTRODUCTION.

TGFα has been purified from both human (Derynck et al., 1985) and rat (Lee et al., 1985) sources, sequence comparison indicating that 92% homology exists between the two species. EGF has been purified from several sources and the highly conserved nature of its primary sequence indicates that the molecule folds into a precise three-dimensional conformation that must be strictly adopted if receptor-binding is to be achieved (Savage et al., 1973). Since it has been shown that TGFα competes with EGF for binding to the EGF receptor (Todaro and DeLarco, 1978), it can be predicted that the tertiary structures of TGFα and EGF are basically equivalent. Indeed, rat TGFα shares 33% homology with murine EGF and 44% with human EGF (Lee et al., 1985). All of the six Cys residues of EGF are conserved at identical positions within TGFα and establish identical disulphide bonds, thereby defining the same three looped regions (Savage et al., 1973). Although the extensive degree of sequence homology existing between EGF and TGFα allows binding to a common receptor, it is not sufficient to allow antigenic cross-reactivity (Figure 5.1).

5.1.1. ^{1}H NMR analysis of the tertiary structure of TGFα.

Recently, preliminary ^{1}H NMR analysis has led to the proposal of a three-dimensional structural model for human TGFα (Montelione et al., 1989) (Figure 5.2). The model predicts that TGFα adopts similar chain folds to EGF and is composed of amino and carboxyl terminal antiparallel β sheets. The larger N-terminal antiparallel β sheet involves Gly[19] -
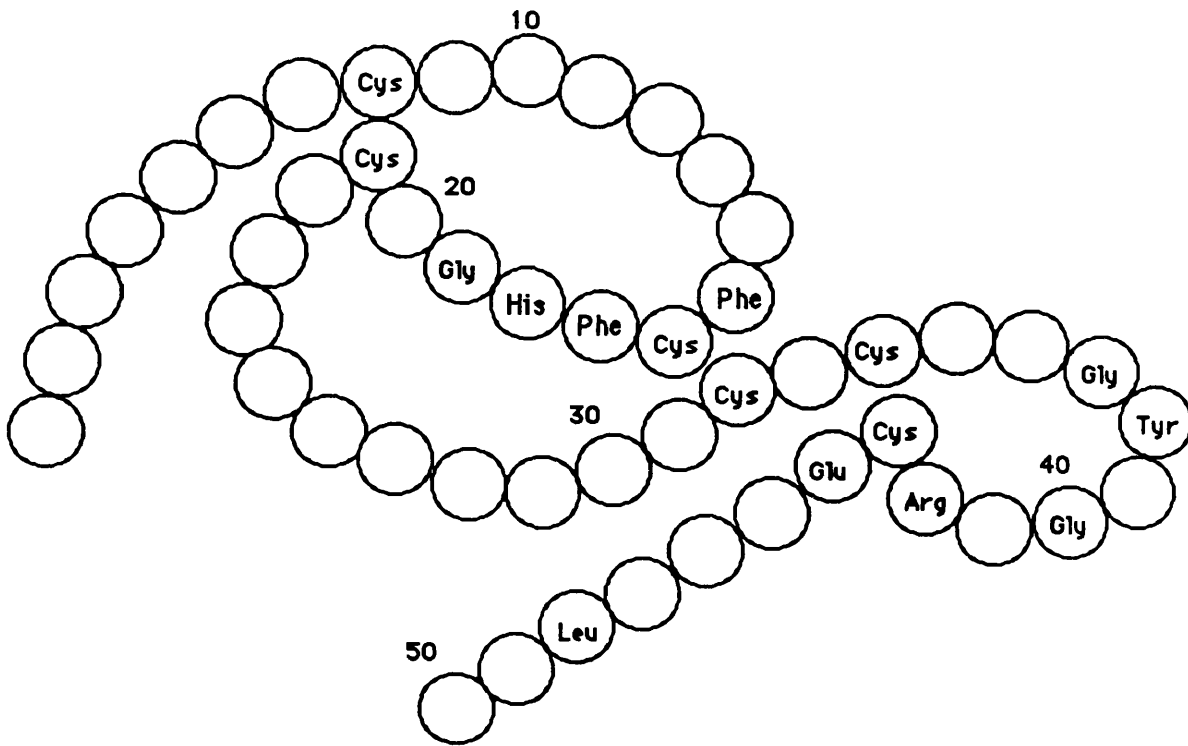
Figure 5.1. The amino acid sequence of human TGF α indicating the residues that are conserved or conservatively changed in the EGF-like family of growth factors.

V25-Q26-E27-D28

H—C(L24)
N—H
O=C
(F23)C—H
H—N
C=O
H—C(R22)
N—H
O=C
(C21)C—H
H—N
C=O
H—C(T20)
N—H
O=C
(G19)C—H

(K29)C—H
O=C
N—H
H—C(P30)
C=O
H—N
(A31)C—H
O=C
N—H
H—C(C32)
C=O
H—N
(V33)C—H
O=C
N—H
H—C(C34)

H35

G40-A41-R42-C43-E44

C=O
H—C(V39)
N—H
O=C
(Y38)C—H
H—N

H—N
(H45)C—H
O=C
N—H
H—C(A46)
C=O
H—N
(D47) C—H

S36-G37
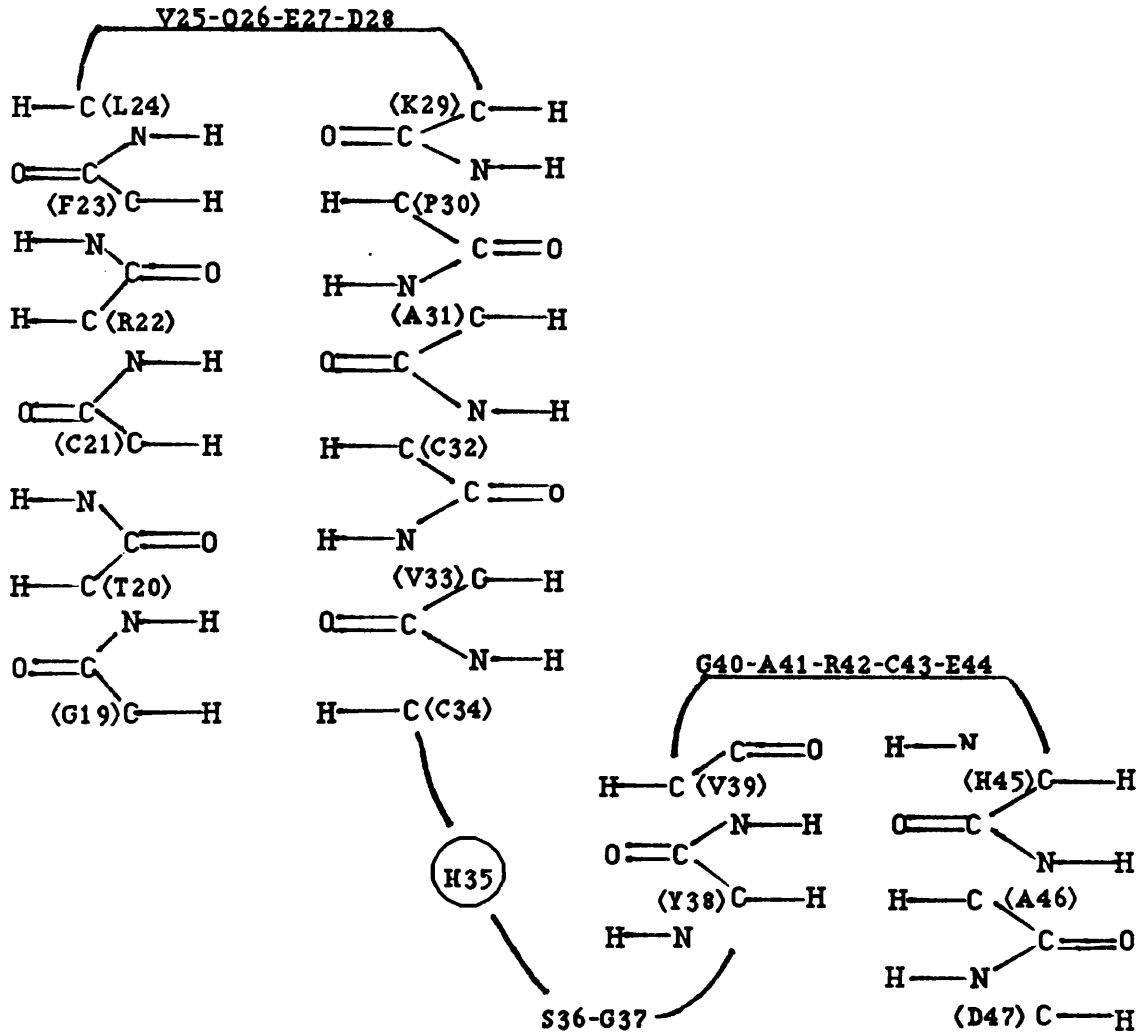
Figure 5.2. Secondary structures in human TGFα, planar schematic representations of the antiparallel β sheets.

Leu^{24} and Lys^{29} - Cys^{34} as the two strands, seperated by a type I β-bend at Val^{25} - Asp^{28}. The region Ser^{11} - Phe^{15} represents a multiple bend region, with Ser^{11} - Gln^{14} involved in a type I β-bend, leading into the first strand of the N-terminal β sheet. A type II β-turn involving His^{35} - Tyr^{38} leads into the smaller antiparallel β-sheet between Tyr^{38}/ Val^{39} and His^{45}/ Ala^{46}. It was determined that the amino terminal (Val^{1} - Asp^{7}) was not involved in any rigid secondary or tertiary structure (Brown et al., 1989; Montelione et al., 1989). Furthermore, there was no evidence for the formation of a third antiparallel strand between the amino terminus and the N-terminal domain β-sheet as was reported for murine EGF (Montelione et al., 1987). These results are in direct agreement with those of Simpson et al. (1985) who observed that amino terminal deletions in rat TGFα did not interfere greatly with the ability to bind to the EGF receptor, suggesting that this region of the molecule was unlikely to contribute to the binding site. However, ^{1}H NMR analysis has not as yet provided sufficient information to determine the chain folding of the carboxyl terminal segment Asp^{47} - Ala^{50}. It can therefore be concluded that many of the secondary structural elements reported for EGF are found in TGFα, folding the molecule into a similar bilobed structure. The only significant differences between the structure of the two molecules are observed in response to changes in pH and in their conformational flexibility (Campbell et al., 1989). The three-dimensional structure of EGF varies little between pH 3 and pH 8 and displays limited flexibility. In contrast, the tertiary structure of TGFα varies according to pH, at pH 6.5 an EGF-like structure is observed, whereas at pH 3.8 there is an interconversion between at least two conformations.

5.1.2. A model for the tertiary structure of TGFα.

The ^{1}H NMR three-dimensional model can be used to predict regions of the molecule that may be involved in the generation of the receptor binding site. However, these predictions can be verified and extended in the light of those residues predicted to play an important structural or functional role, by virtue of their highly conserved nature. It can be predicted that residues involved in receptor binding will be conserved or conservatively changed throughout the EGF-like family of growth factors. As discussed in Chapter 3, there are sixteen such residues that may be responsible for folding the molecule into a suitable conformation for receptor binding (structural) or that may participate directly in receptor binding (functional). Structural residues should be conserved or conservatively changed not only in the growth factor family but also in the EGF-like domains of a wide range of proteins where they have been implicated in directing ligand-receptor interactions (see Chapter 4). However, those residues involved directly in specific interactions with the EGF receptor will be conserved only within those molecules that bind to the receptor. A structural role has been suggested for the three Gly residues which, by virtue of their short side-chains are often involved in tight β turns. The six Cys residues are an absolute requirement for the establishment of the biologically active conformation and are also classified as structural. The only other residue that is conserved throughout the growth factor family and the EGF-like domains is Tyr^{38} (Tyr^{37} in EGF), suggesting a structural role.

The three-dimensional model for human EGF predicts that the remaining conserved residues, Tyr^{13} (Phe^{15}), Leu^{15} (Phe^{17}), His^{16}

(His[18]), Arg[41] (Arg[42]), Gln[43] (Glu[44]) and Leu[47] (Leu[48]) (residues in brackets refer to human TGFα) lie on the same face of the molecule and are involved in the establishment of the receptor recognition site (Campbell et al., 1989). The failure of a panel of synthetic fragments, corresponding to different regions in the primary structure of TGFα, to bind to the receptor supports the proposal that several areas of the molecule contribute to the receptor recognition site (Defeo-Jones et al., 1988). ^{1}H NMR analysis of human EGF has suggested that His[16] and Gln[43] are located at the interface of the N- and C-domains where they have been implicated in maintaining the relative orientation of the two domains (Campbell et al., 1989). Thus, a similar structural role can be envisaged for the corresponding residues in human TGFα (His[18] and Glu[44]). A functional role can therefore be anticipated for the remaining conserved residues of human TGFα, Phe[15], Phe[17], Arg[42] and Leu[48]. The putative involvement of any of these residues in the maintenance of the tertiary structure or the establishment of the receptor recognition site can be verified by mutagenesis, thereby determining the nature of the sidechain responsible.

The elucidation and comparison of the tertiary structures of EGF and TGFα will lead to a better understanding of which regions of the molecules are important for receptor binding. The determination of the conformation of TGFα is basic to the design of antagonists, which could represent important therapeutic tools in the treatment of certain malignancies which are known to express elevated levels of the EGF receptor. Such an antagonist would bind to the EGF receptor, but would be unable to induce proliferation and transformation. It will therefore be necessary to dissociate the interactions responsible for receptor binding from those involved in signal transduction.

The model for the three-dimensional structure of TGFα can only be verified and improved by further ^{1}H NMR analyses and diffraction techniques employing crystals of TGFα. The progress of these investigations is hampered by the requirement of these techniques for large amounts of material, with 20 mg required for each ^{1}H NMR analysis and 100 mg needed to ensure crystallisation. TGFα is secreted at very low levels into the medium by transformed cells. Indeed, only 1.5μg of TGFα was isolated from 136 litres of medium conditioned by a melanoma cell line (Marquardt and Todaro, 1982). Expression systems have been devised for the production of recombinant TGFα in *E. coli* and yeast (Derynck et al., 1984, 1985). However, in common with the expression of recombinant EGF (see Chapter 3), these systems fail to produce biologically active TGFα in the quantites required for crystallisation.

5.1.3. Aims of this study.

There is obviously an absolute requirement for an expression system producing large quantities of native TGFα and mutant peptides before the current model for the three-dimensional structure of TGFα can be extended. The generation of large amounts of pure TGFα will enable further ^{1}H NMR investigations to proceed and TGFα crystals to be grown. The availability of crystals will make the complete elucidation of the tertiary structure of TGFα possible through diffractional analysis. Mutational analysis of residues predicted to play a role in the receptor binding site may be able to distinguish between those residues involved in binding to the receptor and those responsible for its activation, leading to the design of antagonists. The comparison of the tertiary structures of TGFα and EGF, and their co-crystallisation with the EGF

receptor will lead to the elucidation of the receptor-ligand binding site. This study describes the establishment of an expression system for the production of human TGFα in insect cells. This system will generate large quantities of biologically active TGFα for biophysical characterisation.

5.2. RESULTS.

5.2. CONSTRUCTION OF PACC 254.

5.2.1. Construction of Mp19.253.

The processing of the mature 50 amino acid TGFα species from the precursor is extremely specific (Derynck et al., 1984) (see Chapter 4). Both the amino and carboxyl termini processing sites are at the tripeptide Ala - Val - Val with the cleavage occurring between the Ala residue and the first Val residue. In the precursor sequence, amino terminal processing occurs between Ala^{39} and Val^{40} and the carboxyl terminal processing between Ala^{89} and Val^{90} (Figure 5.3). The first step in the construction of the coding sequence was to introduce a stop codon adjacent to Ala^{89} so as to prevent read through into the adjacent coding sequence. The starting material for these manipulations was Mp19.252 so that the re-introduced ATG and the Kozak consensus for translation were present (see Chapter 4). The restriction site for the enzyme BamHI was also introduced at the 3' end of the coding sequence for the subsequent introduction of the sequence, on an EcoRI-BamHI fragment, into the baculovirus expression vector pAcC12. pAcC12 was derived by the insertion of a polylinker into the BamHI site of pAc373 (Cetus Corp.) (Figure 5.4). The oligonucleotide synthesised for the *in vitro* mutagenesis reaction was a 30mer with the sequence:
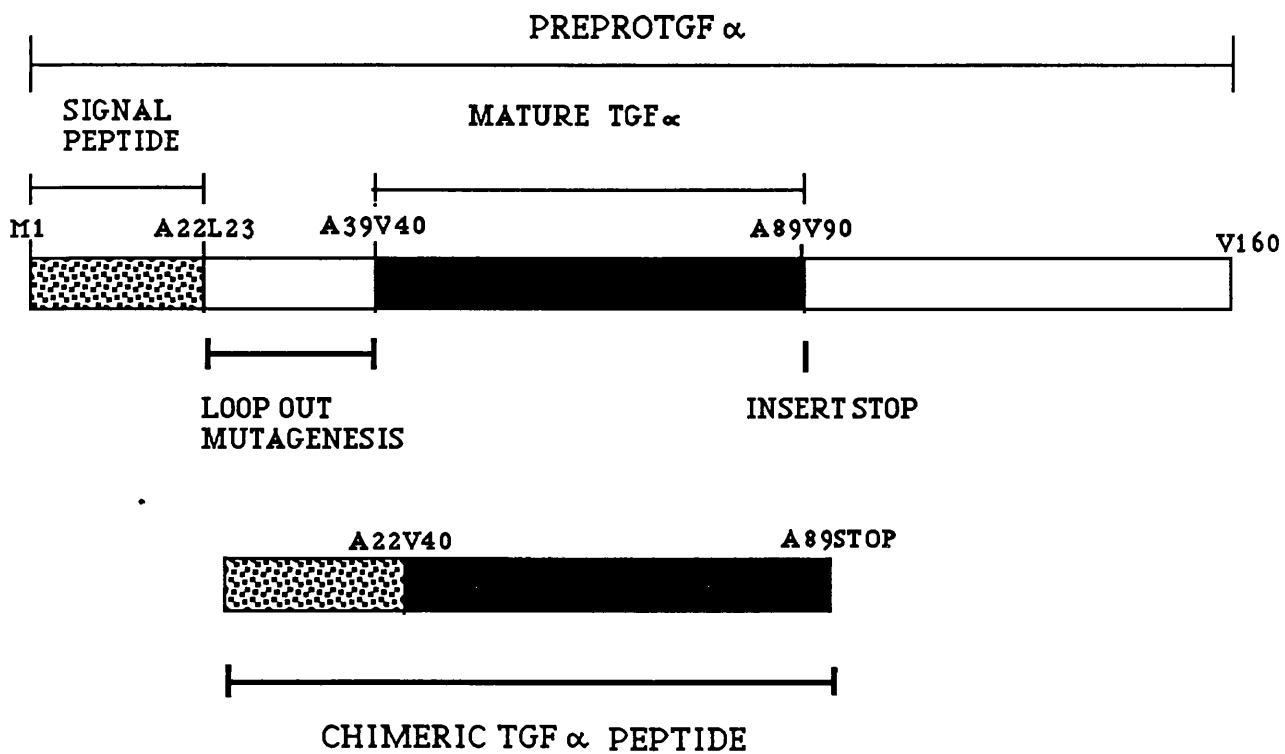
Figure 5.3. The scheme for the generation of the chimeric TGF α molecule between the mature TGF α peptide and the signal peptide.

Figure 5.4.   The restriction endonuclease map of the transfer vector pacC12 (Cetus Corp.).   The plasmid was derived from pAc373 with the insertion of a polylinker at the BamHI site.   The polylinker contains the following unique restriction endonuclease cleavage sites, SmaI, PstI, EcoRI, BglII, SacI and NcoI.
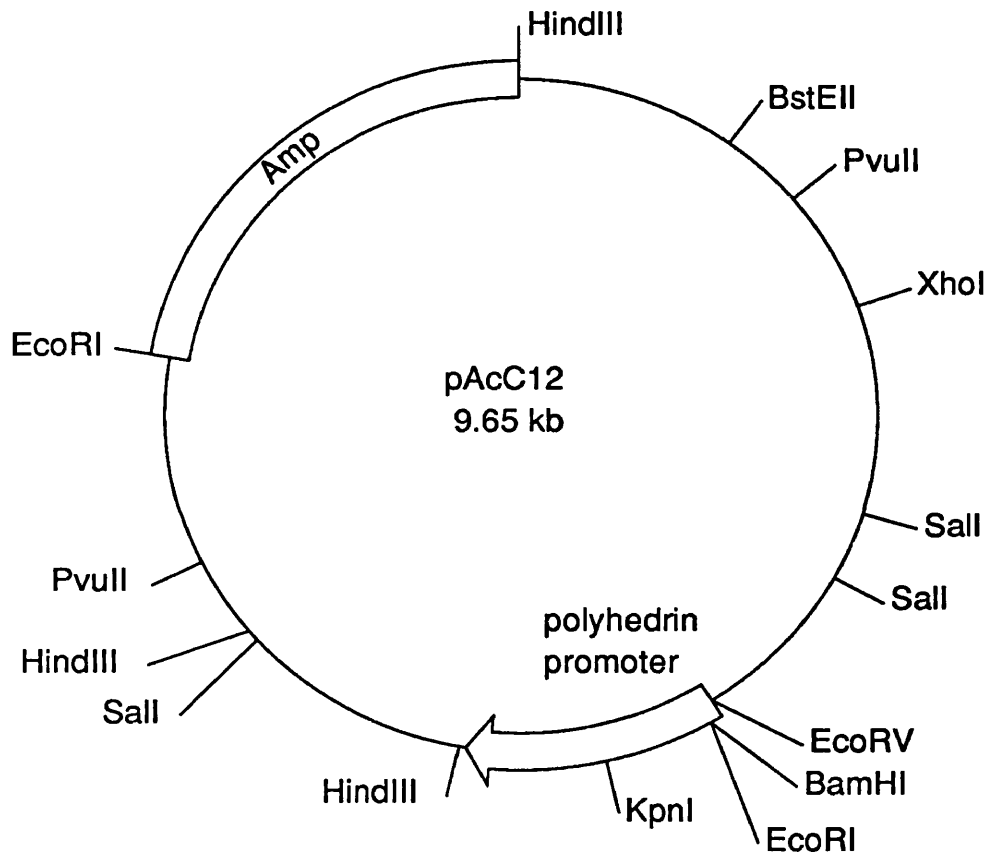
STOP   BamHI

5' CTCCTGGCCTAGGTGGATCCCAGCCAGAAG 3'

ACTUAL SEQUENCE

LeuLeuAla^{89}

5' CTCCTGGCCGTGGTGGCTGCCAGCCAGCCAGAAG 3'

MUTATED SEQUENCE

Ala^{89} OP   BamHI

5' CTCCTGGCCTAGGTGGATCCCAGCCAGAAG 3'

The oligonucleotide designated 253 was synthesised, 20 μg purified by 15% (w/v) PAGE and a stock prepared at 5 OD_{260} units/ml. The *in vitro* mutagenesis reaction was carried out using the *in vitro* mutagenesis kit (Amersham) and one single-stranded Mp19.252 minipreparation as the template and the purified oligonucleotide stock. The mutant DNA was used to transform an aliquot of competent XL-1 blue and the transformation reaction plated onto an L-agar plate, supplemented with ampicillin. Single-stranded M13 DNA was prepared from several plaques and subjected to 'T-tracking'. A potential mutant, designated Mp19.253 was sequenced and then subcloned into the Bluescript vector SK^{+} to give a stable high concentration stock of the DNA.

5.2.2. The generation of Mp19.254.

The intervening DNA sequence between the 3' end of the sequence encoding the signal peptide and the 5' end of the sequence

encoding the mature TGFα species was removed by *in vitro* mutagenesis. The precise site for the cleavage of the signal sequence from the rest of the TGFα precursor is unknown and therefore, the "(-3, -1)" rule for assigning signal sequence cleavage sites was applied (von Heijne 1983, 1986). It has been suggested that the residue in position -1 (relative to the cleavage site) must be small and that at position -3 must not be aromatic, charged or large and polar. These rules for signal peptide cleavage can be applied to the following sequence in the TGFα precursor protein:

$$Cys^{20} \ Gln \ Ala^{22}$$

where residue -1 = Ala and residue -3 = Cys. The 91 bases between the codon for Ala^{22} and the codon for the 5' end of the mature TGFα at Val^{40} were removed by *in vitro* mutagenesis. The oligonucleotide employed was a 24mer which overlapped on either side of the junction by 12 nucleotides, ensuring that the oligonucleotide hybridised strongly.

ACTUAL SEQUENCE

$$Ala^{22} \qquad\qquad\qquad Val^{40}$$

5' GCGTGCCAGGCCTTGGAC (XXX) 13 GCAGCAGTGGTGTCCCATTTT

MUTATED SEQUENCE

EcoR1 Met^1 $Ala^{22}Val^{40} \ Ala^{89}$ BamH1

5'GAATTCAAGCGTTCTACCATG----GCCGTG---GCCTAGGTGGATCCC3'

The 24mer designated 254 was synthesised, 20 μg purified by 15% (w/v/) PAGE and a stock solution prepared at 5 OD_{260} units/ml. The *in*

vitro mutagenesis reaction was performed on a single-stranded minipreparation of Mp19.253 DNA using the oligonucleotide stock solution according to the *in vitro* mutagenesis kit (Amersham). The mutant DNA was used to transform an aliquot of frozen competent XL-1 blue and plated onto an L-agar plate, supplemented with ampicillin. Single-stranded DNA was prepared from twenty-four plaques and analysed by 'T-tracking'. The entire sequence of one of the mutants, designated Mp19.254, was determined to ensure that the coding sequence did in fact contain all three mutations (Figure 5.5).

The 254 insert was 'shot gun' cloned into the Bluescript vector, SK$^+$ following digestion with EcoRI and BamHI so as to generate a stable stock at high concentration for subsequent cloning procedures. Plasmid DNA was isolated according to the minipreperation method and an aliquot digested with EcoRI and BamHI, treated with RNase 1 'A' and analysed by 0.8% (w/v) agarose gel electrophoresis. Eleven of the twelve samples contained the insert and the DNA from one clone designated SK.254, was prepared by the plasmid maxipreperation method (Figure 5.6).

5.2.3. The generation of pAcC.254.

The 254 coding sequence was subcloned into pAcC12 for the expression of the mature TGFα peptide in Sf9 cells. 5 μg of pAcC12 DNA was digested with EcoRI and BamHI and treated with CIP. The DNA was purified by electroelution, phenol: chloroform extracted, ethanol precipitated and resuspended in 20 μl of TE. 5 μg of SK.254 was digested with EcoRI and BamHI and the insert purified in the same way. 5 μl of the purified 254 insert and 0.5 μl of the phosphatased pAcC12 DNA were ligated overnight and 0.1 x volume used to transform an aliquot of

Figure 5.5. M13 dideoxy sequencing of Mp19.254. The first set of four tracks represents Mp19.254 labelled in G, A, T and C respectively and shows the sequence of the oligonucleotides 253 and 254. The second set of four tracks represents a shorter run of the previous samples and shows the sequence of the oligonucleotide 252.
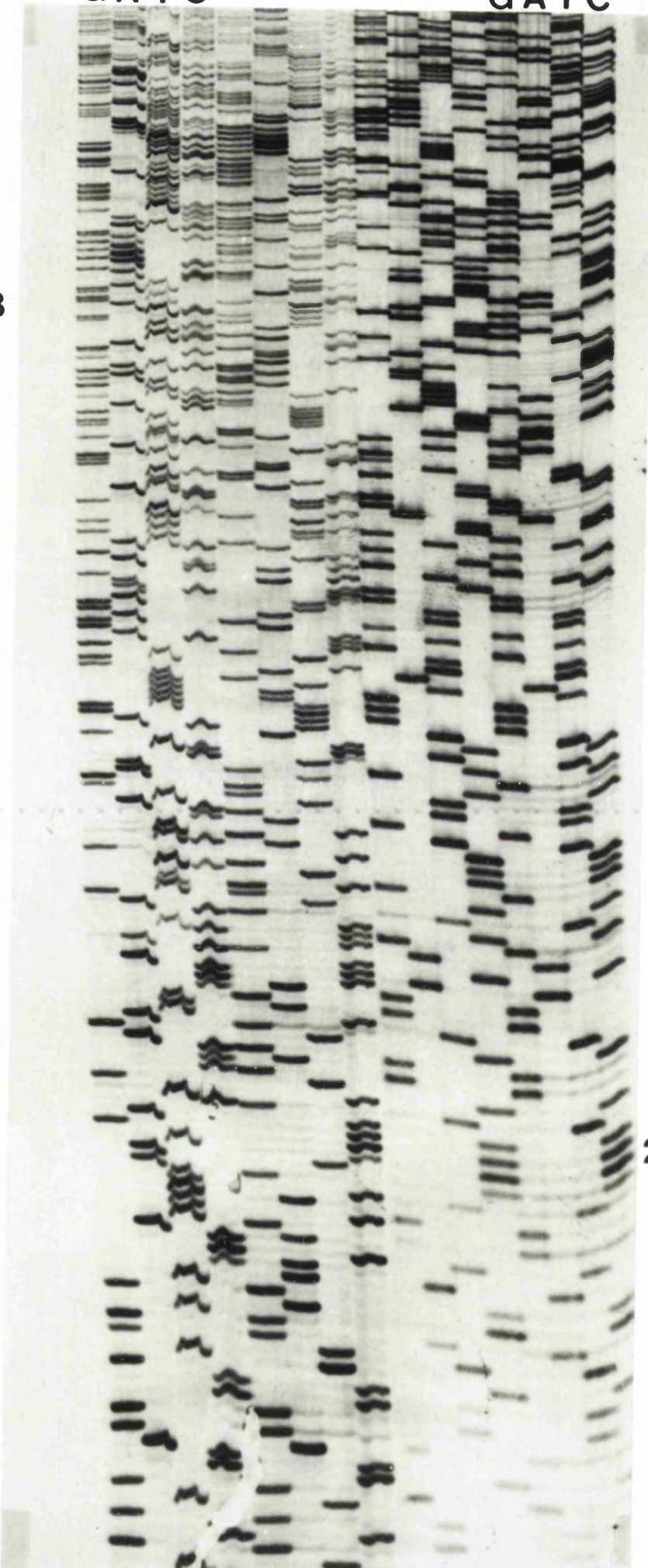
Figure 5.6. The DNA of one double stranded (RF) miniprepcration of Mp19.254 was digested at 37°C for 1 h with EcoRI and BamHI in a final volume of 30 μl. The enzymes were inactivated by incubation at 75°C for 10 min. 1μg of SK+ was also digested with EcoRI and BamHI in a final volume of 30 μl and the enzymes heat-inactivated as before. 0.1 x volume of the digests were combined and ligated overnight at 16°C in a 20 μl ligation volume. 0.25 and 0.1 x volumes of the ligation mix were used to transform an aliquot of SK+ competent cells, X-gal and IPTG added and the transformation plated to a L-agar plate supplemented with ampicillin. Twelve white colonies were picked from the plate on which the colonies were well sepcrated, and plasmid DNA prepared according to the plasmid miniprepcration method. 5 μl of the DNA was digested with EcoRI and BamHI in a final volume of 25 μl and the digests treated with 1 μl of 1 μg/ml RNase 1 'A' for 15 min at 37°C prior to analysis by 0/8% (w/v) agarose gel electrophoresis against 1 kb molecular weight markers (BRL).

Lane 1.   1 kb molecular weight size markers.
Lane 2.   EcoRI/BamHI digestion of SK.254 1.
Lane 3.   EcoRI/BamHI digestion of SK.254 2.
Lane 4.   EcoRI/BamHI digestion of SK.254 3.
Lane 5.   EcoRI/BamHI digestion of SK.254 4.
Lane 6.   EcoRI/BamHI digestion of SK.254 5.
Lane 7.   EcoRI/BamHI digestion of SK.254 6.
Lane 8.   EcoRI/BamHI digestion of SK.254 7.
Lane 9.   EcoRI/BamHI digestion of SK.254 8.
Lane 10.   EcoRI/BamHI digestion of SK.254 9.
Lane 11.   EcoRI/BamHI digestion of SK.254 10.
Lane 12.   EcoRI/BamHI digestion of SK.254 11.
Lane 13.   EcoRI/BamHI digestion of SK.254 12.
Lane 14.   EcoRI/BamHI digestion of SK.253.
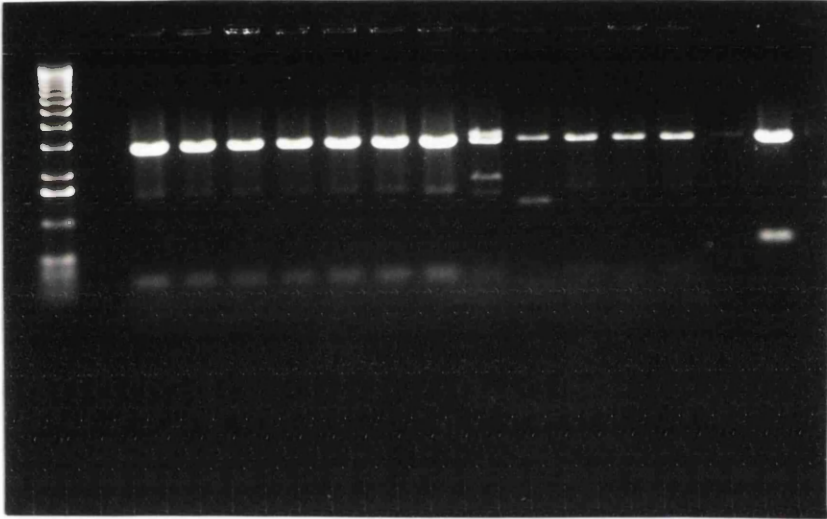
competent XL-1 blue. The transformation was plated onto an L-agar plate supplemented with ampicillin and plasmid DNA prepared by the minipreperation method. An aliquot of the DNA was digested with EcoRI and BamHI, treated with RNase 1 "A" and analysed for the presence of inserts by 0.8% (w/v) agarose electrophoresis. All of the 24 miniprep rations contained the 254 insert and the DNA from one, denoted pAcC.254, was prepared according to the plasmid maxipreparation method.

5.2.4. Transfection of Sf9 cells with pAcC.254.

Sf9 cells were cotransfected with pAcC.254 DNA and AcNPV DNA and extracellular virus particles harvested after 4-6 days. The virus was plated to fresh monolayers and the recombinant virus particles identified by four rounds of plaque hybridisation, using the TGFα cDNA as a probe, followed by two rounds of visual screening.

5.3. The expression of TGFα¡.

Several recombinants were assessed for their ability to direct the synthesis of TGFα¡. 2.5×10^6 Sf9 cells were infected with each of the recombinant viruses and after 72 h, the cells examined for TGFα¡ expression by immunoblot analysis. The cells were lysed with hot sample buffer and analysed by 15% (w/v) SDS-PAGE. The proteins were transferred to nitrocellulose and probed with the anti-TGFα monoclonal antibody (Oncogene Sci Inc). The results in Figure 5.7A indicate that a small peptide of molecular weight approximately 6 kD was specifically recognised by the anti-TGFα monoclonal antibody. No such peptide was recognised in the lysates of wildtype infected cells. All of the recombinant viruses direct the synthesis of TGFα¡ at similar levels.

Figure 5.7A. 2.5×10^6 Sf9 cells were infected with each of eight recombinant TGFα$_i$ viruses and harvested at 72 h post infection. The cells were lysed with hot sample buffer, analysed by 15% (w/v) SDS-PAGE and immunoblotted with the anti-TGFα antibody.

Lane 1.   wild type lysate.

Lane 2.   virus 1.

Lane 3.   virus 2.

Lane 4.   virus 3.

Lane 5.   virus 4.

Lane 6.   virus 5.

Lane 7.   virus 6.

Lane 8.   virus 7.

Lane 9.   virus 8.

Figure 5.7B. 2.5×10^6 Sf9 cells were infected with TGFα$_i$ virus and harvested at 48 h, 72 h and 96 h post infection. The cells were lysed in hot sample buffer and the medium immunoprecipitated with the anti-TGFα antibody. The products were analysed by Tricine-SDS-PAGE and immunoblotted with the anti-TGFα antibody.

Lane 1.   wild type lysate.

Lane 2.   lysate 48 h post infection.

Lane 3.   lysate 72 h post infection.

Lane 4.   lysate 96 h post infection.

Lane 5.   wild type medium.

Lane 6.   medium 48 h post infection.

Lane 7.   medium 72 h post infection.

Lane 8.   medium 96 h post infection.

A
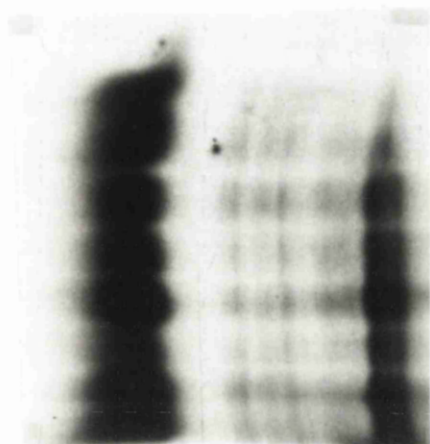
WILD TYPE
1
2
3
4
5
6
7
8

TGFα
6 kD

B
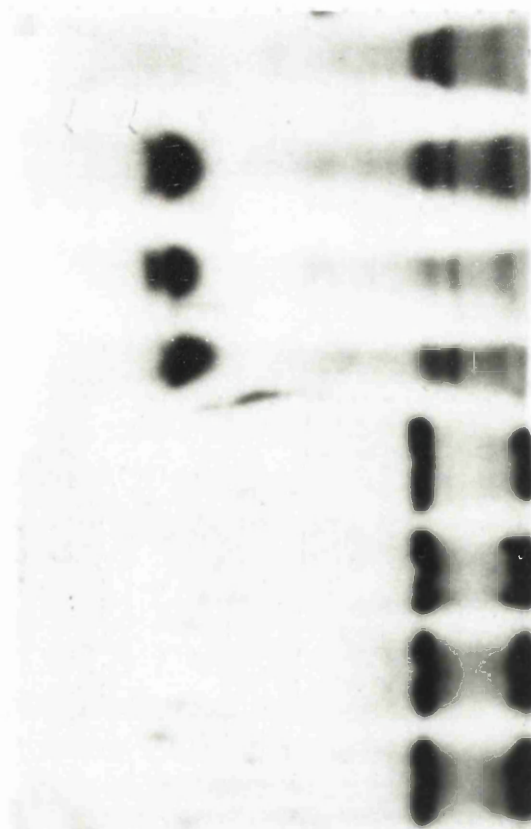
WILD TYPE LYSATE

48H LYSATE

72H LYSATE

96H LYSATE

WILD TYPE MEDIUM

48H MEDIUM

72H MEDIUM

96H MEDIUM

However, it is difficult to determine an accurate figure for the molecular weight of a small peptide by high percentage SDS-PAGE since they migrate as diffuse bands.

The production of TGFα$_i$ and its secretion into the medium was assessed during the post infection period. Aliquots of 2.5×10^6 Sf9 cells were infected with the TGFα$_i$ virus and harvested at 48, 72 and 96 h post infection. The cells were lysed in hot sample buffer and the medium immunoprecipitated with the anti-TGFα monoclonal antibody. The samples were analysed by Tricine-SDS-PAGE (Schagger and von Jagow, 1987). The superiority of this method for resolving proteins in the 5-30kD molecular weight range is due to the introduction of tricine as the trailing ion in place of glycine. In the presence of tricine, the stacking and destacking of small proteins can be achieved at the same pH as the separating gel and at low concentrations of acrylamide. As a result, low molecular weight peptides migrate smoothly between the sample and separating gel and move as tight, discrete bands. The gel was then immunoblotted with the anti-TGFα monoclonal antibody. As can be seen from Figure 5.7B, the TGFα$_i$ migrates as a tight band allowing the molecular weight to be confirmed as 6 kD. The expression of TGFα$_i$ in the lysates is maximal at 48 h post infection and continues to be expressed throughout the following 48 h time period. Although no TGFα$_i$ appears to have been immunoprecipitated from the medium, longer exposure of the blot indicates that TGFα$_i$ is in fact secreted into the medium. The observation that the TGFα$_i$ present in the lysates has a molecular weight of 6 kD indicates that the signal peptide has been removed prior to secretion of TGFα$_i$. The TGFα signal sequence has therefore been recognised by the insect cells allowing the secretion of the TGFα$_i$. The difficulty in detecting the secreted form may be due to

However, it is difficult to determine an accurate figure for the molecular weight of a small peptide by high percentage SDS-PAGE since they migrate as diffuse bands.

The production of $TGF\alpha_i$ and its secretion into the medium was assessed during the post infection period. Aliquots of 2.5×10^6 Sf9 cells were infected with the $TGF\alpha_i$ virus and harvested at 48, 72 and 96 h post infection. The cells were lysed in hot sample buffer and the medium immunoprecipitated with the anti-TGFα monoclonal antibody. The samples were analysed by Tricine-SDS-PAGE (Schagger and von Jagow, 1987). The superiority of this method for resolving proteins in the 5-30kD molecular weight range is due to the introduction of tricine as the trailing ion in place of glycine. In the presence of tricine, the stacking and destacking of small proteins can be achieved at the same pH as the separating gel and at low concentrations of acrylamide. As a result, low molecular weight peptides migrate smoothly between the sample and separating gel and move as tight, discrete bands. The gel was then immunoblotted with the anti-TGFα monoclonal antibody. As can be seen from Figure 5.7B, the $TGF\alpha_i$ migrates as a tight band allowing the molecular weight to be confirmed as 6 kD. The expression of $TGF\alpha_i$ in the lysates is maximal at 48 h post infection and continues to be expressed throughout the following 48 h time period. Although no $TGF\alpha_i$ appears to have been immunoprecipitated from the medium, longer exposure of the blot indicates that $TGF\alpha_i$ is in fact secreted into the medium. The observation that the $TGF\alpha_i$ present in the lysates has a molecular weight of 6 kD indicates that the signal peptide has been removed prior to secretion of $TGF\alpha_i$. The TGFα signal sequence has therefore been recognised by the insect cells allowing the secretion of the $TGF\alpha_i$. The difficulty in detecting the secreted form may be due to

the low levels of the growth factor present in the medium or the low affinity of the antibody.

5.4. The receptor binding activity of TGFα$_i$.

From the previous results it can be determined that insect cells infected with the recombinant virus will synthesise TGFα$_i$. However, the question of whether the peptide is correctly folded into its active conformation must be addressed by assessing its ability to bind to the EGF receptor as demonstrated in an [^{125}I]EGF competition assay. 1.0 x 10^7 Sf9 cells were infected with the TGFα$_i$ virus under serum-free conditions and the medium harvested 72 h post infection. Extracellular virus particles were removed by ultracentrifugation at 100,000g for 30min and the pH of the medium adjusted to 7.4. The EGF standards and the serial dilutions of the TGFα$_i$-conditioned medium were prepared, in duplicate, in IPL41, pH 7.4, 0.1% (w/v) BSA. The EGF standards and conditioned medium samples were added, in the presence of 5 x 10^4 cpm [^{125}I]EGF (specific activity 100 μCi/μg, Amersham), to NR6+ fibroblasts (DiFiore et al., 1987) and the TGFα$_i$ present in the medium evaluated for its ability to compete with the [^{125}I]EGF for binding to the EGF receptor. The results in Figure 5.8 indicate that the TGFα$_i$-conditioned medium is able to compete with the [^{125}I]EGF for receptor binding. This receptor binding capacity is not due to a component of the medium alone. Furthermore, since the cells were cultured in the absence of serum, the response cannot be attributed to the trace amounts of growth factors present in serum. Therefore, cells infected with the TGFα$_i$ virus secrete TGFα$_i$ into the medium which is folded into is active receptor binding conformation. By comparison with the EGF standards, it can be estimated that TGFα$_i$ is present in the medium at a concentration of approximately 100 ng/ml.

Figure 5.8. 1.0 x 10^7 Sf9 cells were infected under serum-free conditions and harvested 72 h post infection. The extracellular virus particles were removed by ultracentrifugation at 100,000g for 30 min, the pH adjusted to 7.4 and BSA added to 0.1% (w/v). 2.0 x 10^4 NR6+ fibroblasts were plated in DMEM, 10% (v/v) FCS to 24 well plates and grown for 2-3 days until just subconfluent. 5 x 10^4 cpm [^{125}I] EGF (specific activity of 100 μCi/μg, Amersham) was added to each well and EGF standards, prepared in PBS, 0.1% (w/v) BSA, or serial dilutions of TGFα_i medium dispensed in duplicate. The cells were incubated at 4°C for 4 h and then lysed in 0.5 M NaOH, 0.1% (w/v) SDS. The lysates were transferred to scintillation vials and counted on the γ channel of a scintillation counter for 1 min. The mean of duplicate values for [^{125}I]EGF bound was plotted against nM EGF or serial dilution.

Panel A.   EGF standard curve using known concentrations of a human EGF standard.

Column 1.   0 nM EGF.

Column 2.   0.2 nM EGF.

Column 3.   1 nM EGF.

Column 4.   2 nM EGF.

Column 5.   5 nM EGF.

Column 6.   10 nM EGF.

Column 7.   100 nM EGF.

Panel B.   Serial dilutions of the TGFα_i-conditioned medium.

Column 1.   undiluted.

Column 2.   serial dilution 1.

Column 3.   serial dilution 2.

Column 4.   serual dilution 3.

Column 5.   serial dilution 4.

EGF standard curve

cpm bound

3000
2000
1000
0

0   0.2   1   2   5   10   100

nM EGF

TGFa medium

cpm bound

3000
2000
1000
0

1   1:2   1:2   1:2   1:2

serial dilution
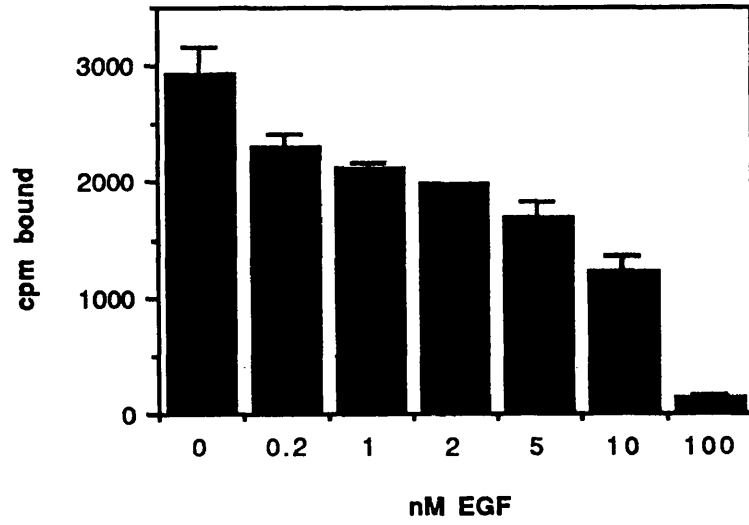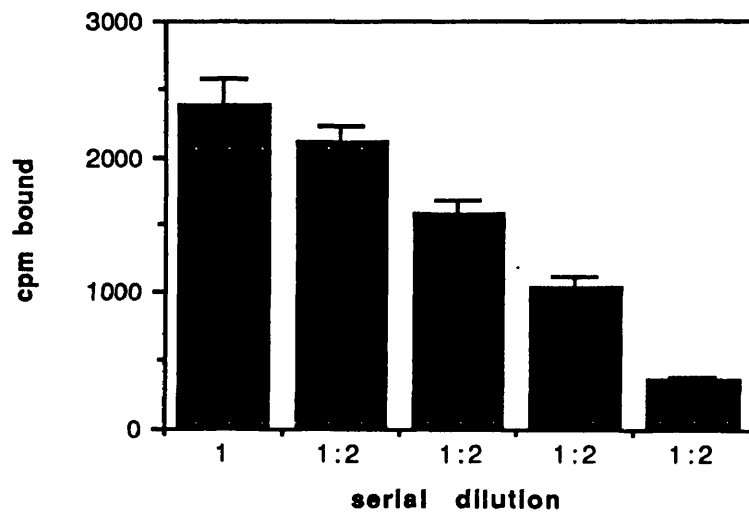
500 ml of conditioned medium was produced in an attempt to partially purify the TGFα_i for further characterisation. 2.0×10^7 Sf9 cells were infected in 50 ml of serum-free medium in each of ten 500 ml flasks. At 72 h post infection, the cells were removed by centrifugation at 7600g for 20 min and frozen at -20°C. The pH of the medium was adjusted to 1.5 and ultracentrifuged at 100,000g for 30 min to remove any particulate matter. The acidified medium was applied to a C_{18} Sephak cartridge (Waters) according to manufacturers' instructions and the eluate applied to a second cartridge. The TGFα_i was eluted in 60% acetonitrile and lyophilised. A stock solution was prepared in 100 mM HCl at a concentration of approximately 100 ng/μl.

5.5. The mitogenic activity of TGFα_i.

The results of the above $[^{125}I]$EGF competition assay indicate that the TGFα_i is able to bind to the EGF receptor. However, it cannot be assumed that the TGFα_i will also illicit a biological response. In order to evaluate the mitogenic capacity of the TGFα_i, a $[^3H]$Thymidine incorporation assay was performed on NR6+ fibroblasts with the non-EGF receptor expressing NR6 cell line (Pruss and Herschman, 1977) as the negative control. The analyses were performed in duplicate, with 0.5 nM and 100 nM EGF standards and 1 μl samples of undiluted and 1/10 and 1/100 dilutions of concentrated TGF$_i$. As can be seen in Figure 5.9, 10% (v/v) FCS caused a maximal incorporation of $[^3H]$Thymidine (sample 4). In addition, the inability of NR6+ fibroblasts to incorporate $[^3H]$Thymidine in the absence of any growth factor (sample 1) or in the presence of concentrated medium, conditioned by uninfected cells (sample 5), indicates that the mitogenic response induced by the TGFα_i sample was as a direct consequence of the secretion of TGFα_i in the

Figure 5.9. 2×10^4 NR6 or NR6+ fibroblasts were plated to 24 well plates in DMEM, 10% (v/v) FCS and incubated for 2-3 days until confluent. The cells were then made quiescent by incubation for 5 days at 37°C in starving medium (DMEM supplemented with 10% (v/v) original medium). The EGF standards and test samples were added in duplicate and the cells incubated for a further 18 h at which time, 1 μCi [^{3}H]Thymidine (specific activity 5 Ci/mmol, Amersham) was added to each well. The incubation was extended for a further 4 h, the cells lysed in 0.5 M NaOH and the DNA precipitated by the addition of 2 x volume 10% (w/v) TCA and incubation at 4°C overnight. The DNA was immobilised on glass scintered filters and counted on the [^{3}H] programme of the scintillation counter. The mean of the values for [^{3}H]Thymidine incorporation was plotted against the test sample as shown.

Panel A.   NR6+ fibroblasts.
Column 1.   0 nM EGF standard.
Column 2.   0.5 nM EGF standard.
Column 3.   100 nM EGF standard.
Column 4.   10% (v/v) FCS.
Column 5.   1 μl concentrated uninfected medium.
Column 6.   1 μl concentrated uninfected medium plus 5 nM EGF.
Column 7.   1 μl undiluted concentrated EGF$_i$.
Column 8.   1 μl 1/10 dilution concentrated EGF$_i$.
Column 9.   1 μl 1/100 dilution concentrated EGF$_i$.
Column 10.   1 μl undiluted concentrated TGFα_i.
Column 11.   1 μl 1/10 dliution concentrated TGFα_i.
Column 12.   1 μl 1/100 dilution concentrated TGFα_i.

Panel B.   As above but with NR6 fibroblasts.

NR6+ THYMIDINE INCORPORATION
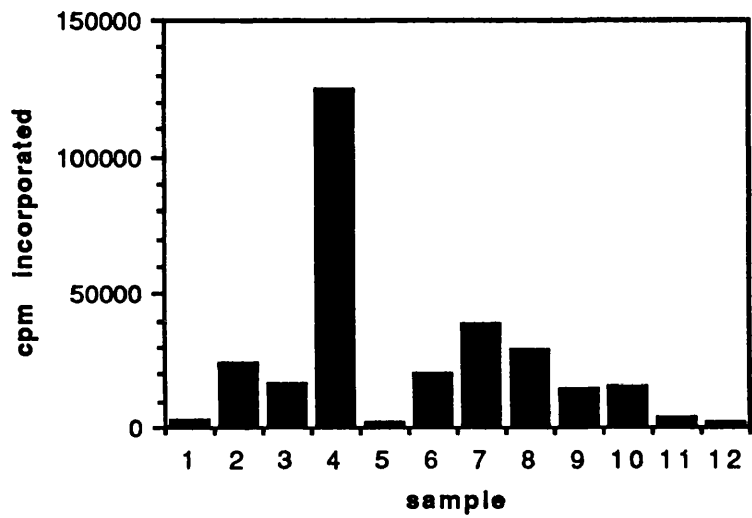
NR6 THYMIDINE INCORPORATION
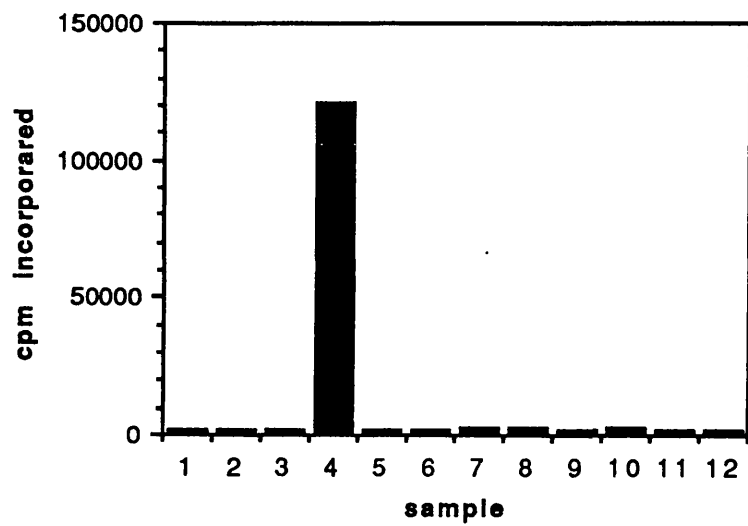
medium. Furthermore, the non-responsiveness of the NR6 cells (Panel B) suggests that the mitogenic response of NR6+ fibroblasts to the $TGF\alpha_i$ is due to the $TGF\alpha_i$ binding to and activating the EGF receptor.

5.6. The $TGF\alpha_i$ activation of the EGF receptor protein-tyrosine kinase.

5.6.1. *In vitro* kinase assay with A431 membranes.

The ability of the $TGF\alpha_i$ to stimulate the EGF receptor protein-tyrosine kinase activity was determined in several ways. Aliquots of A431 membranes were incubated at $4^{o}C$ for 10 min in 50 µl of kinase buffer containing 1 µg of EGF or 1 µl or 5 µl of $TGF\alpha_i$. 0.5 µCi of [γ-^{32}P] ATP (specific activity 10 mCi/ml, Amersham) was added and the reaction incubated at room temperature for 10 min. The reaction products were analysed by 7.5% (w/v) SDS-PAGE and as can be seen in Figure 5.10, $TGF\alpha_i$ binds to the EGF receptor and stimulates the intrinsic protein-tyrosine kinase activity as measured by receptor autophosphorylation.

5.6.2. *In vitro* kinase assay with $EGFR_i$.

It has previously been determined that the $EGFR_i$ protein-tyrosine kinase activity is stimulated by EGF at 48 h post infection (Figure 3.16B). The ability of $TGF\alpha_i$ to stimulate the $EGFR_i$ protein-tyrosine kinase activity was assessed by *in vitro* assay at 48 h post infection. 2.5 x 10^6 Sf9 cells were infected with the $EGFR_i$ virus and harvested at 48 h post infection. The cells were resuspended in 5 ml of $TGF\alpha_i$-conditioned medium or PBS, containing 1 µg/ml EGF or 1 µl of concentrated $TGF\alpha_i$, and incubated at $27^{o}C$ for 10 min. The cells were lysed and immunoprecipitated with the anti-EGF receptor monoclonal antibody, ICR9. An *in vitro* kinase assay was performed and the

Figure 5.10. A431 membranes were resuspended in 50 µl kinase buffer and incubated with 1 µg of human EGF standard and 1 and 5 µl aliquots of concentrated TGFα$_i$ conditioned medium for 10 min at room temperature. 0.5 µCi [γ-^{32}P] ATP (specific activity 10 mCi/ml, Amersham) was added and the reaction incubated at room temperature for 10 min. The reaction products were analysed by 7.5% (w/v) SDS-PAGE and exposed to Hyperfilm (Amersham) for 30 min at -80°C.

Lane 1.   + 1 µg EGF standard.

Lane 2.   -EGF.

Lane 3.   + 1 µl TGFα$_i$.

Lane 4.   + 5 µl TGFα$_i$.
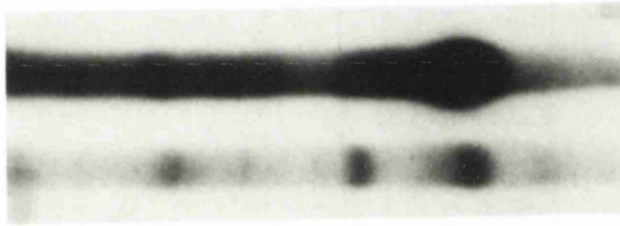
EGF STANDARD

EGF STANDARD

1 μl TGFα CONCENTRATE

5 μl TGFα CONCENTRATE

170 KD   EGFR

reaction products analysed by 7.5% (w/v) SDS-PAGE. Figure 3.17A shows that TGFα$_i$, before and after concentration is able to stimulate the protein-tyrosine kinase activity of the EGFR$_i$. The results suggest that the TGFα$_i$-conditioned medium is able to stimulate the protein-tyrosine kinase activity to a greater extent than the standard EGF, but this is probably due to the presence of a higher concentration of the TGFα$_i$ in the medium than was previously thought.

5.6.3. Phosphotyrosine immunoblot analysis.

The ability of TGFα$_i$ to stimulate the protein-tyrosine kinase activity of the EGFR$_i$ was also assessed by phosphotyrosine immunoblot analysis. The cells were treated as for the *in vitro* kinase assay, but lysed in hot sample buffer containing 500 μM sodium orthovanadate. The lysates were analysed by 7.5% (w/v) SDS-PAGE and immunoblotted with the anti-phosphotyrosine antibody. The results in Figure 3.17B indicate that TGFα$_i$ stimulates the EGFR$_i$ protein-tyrosine kinase activity as measured by increased autophosphorylation on Tyr residues.

5.7. HPLC analysis of TGFα$_i$.

HPLC analysis was performed on a sample of the TGFα$_i$ so as to generate a more accurate measurement of the yield. 100 ml of the conditioned medium was acidified amd applied to the Sephak cartridge as before. The TGFα$_i$ was eluted in 60% acetonitrile and lyophilised to remove the acetonitrile. The sample was resuspended in 0.1% (v/v) TFA and applied to a C$_{18}$ under the same conditions as for the EGF$_i$ sample. As in the case of the EGF$_i$, a similar contaminant that co-eluted off the Sephak cartridge eluted through the entire acetonitrile gradient and absorbed across the whole of the UV range monitored. The 214nm

absorbance specific to TGFα was therefore completely obscured and the yield of TGFαᵢ could not be assessed by HPLC analysis at this stage of the purification.

5.8. DISCUSSION.

A coding sequence encoding the mature TGFα 50 amino acid growth factor linked to the signal sequnce was constructed by *in vitro* mutagenesis (5.2). DNA sequencing confirmed that no unwanted changes had occurred during the mutagenesis procedure (5.2.2). The coding sequence was integrated into the AcNPV genome and a recombinant baculovirus isolated (5.2.5). Insect cells infected with the recombinant virus expressed a 6 kD peptide that was specifically recognised by an anti-TGFα monoclonal antibody (5.3). The expression of TGFαᵢ was apparent by 48 h post infection which is consistent with the transcriptional activation of the polyhedrin promoter (for review see, Summers and Smith, 1987). The TGFαᵢ continued to be expressed for a further 48 h until the cells lyse. The presence of the TGFαᵢ in the medium indicates that the TGFα signal peptide is efficiently recognised and removed by the insect cells.

The conditioned medium was harvested at 72 h post infection in an attempt to maximise the expression before the cells lysed. It is possible that the lysed cells will release degradative enzymes into the medium. Furthermore, cells lysis would lead to the accumulation of cellular components which interfere with subsequent purification. Preliminary results indicate that TGFαᵢ is secreted into the medium at 100 ng/ml. This value is based on the relative concentration of TGFα in the [^{125}I]EGF assay and represents the amount of TGFα that is folded correctly and is therefore able to bind to the EGF receptor. A large

amount of TGFα_i remains within the cell, possibly due to the exhaustion of the capacity of the transport system by the high expression levels (5.3). TGFα_i is acid stable and therefore the material remaining in the cell pellet can be acid extracted, thereby increasing the yield significantly (Cohen and Carpenter, 1975).

It is possible that the yield of the TGFα_i could be improved at the level of transcription or translation. The 5' untranslated region of the construct comprises 45 bases including the length of the polylinker. It has been determined that the 5' untranslated region can reduce the efficiency of transcription or translation if it is excessively long (several hundred nucleotides) or includes regions of potential secondary structure (Lucklow and Summers, 1988). It is unlikely that the 5' untranslated sequence of the TGFα construct contains any potential secondary structural motifs since it was designed on the basis of the Kozak consensus sequence (Kozak, 1984, 1987). There is no evidence to suggest that the insect cell translation start sites conform to those operating in higher eukaryotes, but it is extremely unlikely that the introduction of a Kozak consensus sequence would impair translational efficiency. Furthermore, the pAcC12 vector has been used successfully to express several eukaryotic proteins (Cetus Corp.).

It is likely that the yield of TGFα_i could be improved greatly by increasing the cell density and improving the culture conditions. The estimate of the concentration of TGFα_i was derived from the [^{125}I]EGF competition assay based on the conditioned medium from a small scale culture of 1.0×10^7 cells in 10 ml of medium in a 250 ml flask. Sf9 cells adapt readily to suspension culture in Techne stirring vessels. Indeed, using a cell density of 5.0×10^6 cells/ml, 2 mg/litre of the purified

extracellular domain of the EGF receptor has been achieved (C Greenfield, personal communication).

Relatively few alternative systems exist for the expression of TGFα. Derynck et al. (1984) devised a system for the production of human TGFα in *E. coli*. The TGFα was produced as a fusion protein with the first seventeen residues of the Trp leader sequence, mature TGFα released by CNBr treatment at the connecting Met residue. However, as with many foreign proteins expressed in *E. coli*, a high proportion of the molecules were incorrectly folded and therefore biologically inactive. Following glutathione treatment to reshuffle the disulphide bonds, biologically active TGFα was extracted at a yield of 1 mg/g of *E. coli*. An alternative expression system in yeast, using the α pheremone mating factor, resulted in the secretion of 90% of the TGFα in a biologically active form but only at a yield of 8 ng/ml (Derynck et al., 1985). The insect cell system therefore possesses many advantages over the two previous expression systems. Not only is there the potential to generate a high yield of TGFα, but it is also secreted into the medium in a biologically active form. In contrast to other eukaryotic expression systems, insect cells adapt readily to large scale culture and will grow in synthetic medium. Furthermore, the cells can be cultured in the absence of serum, thereby facilitating purification and reducing the cost dramatically.

The TGFα$_i$ is able to compete with [^{125}I]EGF for binding to the EGF receptors displayed on the surface of NR6+ fibroblasts (5.4). Furthermore, TGFα$_i$ stimulates DNA synthesis in quiescent fibroblasts, as measured by the incorporation of [^{3}H]Thymidine (5.5). Therefore, in addition to expressing TGFα, the insect cells direct the formation of the correct disulphide pairings, thereby folding the molecule into its

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biologically active three-dimensional conformation. The EGF receptor-binding activity of TGFα$_i$ was further demonstrated by its ability to activate the receptor protein-tyrosine kinase as measured by *in vitro* kinase assays (5.6.1, 5.6.2) and *in vivo* phosphotyrosine immunoblot analysis (5.63).

HPLC analysis was performed in an attempt to obtain a more accurate measurement of the yield of TGFα$_i$. However, the TGFα-specific absorbance was masked by the presence of a contaminant. As was discussed in Chapter 3, IPL41 contains several components that could be responsible for the contaminating material. It is most likely to be the lipid emulsion and cod liver oil, which could be removed by introducing a gel permeation step or extracting with a non polar organic solvent prior to the C$_{18}$ cartridge.

The existing ^{1}H NMR data, coupled with information as to the residues which are conserved throughout the EGF family of growth factors can predict the residues which may be involved in binding to and activating the EGF receptor. These predictions can then be evaluated by mutational analysis. Preliminary mutational analysis has focused initially on the highly conserved residue Leu[48] (corresponding to Leu[47] in EGF) in human TGFα (Lazar et al., 1988). In addition, the relevance of Asp[47], which is precisely conserved between EGF and TGFα has been assessed. Mutants possessing substitutions of Ala, Asn, Ser or Glu in place of Asp[47] were active in receptor binding and colony formation assays, indicating that neither the negative charge nor the polarity of the Asp residue were essential. Indeed, both the Myoxoma growth factor and Shope virus growth factor have an Asn residue at position 47 (Upton et al., 1987). Conversely, mutation of Leu[48] to an Ala or Met residue or to the related Ile residue destroyed all activity. These

results indicate that Leu^{48} is essential through its exact geometry for biological activity. Indeed, mutational analysis of the corresponding residue in human EGF (Leu^{47}) has confirmed the absolute requirement for a Leu residue at this position (Ray et al., 1988; Engler et al., 1988). Furthermore, ^{1}H NMR analysis of EGF indicated that, although the corresponding residues, Asp^{46} and Leu^{47} are both solvent accessible, their sidechains point in different directions in the β-sheet. These observations indicated that these residues potentially play very different roles in the establishment of the biologically active conformation of EGF (Montelione et al., 1987).

Tyr^{38}, which is precisely conserved in the EGF-family of growth factors, was mutated to a Phe residue with no loss of receptor binding or mitogenicity (Defeo-Jones et al., 1988). However, substitution of Tyr^{38} by an Ala residue resulted in total loss of activity, suggesting the absolute requirement for the presence of an aromatic residue at position 38. Lazar et al. (1989) also performed a series of substitutions for Tyr^{38} confirming the requirement for an aromatic residue at position 38. Interestingly, whilst mutation to a Trp residue gave similar relative potencies in receptor binding and mitogenic assays, they observed that mutation to a Phe residue generated a peptide which displayed only 50% activity relative to the wild type TGFα in the receptor binding assay, but was as potent as the wild type in colony formation and [^{3}H]Thymidine incorporation assays. These observations indicate that Tyr^{38} is involved directly in binding to the receptor. Indeed, it has been suggested that the corresponding residue in human EGF (Tyr^{37}) may play a functional role due to the solvent-accessiblity of its side chain (Cooke et al., 1987; Montelione et al., 1987). Thus, it may indeed be possible to dissociate residues involved in receptor binding

from those associated with the generation of the mitogenic signal, leading to the design of antagonists. Such antagonists will be clinically important for the treatment of malignancies which express elevated levels of the EGF receptor. Furthermore, such antagonists could be employed to disrupt the TGFα autocrine cycle operating in many tumours.

The isolation of a recombinant baculovirus directing the expression of human TGFα means that the expression system can now be scaled up using the 6 and 60 litre bioreactor on site facilities. The availability of large amounts of TGFα will allow the complete determination of its tertiary structure through continued ^{1}H NMR investigations in conjunction with the analysis of crystals through diffractional methods. Comparison of the tertiary structures of EGF and TGFα, and their co-crystallisation with the EGF receptor will lead to the elucidation of the receptor ligand-recognition site.

CHAPTER SIX.

THE C-ERBB-2/NEU GENE PRODUCT.

6.1. INTRODUCTION.

6.1.1. The identification of neu.

The injection of pregnant rats with the carcinogen ethylnitrosourea at day 15 of gestation resulted in the appearence of neuro/glioblastomas in their offspring (Schubert et al., 1974). DNA extracted from these ethylnitrosourea-induced neuroblastomas caused transformation when transfected into NIH 3T3 fibroblasts, the transfectants generating fibrosarcomas when injected into newborn mice (Padhy et al., 1982). Sera from these mice specifically recognised a 185 kD cell surface glycoprotein in cell lysates prepared from the neuroblastomas and the transfection-derived foci, demonstrating that the synthesis of $p185^{neu}$ was directed by the neuroblastoma-inducing sequence. The observation that the EGF receptor and $p185^{neu}$ had similar molecular weights and were both phosphorylated cell-surface glycoproteins led Schechter et al. (1984) to screen neuro/glioblastoma samples with the EGF receptor-related v-erbB sequence. They detected distinct nucleic acid sequence homology with v-erbB and furthermore, determined that antisera raised against the EGF receptor displayed cross-reactivity with $p185^{neu}$. Detailed analysis revealed that the two genes resided on different chromosomes, the EGF receptor gene on chromosome 7 and neu on chromosome 17 and, hence the neu gene is related to, but distinct from the gene encoding the EGF receptor (Schechter et al., 1985). Exposure of neu-transfected NIH 3T3 fibroblasts to monoclonal antibodies raised against $p185^{neu}$ resulted in the rapid,

reversible loss of total cellular $p185^{neu}$ and reversion to a nontransformed phenotype, suggesting the absolute requirement for $p185^{neu}$ in the maintenance of neu-induced transformation (Drebin et al., 1984, 1985). The significant similarities between $p185^{neu}$ and the EGF receptor, a member of the family of receptor protein-tyrosine kinases, led Stern et al. (1986) to investigate whether $p185^{neu}$ was also associated with a protein-tyrosine kinase activity. Indeed, in an *in vitro* kinase assay, an immune complex of $p185^{neu}$ was labelled by autophosphorylation.

6.1.2. The structure of $p185^{neu}$.

The neu cDNA sequence, isolated from a neu-transformed NIH 3T3 cDNA library, was found to be biologically active in a focus formation assay (Bargmann et al., 1986a). The cDNA sequence predicted that, like the EGF receptor, $p185^{neu}$ comprised an extracellular domain containing two Cys-rich repeat clusters, a membrane spanning segment and a cytoplasmic protein-tyrosine kinase domain (see Figure 6.1). Indeed, $p185^{neu}$ displayed 50% amino acid sequence homology with the EGF receptor, with over 80% homology occurring in the protein-tyrosine kinase domain. Concurrently, cDNA sequences for neu and it corresponding proto-oncogene were isolated from libraries prepared from a rat neuro/glioblastoma cell line and rat liver respectively (Hung et al., 1986).

The observation that the normal form of $p185^{neu}$ was structurally similar to its transforming counterpart indicated that the oncogenic activation of neu did not involve major structural alterations in the gene product (Stern et al., 1986). It was suggested that the mutation(s) activating $p185^{neu}$ affected its relative abundance at the

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EXTRACELLULAR DOMAIN

V659

T654   T664

CYTOPLASMIC DOMAIN

30 aa

Y 1068   Y 1148   Y 1200   Y 1127

Y 1173   Y 1226

EGF receptor   p185$^{c\text{-erbB-2}}$
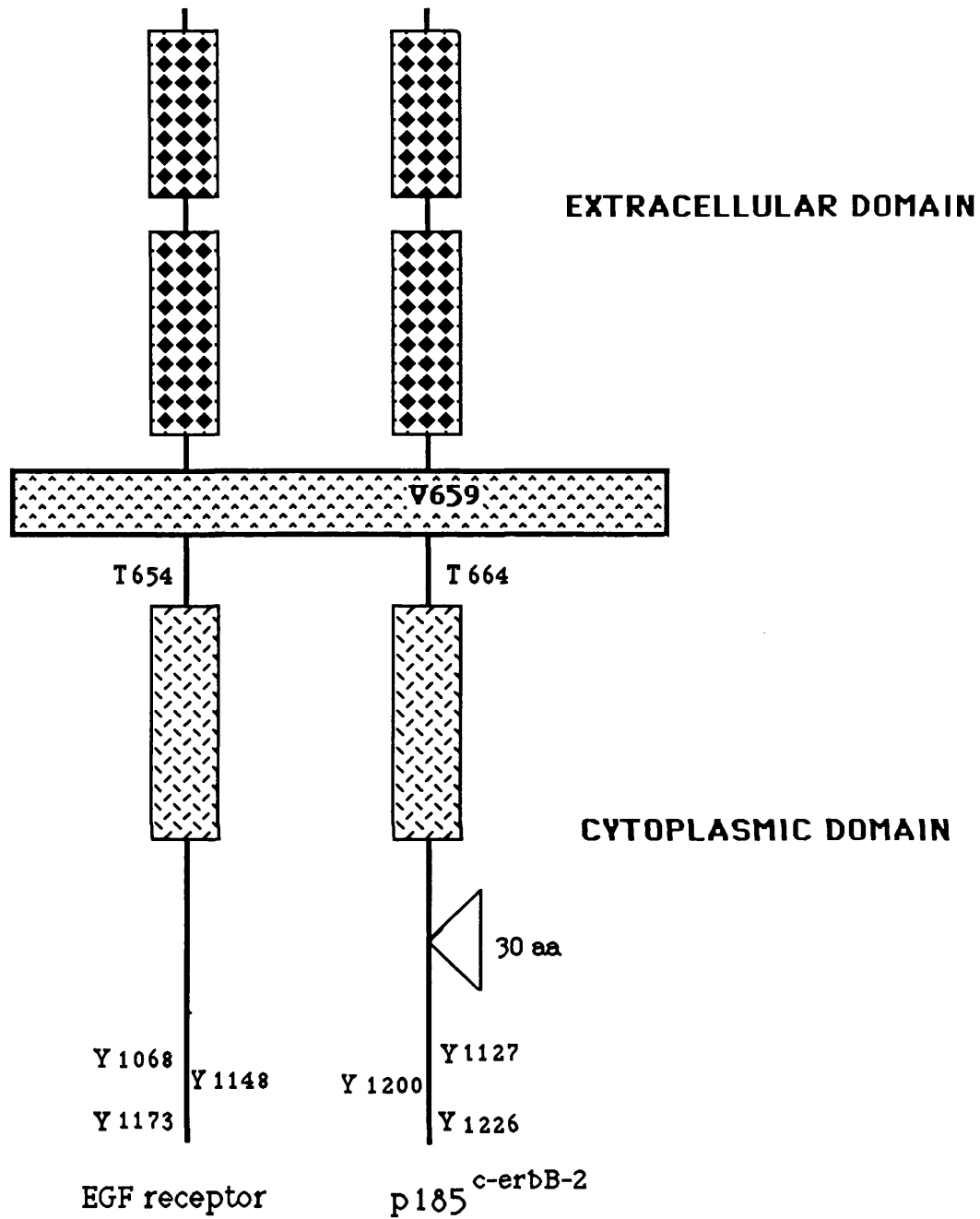
FIGURE 6.1. Schematic representation of the structure
of the EGF receptor and p185$^{c\text{-erbB-2}}$

Cys rich repeats

Kinase domain

cell surface rather than directly altering its structure. However, it was determined that amplification of the normal neu gene in NIH 3T3 cells resulted in the production of normal $p185^{neu}$, at levels comparable to those of activated $p185^{neu}$ in transformed cell lines, without concomittant cellular transformation, suggesting that high level expression of $p185^{neu}$ was not sufficient for its activation (Hung et al., 1986). It was therefore suggested that the mutational activation of neu arose from subtle changes in the structure of $p185^{neu}$, a proposal supported by the evidence that ethylnitrosourea is responsible for the generation of point mutations (Margison and O'Conner, 1979).

6.1.3. The mutational activation of neu.

Comparison of the sequences of the cDNAs for normal and transforming neu determined the presence of a single amino acid substitution at position 664 within the transmembrane domain (Bargmann et al., 1986b). Hence, the replacement of Val^{664} with a Glu residue appears sufficient to cause the activation of $p185^{neu}$, resulting in the generation of rat neuroblastomas (Padhy et al., 1982) and mouse breast carcinomas (Muller et al., 1988). Mutational analysis indicated that the protein-tyrosine kinase activity is necessary but not solely responsible for transformation since a highly truncated neu gene product, containing only the protein-tyrosine kinase and transmembrane domains, still varied in biological activity depending on the nature of the residue at position 664 (Bargmann et al., 1986b). Thus, the transmembrane substitution acts through, or in association with, the protein-tyrosine kinase activity to illicit cellular transformation. Initial evidence from an autophosphorylation assay on solubilised, immunoprecipitated protein suggested that there was no obvious

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difference between the protein-tyrosine kinase activities of normal and transforming neu (Stern et al., 1986). However, experiments involving the phosphorylation of $p185^{neu}$ in membranes have since determined that the level of $p185^{neu}$ phosphorylation in neu-transformed cells is significantly higher than that in cells containing only the normal form (Bargmann and Weinberg, 1988a). This preferential phosphorylation of transforming $p185^{neu}$ is a consequence of autophosphorylation, since it occurs on Tyr residues and requires an intact protein-tyrosine kinase domain. A kinase-inactive mutant possessing a 4 amino acid insertion within the protein-tyrosine kinase domain is transported efficiently to the cell surface where it displays only 1% of the autophosphorylation potential of the wild type $p185^{neu}$ and fails to induce cellular transformation (Bargmann and Weinberg, 1988a). These results suggested that transforming $p185^{neu}$ is associated with an active protein-tyrosine kinase which is an intrinsic property of the transforming protein.

6.1.4. Mutational analysis of Val ^{664}.

Bargmann and Weinberg (1988a) investigated the nature of the activating mutations at position 664. Of the eight mutations introduced, only mutation to Glu or Gln was fully activating, while mutation to Asp was only weakly activating. It is unlikely that neu is activated by the perturbation of a specific property of Val^{664}, it is more likely that the activation arises as a result of the specific introduction of Glu/Gln residues. As a consequence of its membrane localisation, Glu^{664} has the potential to be negatively charged, suggesting the activating mutation may insert a charged residue into the otherwise hydrophobic environment of the transmembrane domain, thereby distorting its conformation. However, since the mutation of Val^{664} to Lys/His is not

activating and mutation to Asp is only weakly activating, it is unlikely that the presence of a negative charge in the transmembrane domain of $p185^{neu}$ is activating per se. Indeed, mutation to the uncharged residue Gln is much more transforming than mutation to an Asp. That the nature of the activating mutation is highly specific was demonstrated by the observation that the introduction of a Glu at position 663 or 665 was not transforming. It has been suggested that the presence of a Glu/Gln at position 664 specifically enhances or decreases an intermolecular association that is essential for receptor activation, the interaction being more sterically favourable with Glu/Gln than the smaller Asp. Furthermore, virtually the entire extracellular domain of the activated neu can be truncated without inhibiting its transforming capacity, indicating that the transmembrane domain does not function to activate $p185^{neu}$ by perturbing its ligand specificity or affinity (Bargmann and Bargmann, 1988b).

6.1.5. The identification of c-erbB-2.

c-erbB-2, the human homologue of neu was initially identified by the observation that a v-erbB homologous sequence was amplified in a human mammary carcinoma (King et al., 1985). The screening of a human genomic library with a v-erbB probe led to the isolation of a clone which was found to be amplified in a human salivary gland adenocarcinoma (Semba et al., 1985). Concurrently, genomic and cDNA clones encoding an EGF receptor-related protein-tyrosine kinase receptor were isolated which shared chromosomal localisation with neu (Coussens et al., 1985; Yamamoto et al., 1986). A 4.8 kb c-erbB-2 specific mRNA was widely expressed in normal foetal and adult tissue, including the kidney, liver, skin, lung, stomach, colon and uterus and in a variety of malignant tissues, including a mammary carcinoma, (King et al.,

1985), a salivary gland adenocarcinoma (Semba et al., 1985) and a gastric carcinoma cell line (Yamamoto et al., 1986). Antibodies raised against a peptide from the carboxyl-terminus of c-erbB-2, immunoprecipitated a phosphoprotein of 185 kD from lysates of adenocarcinoma cells (Akiyama et al., 1986). Furthermore, monoclonal antibodies raised against the extracellular domain of p185$^{c-erbB-2}$ inhibited the growth of breast tumour-derived cell lines overexpressing p185$^{c-erbB-2}$ and prevented c-erbB-2-transformed NIH 3T3 fibroblasts forming colonies in soft agar (Huziak et al., 1989).

6.1.6. The activation of c-erbB-2.

The gene for c-erbB-2 is amplified in a gastric cancer cell line (Fukushige et al., 1986) and in as many as 30% of human breast carcinomas (Kraus et al., 1987), although amplification is not necessarily a prerequisite of transformation. Transfection of NIH 3T3 cells with the full length c-erbB-2 sequence led to transformation (Difiore et al., 1987b). Furthermore, p185$^{c-erbB-2}$ displaying an amino terminal deletion was able to induce transformation at levels of expression 5 to 10 fold lower than those necessary to achieve transformation with the normal protein. Thus, it appears that molecular alteration in the c-erbB-2 sequence, as well as overexpression of the normal gene, is able to cause transformation. The lesion responsible for the activation of the neu oncogene was introduced into the c-erbB-2 coding sequence at the corresponding position (Val^{659}) to determine whether this mutation would be activating (Segatto et al., 1988). Such a mutational event would involve two nucleotide substitutions, an event unlikely to occur spontaneously in human tumours and therefore the mutation to an Asp residue requiring only a single base mutation was also introduced. When the

coding sequences were expressed under the control of the Mo-MuLV LTR and transfected into NIH 3T3 cells, although the rate of synthesis and localisation of the c-erbB-2 mutant proteins was similar to that of the wild type protein, they displayed an enhanced *in vitro* kinase activity. Indeed, both of the mutations were observed to increase the transforming capacity to an extent equivalent to that of the amino-terminal truncation. However, the Val to Glu mutation was not observed in samples from human breast tumours (Slamon et al, 1989) which is consistent with the concept that overexpression of a normal c-erbB-2 gene product, rather than mutational activation may be an important pathological event in some tumours.

6.1.7. Amplification of c-erbB-2 in breast adenocarcinomas.

A number of investigations have indicated that c-erbB-2 gene is amplified and overexpressed in a significant proportion of primary adenocarcinomas of the breast (Slamon et al., 1987; Berger et al., 1988; Maguire and Greene, 1989). Slamon et al. (1987) screened 189 primary human breast carcinoma samples and determined that c-erbB-2 was amplified in approximately 30% of cases. Furthermore, a significant correlation of amplified c-erbB-2 with a negative prognosis and a high probability of relapse was observed. There is, however, considerable variability in the reported incidence of gene amplification and its correlation with clinical outcome, arising as a consequence of the small number of cases evaluated in each study and the fact that only one of the parameters of DNA, RNA or protein levels was evaluated in each survey. Further complications are introduced by the dilution of tumour cells with normal vascular, stromal and inflammatory cells, which together account for more than 50% of the tissue sample. Slamon et al. (1989) instigated a study on 668 cases of primary breast carcinoma by

Southern, Northern and Western blotting techniques and immunohistochemical analysis of tissue sections. The results of this extensive study confirmed the initial observation of 30% gene amplification correlating with negative prognosis.

6.1.8. The identification of c-erbB-3.

Recently, a third distinct member of the erbB gene family has been identified by reduced stringency hybridisation of v-erbB to a normal human genomic library (Kraus et al., 1989). cDNA sequence information predicted a 148 kD membrane-associated protein with striking structural homologies to both the EGF receptor and p185$^{neu/c-erbB-2}$ including overall size, the presence of the signature Cys clusters in the extracellular domain and the uninterrupted cytoplasmic protein-tyrosine kinase domain. However, distinct regions of the coding sequence possessed a high degree of divergence, in particular the carboxyl terminus and a short stretch of 29 amino acids carboxy-terminal to the ATP binding site. This region differs from the corresponding region in p185$^{neu/c-erbB-2}$ and the EGF receptor at 28 and 25 residues respectively. These regions of high divergence may therefore reflect the functional specificity of the receptor-like molecules. The involvement of EGF receptor and c-erbB-2 gene amplification and/or overexpression in human squamous carcinomas and breast carcinomas respectively has been well documented (Libermann et al., 1985; Slamon et al., 1989). The observation that the c-erbB-3 gene is overexpressed in certain human mammary carcinoma cell lines implies that c-erbB-3 may also play a role in the generation and maintenance of certain human malignancies.

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Concurrently, a second group cloned the c-erbB-3 (designated HER3) gene (Plowman et al., submitted). They identified a subclone of the A431 cell line which displayed a 30-fold increase in sensitivity to amphiregulin (AR). A cDNA library was prepared and screened with a degenerate oligonucleotide to a stretch of ten amino acids unique to the protein-tyrosine kinase domain of the EGF receptor and $p185^{neu}$. Subsequent screening identified a human breast carcinoma cell line as a more abundant source of the c-erbB-3 mRNA and a series of overlapping cDNA clones encompassing the entire coding sequence were isolated. Upon cotransfection of COS cells with the c-erbB-3 and the AR precursor, a cerbB3 receptor-specific monoclonal antibody recognised a 160 kD phosphoprotein indicating that AR was binding to and activating the c-erbB-3 gene product. Furthermore, the c-erbB-3 gene product did not bind EGF nor TGFα. This disparity in the response of the c-erbB-3 gene product to the different ligands may arise as a direct consequence of the less conserved regions at the carboxy-terminus and within the protein-tyrosine kinase domain.

6.2. NEU/C-ERBB-2 AND SIGNAL TRANSDUCTION.

6.2.1. Transmembrane signalling.

Although the structure of $p185^{neu/c-erbB-2}$ suggests that it is a member of the receptor protein-tyrosine kinase family, neither its extracellular ligand nor intracellular substrates have been identified. The requirement for the membrane localisation of activating $p185^{neu}$ in order to illicit its transforming capacity, was demonstrated by the construction of a chimeric molecule between the cytoplasmic domain of $p185^{neu}$ and the transmembrane and extracellular portions of the immunoglobulin heavy chain (Flanagan and Leder, 1988). The cellular

localisation of the chimeric polypeptide was controlled by coexpression with the immunoglobulin light chain, in the absence of the light chain, the fusion protein was expressed intracellularly and had no transforming activity. In contrast, in the presence of the light chain, the chimera was expressed at the cell surface where it directed the production of foci. These observations indicated that the neu transmembrane domain is not specifically required for transformation.

NIH 3T3 cells transfected with the normal version of neu expressed $p185^{neu}$ which was non-transforming. The EGF receptor expressed by transfected NIH 3T3 cells was only transforming in the presence of EGF (Velu et al., 1987). In contrast, in the presence of EGF, NIH 3T3 cells doubly transfected with both normal neu and the EGF receptor formed foci in culture and were tumourigenic in nude mice, demonstrating the synergistic action of the two receptors in the transformation of NIH 3T3 cells. Rat-1 fibroblasts expressing both the EGF receptor and neu demonstrate EGF-stimulatable tyrosine phosphorylation of $p185^{neu}$ (Stern and Kamps, 1988; Kokai et al., 1988) although it has been shown that $p185^{neu}$ does not bind EGF (Akiyama et al., 1986). This tyrosine phosphorylation is EGF-specific since neither PDGF nor insulin treatment is able to initiate such a response. The EGF-stimulatable phosphorylation of $p185^{neu}$ and the EGF receptor occurred with similar kinetics and EGF concentration dependency, both phosphorylation events prevented by the EGF-dependent downregulation of the EGF receptor. These results indicated that $p185^{neu}$ may act as a substrate for the EGF receptor protein-tyrosine kinase activity, leading to the activation of the intrinsic $p185^{neu}$ kinase. Indeed, incubation of cells with EGF prior to lysis stimulated the tyrosine phosphorylation of $p185^{neu}$ which had been

immunoprecipitated with a $p185^{neu}$-specific antibody. The involvement of the EGF receptor in the activation of the $p185^{neu}$ kinase was further demonstrated by the fact that Rat-1 cells no longer expressing the EGF receptor do not phosphorylate $p185^{neu}$ (Kokai et al., 1988). A potential explanation for these observations is that EGF stimulates the formation of EGF receptor-$p185^{neu}$ heterodimers, thereby activating the $p185^{neu}$ protein-tyrosine kinase activity. It has therefore been proposed that $p185^{neu}$ functions as a growth factor receptor serving to transduce signals across the plasma membrane, as a consequence of dimer formation, in an analogous manner to the proposed EGF receptor signal transduction pathway (Yarden and Schlessinger, 1987). The recent demonstration that activated $p185^{neu}$ is organised primarily at the plasma membrane in an aggregated from, but that the normal $p185^{neu}$ is not, lends support to this model for signal transduction (Weiner et al., 1989).

6.2.2. Aims of this study.

The expression of $p185^{c\text{-}erbB\text{-}2}$ in the baculovirus system would establish a system for the evaluation of a putative receptor protein-tyrosine kinase analogous to the EGF receptor. The availability of insect cell expression systems for both the EGF receptor and $p185^{c\text{-}erbB\text{-}2}$ will allow comparative analysis of the mechanism of signal transduction by growth factors. Furthermore, insect cells co-infected with the EGF receptor and c-erbB-2 viruses will express both the EGF receptor and $p185^{c\text{-}erbB\text{-}2}$ in sufficient quantites to permit investigation into the formation of homo and heterodimers, thereby allowing the model of receptor dimerisation as a mechanism for signal transduction to be evaluated. The production of large quantities of $p185^{c\text{-}erbB\text{-}2}$ will

facilitate the identification of the p185 ligand from the conditioned media of certain transformed cells by affinity chromatography.

6.3. RESULTS.

6.3. The isolation of a c-erbB-2 recombinant baculovirus.

The vector for the expression of c-erbB-2 in insect cells was constructed by Ian Hiles. The full length 4.4 kb c-erbB-2 cDNA (Yamamoto et al., 1986) has a unique NcoI site encompassing the ATG of the translation start site. A series of baculovirus expression plasmids, pACc3-pACc5 contain the region around the polyhedrin translation start site in addition to the promoter and 5' untranslated sequences (Cetus Corp.). The translation start site includes a NcoI site and therefore, coding sequences possessing a NcoI site at their translation start can be introduced in frame into one of these vectors. The efficiency of translation of the gene should be very high, since the wild type polyhedrin upstream sequences are employed. Furthermore, since the inserted DNA does not contain any upstream sequence, transcription and translation will not be impaired by any regions of potential secondary structure. The c-erbB-2 plasmid was digested with DraI and filled in with Klenow enzyme, before digestion with NcoI. The insert was then cloned into pAcC3 digested with NcoI and SmaI (Figure 6.2).

The c-erbB-2 expression vector was cotransfected with AcNPV DNA and the virus particles harvested 4-6 days later. Potential recombinant viruses were identified by plaque hybridisation employing the cDNA as probe. The filters were washed to a stringency of 0.1 x SSC, 0.1% (w/v) SDS at 65°C and areas of positive hybridisation identified after exposure to Hyperfilm (Amersham). Following four

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Figure 6.2. The restriction endonuclease map of the transfer vector pAcC3 (Cetus Corp.). A unique NcoI site was created at the translation start site of the polyhedrin gene, and a short polylinker containing the SmaI recognition sequence inserted, leaving the NcoI site intact.

Amp

HindIII

BstEII

PvuII

XhoI

EcoRI

pAcC3
9 kb

SalI

PvuII

SalI

HindIII

polyhedrin
promoter

SalI

EcoRV

HindIII

NcoI

SmaI
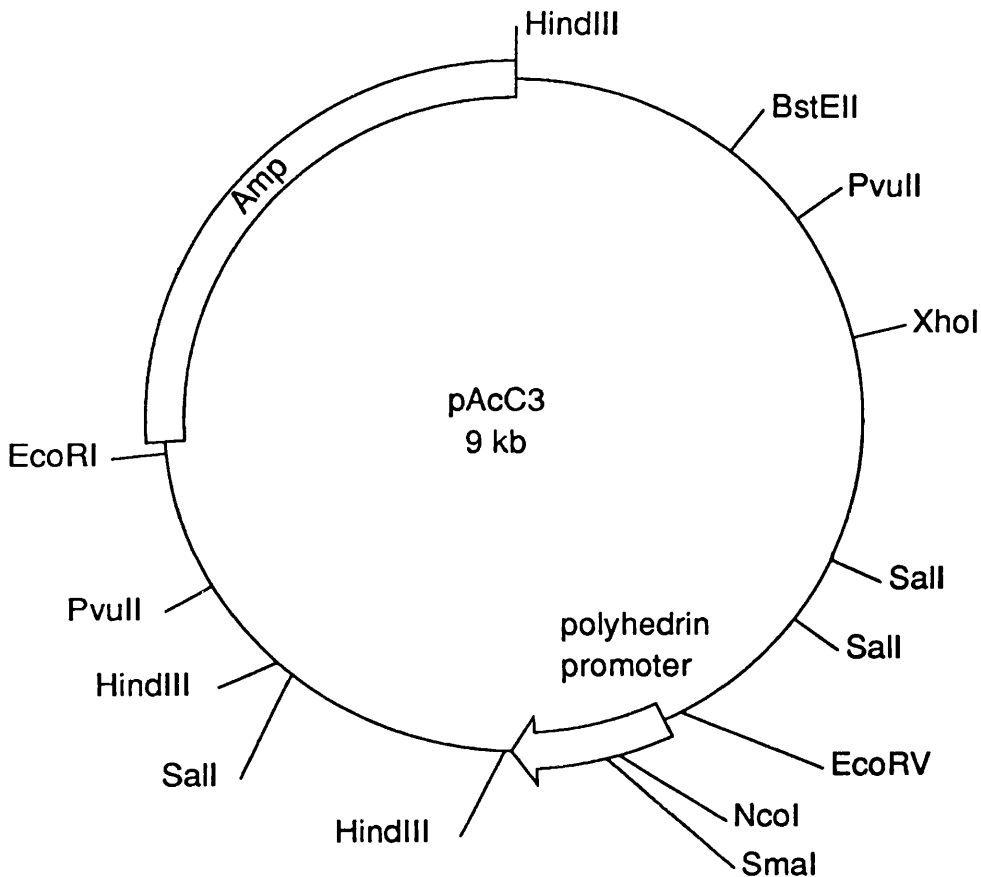
rounds of plaque hybridisation and two rounds of visual screening, several pure recombinant viruses were isolated.

6.4. The expression of c-erbB-2$_i$.

2.5×10^6 Sf9 cells were infected with potential recombinant viruses and at 48 h post infection, the lysates were analysed by 7.5% (w/v/) SDS-PAGE and immunoblotting with the anti-21N monoclonal antibody (Gullick et al., 1987). As can be seen from Figure 6.3A, a high moecular weight polypeptide is specifically identified by the anti-21N antibody. No such polypeptide is expressed by wild type infected cells. By comparison with p185$^{c-erbB-2}$ expressed in the human mammary carcinoma cell line SKBR3 (Kraus et al., 1987), the c-erbB-2 gene product expressed by the infected insect cells has a slightly lower molecular weight. A similar discrepancy was observed in the molecular weights of the EGF receptor in A431 cells and the EGFR$_i$ expressed in insect cells (Greenfield et al., 1987). This low molecular weight of the EGFR$_i$ expressed in insect cells was attributed to the inability of the cells to incorporate complex oligosaccharides at the sites of glycosylation. Therefore, the lower molecular weight of p185$^{c-erbB-2}$ expressed in insect cells is most likely due to incomplete glycosylation.

The expression of p185$_i$ was monitored in the post infection period from 24-72 h. Aliquots of 2.5×10^6 Sf9 cells were infected with the c-erbB-2$_i$ virus and the cells lysed at 24, 48 and 72 h post infection. The lysates were immunoprecipitated in duplicate, with the anti-p185 monoclonal antibody, ICR13 and preimmune serum added to one of the samples. An *in vitro* kinase assay was performed on the lysates and the products of the reaction analysed by 7.5% (w/v) SDS-PAGE. The results in Figure 6.3B reveal that expression of p185$_i$, as indicated by its basal

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Figure 6.3.A. 2.5 x 10^6 Sf9 cells were infected with each of the recombinant c-erbB-2$_i$ viruses. The cells were lysed with Triton x 100 lysis buffer at 48 h post infection, analysed by 7.5% (w/v) SDS-PAGE and immunoblotted with the anti-21N antibody.

Lane 1.   wild type.
Lane 2.   virus 1.
Lane 3.   virus 2.
Lane 4.   virus 3.
Lane 5.   virus 4.
Lane 6.   SKBR3.

Figure 6.3B. 2.5 x 10^6 Sf9 cells were infected with the c-erbB-2$_i$ virus and harvested at 24, 48 and 72 h post infection. The cells were lysed in Triton x 100 lysis buffer and duplicate samples, preimmune serum added to one of the samples, immunoprecipitated with the anti-p185 antibody, ICR13. The immunoprecipitates were resuspended in 50μl kinase buffer, 0.5 μCi [γ-^{32}P]ATP (specific activity 10 mCi/ml, Amersham) added and incubated for 10 min at room temperature. The reaction products were analysed by 7.5% (w/v) SDS-PAGE and the gel fixed, dried and exposed to Hyperfilm (Amersham) overnight at -80°C.

Lane 1.   wild type + ICR13.
Lane 2.   wild type + NRS.
Lane 3.   24 h post infection + ICR13.
Lane 4.   24 h post infection + NRS.
Lane 5.   48 h post infection + ICR13.
Lane 6.   48 h post infection + NRS.
Lane 7.   72 h post infection + ICR13.
Lane 8.   72 h post infection + NRS.

A
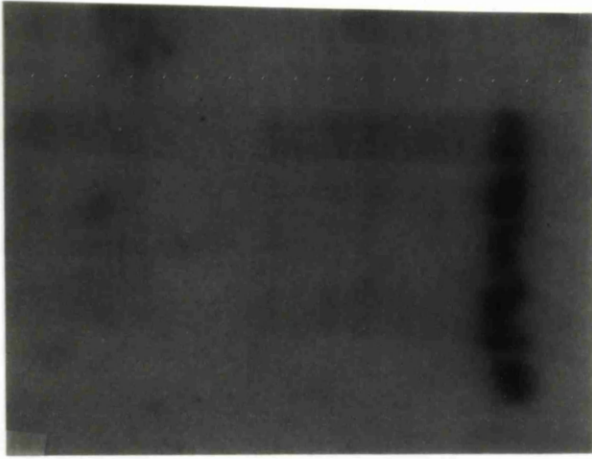
+ WILD TYPE

+ c-erbB-2   1

+ c-erbB-2   2

+ c-erbB-2   3

+ c-erbB-2   4

+ SKBR3

185 kD p185

B
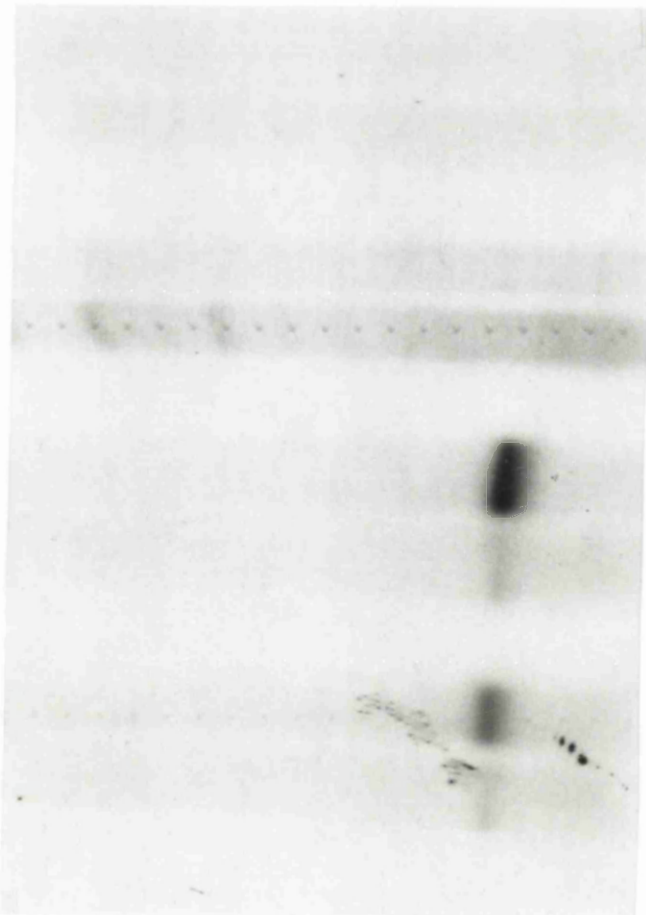
+ WILD TYPE/ ICR13

+ WILD TYPE/ NRS

+ DAY 1/ ICR13

+ DAY 1/ NRS

+ DAY 2/ ICR13

+ DAY 2/ NRS

+ DAY 3/ ICR13

+ DAY 3/ NRS

autophosphorylation state, attains a maximum level by 48 h post infection. However, it proved necessary to expose the gel overnight, suggesting that p185$_i$ was not efficiently expressed by the infected insect cells.

Anti-21N antibody immunoblot analysis of whole cell lysates, suggested that the expression levels of p185$_i$ were in fact higher than was indicated by the analysis of Triton x 100 lysates (Figure 6.4A, lane 1). In order to determine whether a high proportion of p185$_i$ is not solubilised from the cell membrane by Triton x 100 treatment, the cell debris discarded after centrifugation of the lysates were analysed. Infected cells were harvested 48, 72 and 96 h post infection and lysed in Triton x 100 lysis buffer. The lysates and the cell debris were analysed by 7.5% (w/v) SDS-PAGE and immunoblotting with the anti-21N antibody. It was estimated that approximately 75% of the p185$_i$ was not released from the cell membranes by Triton x 100 treatment (Figure 6.4A). Therefore the problem of low recovery of p185$_i$ was not due to a low expression level but a consequence of a low solubilisation efficiency. Infected cells were therefore treated with 1% (w/v) CHAPS lysis buffer. 2.5 x 10^6 Sf9 cells were harvested at 48, 72 and 96 h post infection and lysed with CHAPS lysis buffer. The lysates and cell debris were analysed by 7.5% (w/v) SDS-PAGE and immunoblotted with the anti-21N antibody. As can be seen in Figure 4.6B, 1% (w/v) CHAPS treatment resulted in the solubilisation of 95% of the p185$_i$.

6.5. The membrane localisation of p185$_i$.

In order to establish whether the p185$_i$ is localised in the plasma membrane of the insect cells, cells infected with the c-erbB-2$_i$ virus were subject to immunofluorescence with a mouse anti-p185$^{c\text{-}erbB\text{-}2}$

Figure 6.4A. 2.5×10^6 Sf9 cells were infected with the c-erbB-2 virus and harvested 48, 72 and 96 h post infection. The cells were lysed in Triton x 100 lysis buffer. The lysates and cell debris were analysed by 7.5% (w/v) SDS-PAGE and immunoblotting with the anti-21N antibody.

Lane 1.   Triton x 100 lysate of whole cells.

Lane 2.   pellet 48 h post infection.

Lane 3.   supernatant 48 h post infection.

Lane 4.   pellet 72 h post infection.

Lane 5.   supernatant 72 h post infection.

Lane 6.   pellet 96 h post infection.

Lane 7.   supernatant 96 h post infection.

Figure 6.4B. 2.5×10^6 Sf9 cells were infected with the c-erbB-2 virus and the cells harvested at 48, 72 and 96 h post infection. The cells were lysed with 1% (w/v) CHAPS lysis buffer and the lysates and cell-debris analysed by 7.5% (w/v) SDS-PAGE. The gel was immunoblotted and probed with the anti-21N antibody and exposed to Hyperfilm (Amersham) overnight at -80°C.

Lane 1.   wildtype infection.

Lane 2.   pellet 48 h post infection.

Lane 3.   lysate 48 h post infection.

Lane 4.   pellet 72 h post infection.

Lane 5.   lysate 72 h post infection.

Lane 6.   pellet 96 h post infection.

Lane 7.   lysate 96 h post infection.

A
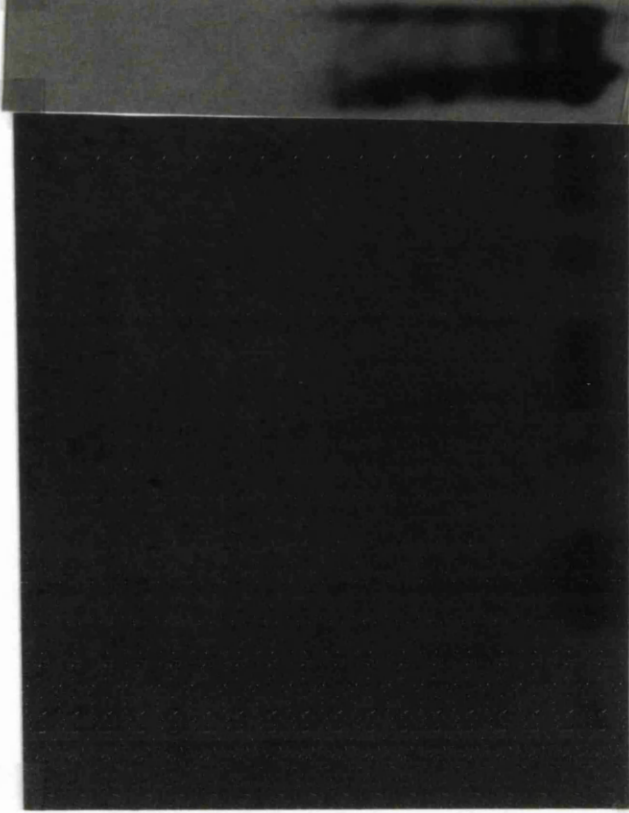
+   TRITON PELLET

+   48 H PELLET

-

+   48 H SUPERNATANT

-

+   72 H PELLET

+   72 H SUPERNATANT

-

+   96 H PELLET

-

+   96 H SUPERNATANT

B
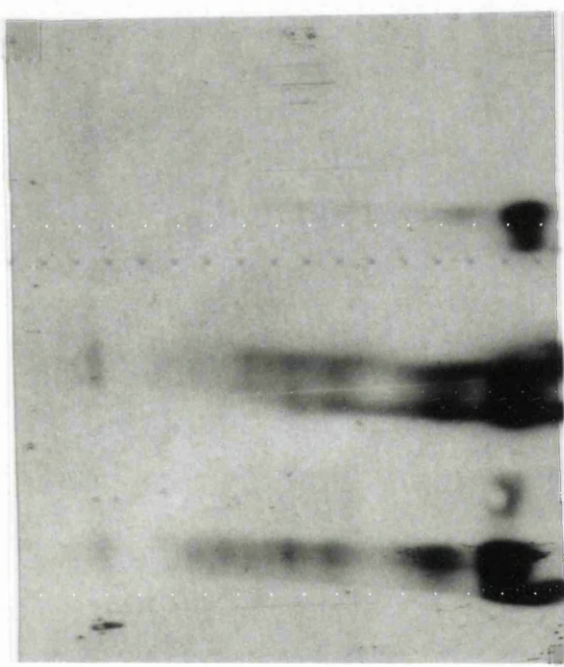
+   WILD TYPE

-

+   48 H PELLET

+   48 H LYSATE

-

+   72 H PELLET

+   72 H LYSATE

-

+   96 H PELLET

+   96 H LYSATE

185 kD

monoclonal antibody specific for the external domain (Oncogene Sci Inc). 2.5×10^6 Sf9 cells were infected with the c-erbB-2i virus and the cells harvested at 48 h post infection when the expression level of p185i was high and when the cells were still viable. The cells were pelleted, washed in 10 ml of IPL41 and resuspended in 0.5 ml of PBS containing 10µg/ml anti-p185 antibody. The cells were incubated at 4°C for 40 min and washed as before. The pellet was resuspended in 0.5 ml of PBS containing a 1/100 dilution of 1 mg/ml goat anti-mouse IgG-FITC (Southern Biotech.) and incubated in the dark at 4°C for 40 min. The cells were washed twice as before and resuspended in 1 ml of PBS and analysed for immunofluoresence by fluorescence microscopy. Analysis of the p185i-expressing cells indicated the presence of a large amount of cell membrane-associated fluorescence. This surface fluorescence was specific for cells infected with the c-erbB-2i virus since wild type infected cells treated in an identical manner displayed no such fluorescence. Furthermore, the ability of the antibody, which was raised against an epitope in the extracellular domain of p185, to recognise p185i indicates that it is orientated correctly within the membrane. The membrane-association of the p185i was documented by fluorescence-activated cell sorting (FACS) performed on the immunofluorescence sample. Figure 6.5 indicates the presence of a high percentage of fluorescent cells in the c-erbB-2i virally infected population but not in that infected with wild type virus (FACS analysis performed by Sue Barnett). Thus, insect cells infected with the c-erbB-2i virus express plasma membrane-associated p185 which is correctly orientated with respect to the membrane.

Figure 6.5. 2.5×10^6 Sf9 cells were infected with the c-erbB-2$_i$ virus and harvested at 48 h post infection.   The cells were washed in 10ml IPL41 and resuspended in 0.5 ml PBS containing 10 μg/ml anti-p185 antibody (Oncogene Sci Corp).   The cells were incubated at 4^oC for 40 min and then washed as before.   The cells were resuspended in 0.5 ml PBS containing a 1/100 dilution of 1 mg/ml goat anti-mouse IgG-FITC (Southern Biotech.) and incubated at 4^oC for 40 min.   The cells were washed twice as before and resuspended in 1 ml PBS.

cell number

Fluorescence intensity ⟶
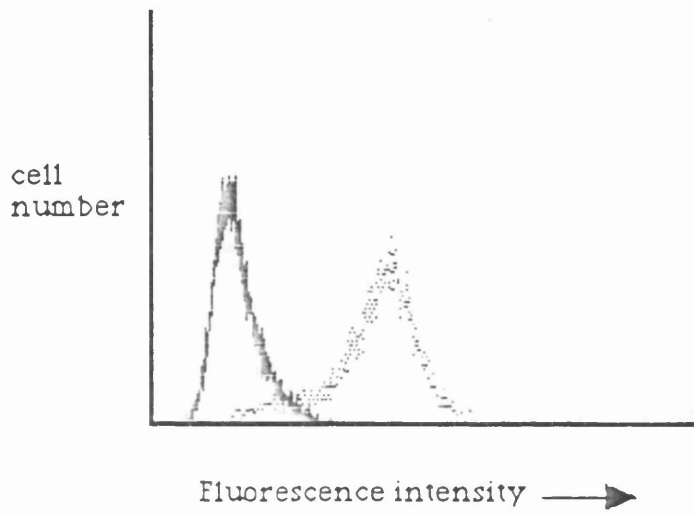
▭ Sf9 cells infected with the c-erbB-2 virus.

▪ Sf9 cells infected with wildtype virus.

6.6. The coexpression of $p185_i$ and $EGFR_i$.

It has been observed that EGF treatment of Rat-1 fibroblasts cotransfected with EGF receptor and $p185^{neu}$ constructs results in $p185^{neu}$ phosphorylation, indicating that $p185^{neu}$ acts as a substrate for the EGF receptor protein-tyrosine kinase activity (Kokai et al., 1988). A potential explanation for these observations is that EGF stimulates the formation of EGF receptor-$p185^{neu}$ heterodimers, thereby activating the $p185^{neu}$ protein-tyrosine kinase.

The major problem associated with demonstrating the presence of EGF receptor-p185 dimers in cell lines is the relatively low level of receptor expression.   In an attempt to overcome this sensitivity problem, the question of heterodimer formation was addressed using the baculovirus expression system.   Coinfection of Sf9 cells with the $EGFR_i$ and c-$erbB$-2_i viruses led to the expression of both proteins at relatively high levels.   The resulting dimers were stabilised by treatment with the chemical cross linker EDAC and analysed by 3-11% (w/v) gradient SDS-PAGE. 1.0×10^7 Sf9 cells were infected with either the $EGFR_i$ or the c-$erbB$-2_i virus or both.   The cells were harvested 72 h post infection, resuspended in PBS containing 1 μg/ml EGF and incubated at 4^oC for 1 h. The cells were then resuspended in 50 mM MES (pH 7.5), 50 mM EDAC and incubated at room temperature for 30 min.   The cells were washed extensively, lysed and immunoprecipitated with either the EGF receptor-specific monoclonal antibody, R1 or the p185-specific antibody, ICR13.   The $p185_i$ and $EGFR_i$ were then subjected to *in vitro* kinase assay and the products of the reaction analysed by 3-11% (w/v) gradient SDS-PAGE.   The results in Figure 6.6A show that in the lysates of the coinfected cells, immunoprecipitation by R1 and ICR13 led to the

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Figure 6.6A. 1.0×10^7 Sf9 cells were infected with the EGFR$_i$ or c-erbB-2$_i$ virus or both. The cells were harvested at 72 h post infection, resuspended in PBS containing 1 µg/ml EGF and incubated at 4°C for 1 h. The cells were then resuspended in 50 mM (pH 7.5) MES, 50 mM EDAC, incubated at room temperature for 30 min and lysed in Triton x 100 lysis buffer. The lysates were divided into two aliquots, one immunoprecipitated with the anti-EGF receptor antibody, R1 and the other with the anti-p185 antibody, ICR13. The immunoprecipitates were resuspended in 50 µl kinase buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 0.1 mM MnCl$_2$, 10 mM MgCl$_2$, 0.2% (w/v) Triton x 100, 100 µM sodium orthovanadate), 1 µCi [γ-^{32}P]ATP (s.a. 10 mCi/ml, Amersham) added and the reaction incubated at 4°C for 10 min. The reaction products were analysed by 3-11% (w/v) gradient SDS-PAGE, the gel fixed, dried under vacuum and exposed to Hyperfilm (Amersham).

Lane 1.   A431 immunoprecipitated with R1 (short exposure).
Lane 2.   A431 immunoprecipitated with R1 (long exposure).
Lane 3.   uninfected Sf9 cells immunoprecipitated with ICR13.
Lane 4.   uninfected Sf9 cells immunoprecipitated with R1.
Lane 5.   coinfected Sf9 cells immunoprecipitated with ICR13.
Lane 6.   coinfected Sf9 cells immunoprecipitated with R1.
Lane 7.   c-erbB-2$_i$ infected Sf9 cells precipitated with ICR13.
Lane 8.   c-erbB-2$_i$ infected Sf9 cells precipitated with R1.
Lane 9.   EGFR$_i$ infected Sf9 cells precipitated with ICR13.
Lane 10.   EGFR$_i$ infected Sf9 cells immunoprecipitated with R1 (long exposure).
Lane 11.   EGFR$_i$ infected Sf9 cells immunoprecipitated with R1 (short exposure).

Figure 6.6B. Duplicate Sf9 cultures were infected as before and one treated with 1 µg/ml EGF. The cells were lysed and immunoprecipitated and the immunoprecipitates resuspended in 50 µl kinase buffer containing 1 mM MgCl$_2$. The in vitro kinase assay was performed and the reaction products analysed as before.

Lane 1.   coinfected Sf9 cells (-EGF) precipitated with R1.
Lane 2.   coinfected Sf9 cells (+EGF) precipitated with R1.
Lane 3.   coinfected Sf9 cells (-EGF) precipitated with ICR13.
Lane 4.   coinfected Sf9 cells (+EGF) precipitated with ICR13.

	A431 CONTROL		INFECTION								
			NO VIRUS		BOTH		P185		P170		
			ANTIBODY								
	R1	R1	ICR	R1	ICR	R1	ICR	R1	ICR	R1	R1
	1	2	3	4	5	6	7	8	9	10	11

370 kD →
355 kD →
340kD →

185 kD →
170 kD →
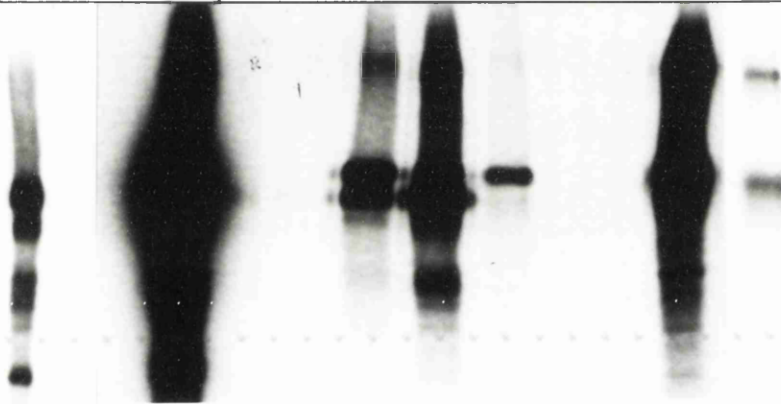
EGF	-	+	-	+
	R1	R1	ICR	ICR
	12	13	14	15

355 kD →
340 kD →

185 kD →
170 kD →
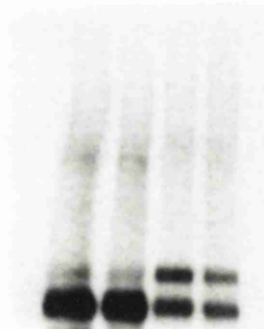
identification of high molecular weight dimers (lane 5 and 6). The composition of the dimers was estimated by comparison with the sizes of the two homodimer bands (lanes 7 and 11). R1 immunoprecipitated mainly $EGFR_i$ homodimers (340 kD), but also $EGFR_i$ -$p185_i$ heterodimers (355 kD), whereas ICR13 immunoprecipitated mainly $EGFR_i$ -$p185_i$ heterodimers and to a lesser extent, $p185_i$ homodimers (370 kD). Heterodimers that had not been sucessfully cross linked were resolved into their monomeric components under the denaturing conditions of the experiment.

In order to ascertain whether the dimerisation was EGF dependent, the experiment was repeated in the presence or absence of $1\mu g/ml$ EGF. Furthermore, in an attempt to reduce the background level of kinase activity, a 10 x lower concentration of $MgCl_2$ (1 mM instead of 10 mM) was used in the kinase buffer (George Panayatou, personal communication). Initial findings suggested that dimerisation was independent of EGF treatment since dimers were observed in both the treated and untreated samples (see Figure 6.6B). However, this experiment was performed at 72 h post infection where is had also been observed that the protein-tyrosine kinase activity of the EGF receptor is EGF-independent (Figure 3.16B). It was suggested that by 72 h post infection, the concentration of the EGF receptor at the membrane was high enough to promote dimer formation and protein-tyrosine kinase activation in the absence of EGF. The detection of both $EGFR_i$ and $p185_i$ homodimers and $EGFR_i$-$p185_i$ heterodimers at 72 h post infection confirms this proposal. The EGF dependency of dimer formation should therefore be investigated at 48 h post infection when the receptor levels are still low enough to require the presence of ligand to promote dimerisation. The immunoprecipitation of heterodimers from the EGF

treated cells would indicate that dimer formation was EGF-dependent. Furthermore, it should be possible to determine whether the protein-tyrosine kinase is active in its dimeric form by *in vitro* kinase assay.

6.7. DISCUSSION.

The coding sequence for the full length human c-erbB-2 molecule has been introduced into the baculovirus genome resulting in the isolation of a recombinant baculovirus (6.1.). Infection of insect cells with this construct directs the expression of a polypeptide which is recognised specifically by both the anti-21N monoclonal antibody and the p185-specific ICR13 antibody. The molecular weight of the polypeptide was slightly lower than the expected 185 kD, although it was not possible to determine the exact molecular weight by 7.5 % (w/v) SDS-PAGE. Similarly, when the EGF receptor was expressed in the baculovirus system, the molecular weight was estimated as 155 kD, 15 kD smaller than the 170 kD species expressed in A431 cells (Greenfield et al., 1987). It was proposed that the $EGFR_i$ was smaller as a result of incomplete glycosylation since insect cells are unable to incorporate complex oligosaccharides (Hsieh and Robbins, 1984). Thus, the lower molecular weight of $p185_i$ can be attributed to incomplete glycosylation. Interestingly, 3-11% (w/v) gradient SDS-PAGE analysis of the $EGFR_i$ and the EGF receptor from A431 (Figure 6.6) demonstrated that the molecular weight of the $EGFR_i$ was closer to 170 kD than 155 kD as was estimated by Greenfield et al. (1987). Indeed, the discrepancy in molecular weights between the $p185_i$ and $p185^{c-erbB-2}$ expressed in SKBR3 cells does not appear to be as high as 15 kD. *In vitro* kinase assays indicated that the protein possesses a basal level of phosphorylation (6.2.). These results suggest that the authenic p185 molecule has been expressed in insect cells. Furthermore, immunofluorescence data and FACS analysis

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indicate that the p185$_i$ is membrane-associated and orientated correctly with respect to the membrane (6.3.).

It is still unclear how the signal generated by the binding of an exogenous ligand to the extracellular domain of a receptor is transduced through the transmembrane segment thereby activating the intracellular protein-tyrosine kinase. An allosteric oligomerisation model has been proposed in which receptor oligomerisation promotes the activation of the kinase via subunit interaction between adjacent cytoplasmic domains (Yarden and Schlessinger, 1987). According to the model, monomeric receptors are in equilibrium with dimeric receptors, the dimeric form having a higher affinity for ligand binding. Thus, receptor oligomerisation is driven by the preferential binding of ligand to the dimeric form. The extensive sequence homology between p185$^{neu/c-erbB-2}$ and the EGF receptor has led to the proposal that p185$^{neu/c-erbB-2}$ is a member of the receptor protein-tyrosine kinase family. In an analogous fashion to the EGF receptor, oligomerisation transduces the ligand-induced signal generated in the extracellular domain of p185$^{neu/c-erbB-2}$ across the membrane resulting in the activation of the intrinsic protein-tyrosine kinase. The overexpression of p185$^{c-erbB-2}$ and its activation by its cognate ligand via an autocrine mechanism, or a mutational event rendering the protein-tyrosine kinase activity independent of ligand binding could therefore lead to transformation. The recent demonstration that the transforming p185^{neu} is organised at the plasma membrane primarily in an aggregated form, but that the normal p185^{neu} is not, lends support to the oligomerisation model for signal transduction (Weiner et al., 1989).

EGF treatment of Rat-1 fibroblasts cotransfected with EGF receptor and p185 constructs results in p185 phosphorylation,

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indicating that p185 acts as a substrate for the EGF receptor protein-tyrosine kinase activity (Kokai et al., 1988). A potential explanation for these observations is that EGF stimulates the formation of EGF receptor-p185 heterodimers, thereby activating the p185 protein-tyrosine kinase. The EGF-induced stimulation of heterodimers formation between the EGF receptor and p185 have been demonstrated in both A431 and SKBR3 cells (E Lucassen, personal communication). The major problem concerning the detection of heterodimers in these cell lines is due to the relatively low level of receptor expression. Although A431 cells express 2.5×10^6 EGF receptors per cell (Haigler et al., 1978) they express only 1.0×10^6 p185$^{c\text{-}erbB\text{-}2}$ molecules per cell. The SKBR3 cell line expresses only 1.0 $\times 10^6$ p185$^{c\text{-}erbB\text{-}2}$ molecules and 9.0×10^4 EGF receptors per cell respectively (E Lucassen, personal communication).

The question of receptor oligomerisation was addressed in insect cells by coinfection with the EGF receptor and c-erbB-2 viruses (6.4.). The formation of heterodimers was determined by the molecular weight on 6-11% (w/v) gradient SDS-PAGE of the resultant dimers (355 kD) relative to that of the EGFR$_i$ homodimers (340 kD) and the p185$_i$ homodimers (370 kD). Furthermore, antibodies specific for either the EGF receptor or p185 were able to immunoprecipitate this dimer species. The high level of expression of the EGFR$_i$ and p185$_i$ achieved in the insect cells makes the detection of dimers relatively straight forward. The identification of both EGFR$_i$ homodimers and EGFR$_i$-p185$_i$ heterodimers lends support to the oligomerisation model that has been proposed to explain the mechanism of growth factor signal transduction (Yarden and Schlessinger, 1987). The extremely high level of the receptors at the insect cell surface is not truely representative of the physiological state existing in fibroblasts and therefore, although these

observations seem to support the oligomerisation model, alternative intramolecular mechanisms can not be discounted.

Interestingly, infection with the c-erbB-2 virus alone directed the formation of $p185_i$ homodimers independently of the action of a putative ligand. It is possible that the concentration of $p185_i$ at the membrane is sufficiently high to promote dimer formation in the absence of ligand. Indeed, it has been demonstrated that the $EGFR_i$ protein-tyrosine kinase activity is EGF-independent at 72 h post infection when it is proposed that the concentration of the receptor is such that dimerisation occurs in the absence of EGF (Figure 3.16B). The oligomerisation of transforming $p185^{neu}$ but not the normal $p185^{neu}$ has been demonstrated (Weiner et al., 1989). It has been proposed that the mutation in the transmembrane domain of activated $p185^{neu}$ mimicks ligand-induced tyrosine-kinase activation (Bargmann and Bargmann, 1988b), whereas, in human breast carcinomas (Slamon et al., 1987) the transformed phenotype arises as a consequence of the overexpression of $p185^{c-erbB-2}$ and presumably its ligand. The amplification of $p185^{c-erbB-2}$ in NIH 3T3 cells is unable to induce transformation, indicating that the overexpression of $p185^{c-erbB-2}$, in the absence of ligand is not sufficient for activation (Hung et al., 1986; Velu et al., 1987). The observation of $p185_i$ homodimers in infected Sf9 cells indicates that the level of $p185_i$ at the insect cell membrane is sufficiently high enough to promote dimerisation. Thus the availability of recombinant baculoviruses directing the expression of both the EGF receptor and p185 in insect cells generates an *in vivo* system in which the mechanism of signal transduction of analogous growth factor receptors can be compared.

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Since the ligand for $p185^{c-erbB-2/neu}$ has not yet been identified, it has proved difficult to analyse the biochemical properties and biological function of the putative receptor. The search for the ligand was instigated by the construction of chimeric molecules involving the EGF receptor. One such chimera was constructed from the ligand binding, transmembrane and juxtamembrane domains of the EGF receptor fused to the protein-tyrosine kinase and carboxyl terminal domains of the neu sequence (Lehvaslaiho et al., 1989). EGF treatment of NIH 3T3 cells transfected with the construct induced tyrosine phosphorylation of the chimeric receptor leading to transformation and growth of colonies in soft agar. In contrast, when expressed at similar levels, normal $p185^{neu}$ showed no protein-tyrosine kinase activity nor transformation properties. These results indicated that normal $p185^{neu}$ possesses mitogenic and transformation potential only in the presence of a ligand which stimulates the protein-tyrosine kinase activity.

In an alternative approach, the extracellular ligand-binding domain of the EGF receptor was fused to the transmembrane and cytoplasmic domains of $p185^{neu}$ and transfected into NIH 3T3 cells (Lee et al., 1989). The chimera was specifically targeted to the cell surface where it exhibited typical high and low affinity binding sites for EGF. EGF binding induced the activation of the heterologous $p185^{neu}$ protein-tyrosine kinase activity leading to autophosphorylation, kinase activation characteristically subject to transmodulation by TPA. Furthermore, the activation of the $p185^{neu}$ protein-tyrosine kinase generated mitogenic and transforming signals in response to EGF or TGFα treatment. These results led to the suggestion that, in common with the EGF receptor, the extracellular domain of $p185^{neu}$ displays

high and low affinity ligand binding sites, ligand binding potentially regulated by TPA. In transfected NIH 3T3 cells, the chimeric receptor required EGF binding to activate the protein-tyrosine kinase activity, whereas transformation induced by wild type $p185^{neu}$ was independent of exogenous ligand (Difiore et al., 1987b). These results led to the proposal that NIH 3T3 cells synthesise the ligand for $p185^{neu}$, thereby illiciting receptor activation via an autocrine mechanism (Lee et al., 1989). These results are in direct opposition to those of several other groups that have demonstrated that the high level of normal $p185^{neu}$ expression in NIH 3T3 cells is insufficient to cause transformation, although differences in the $p185^{neu}$ expression level may account for these differences (Hung et al., 1986; Velu et al., 1987; Lehvaslaiho et al., 1989). There has as yet been no report of the purification of this putative ligand for the c-erbB-2/neu receptor from NIH 3T3-conditioned medium.

A candidate for the $p185^{neu}$/c-erbB-2 ligand was identified in the conditioned medium of ras-transformed fibroblasts (Yarden and Weinberg, 1989). Medium conditioned by ras transformants induced down-modulation of $p185^{neu}$ and activated the *in vitro* protein-tyrosine kinase activity. Furthermore, a rapid increase in $p185^{neu}$ autophosphorylation was specifically induced by this conditioned medium and by no other conditioned media analysed. It is feasible that transmodulation by the EGF receptor was responsible for the activation rather than a direct stimulation of $p185^{neu}$ since ras-transformed cells synthesise TGFα (Sporn and Roberts, 1985). In order to exclude this possibility, a neu expression vector was transfected into haematopoetic cells which do not express the EGF receptor. The resulting transfectants responded mitogenically to the ras-transformant conditioned medium

indicating that the autophosphorylation of p185^{neu} arose as a direct consequence of the ligand-induced activation of its intrinsic protein-tyrosine kinase activity.

Thus, although there have been several reports describing the preliminary identification of the ligand for p185$^{neu/c-erbB-2}$, it has not as yet been purified. The availability of large quantities of p185 will allow affinity chromatographic techniques to be performed on the conditioned media from a variety of transformed cells in an attempt to purify the p185 ligand. The identification of the ligand for p185$^{neu/c-erbB-2}$ and the subsequent determination of its three-dimensional structure would lead to the design of antagonists. The availability of such antagonists would have great clinical implications for the treatment of human breast and ovarian carcinoma.

CHAPTER SEVEN.
GENERAL DISCUSSION.

7.1.   Growth factor receptor signal transduction.

In the introduction to this thesis, I have attempted to outline the mechanisms operating during the normal course of growth factor signal transduction, using the EGF receptor as a model system. Exogenous growth factor binds to the extracellular ligand-binding domain, thereby perturbing the tertiary conformation of the extracellular domain of the receptor (Greenfield et al., 1989). This conformational change is relayed to the intracellular domain, via the transmembrane segment, where it stimulates the intrinsic protein-tyrosine kinase activity. The activation of the receptor protein-tyrosine kinase initiates the phosphorylation of a number of substrates, involved in the generation of a cascade of intracellular signalling events, which ultimately culminate in DNA replication and cell division in the target cells. The absolute requirement for an active protein-tyrosine kinase in the transduction of the mitogenic signal has been demonstrated (Prywes et al., 1986; Honneger et al., 1987a, b). The control of the protein-tyrosine kinase activity is achieved in three ways, by binding of exogenous ligand, by the action of protein kinase C and by the stearic constraints imposed on the kinase domain by the carboxyl terminal domain. In the first way, ligand binding causes down-regulation of the receptor via internalisation of the ligand-receptor complex. The ligand is delivered to lysosomes and the receptor recycled to the cell surface. Following several rounds of recycling, the receptor is also delivered to the lysosomes for degradation. The phosphorylation of the EGF receptor on Thr[654], mediated through protein kinase C, may allosterically regulate the affinity of ligand-binding and the activation of the protein-tyrosine kinase (Schlessinger et al., 1986). In the third way, receptor

autophosphorylation removes the stearic constraints imposed on the protein-tyrosine kinase by its interaction with the carboxyl terminal domain (Bertics and Gill, 1985). The tight control exerted over the receptor protein-tyrosine kinase ensures that it is activated only in response to ligand binding.

7.2. Oncogenesis and the subversion of the normal growth factor signalling pathway.

Subversion of this growth factor signalling pathway leads to uncontrollable DNA replication and cellular proliferation in target cells, resulting in cellular transformation and the formation of tumours *in vivo* (for review see Bishop, 1985, 1987; Sporn and Roberts 1985, 1988). It is obvious that the disruption of the signalling pathway at any point could lead to continuous and uncontrollable DNA replication and cell division. Aberrant expression of a growth factor or the disruption of the normal controlled expression of a growth factor could result in the continual stimulation of the cognate receptor. Retroviral infection may lead to the direct expression of a growth factor, as in the case of SSV which directs the expression of the PDGFB -homologous peptide, $p28^{v-sis}$ (Waterfield et al., 1983; Doolitle et al., 1984). Alternatively, oncogenic activation may result in the production of endogenous growth factors that act in an autocrine fashion on the producer cells via interaction with their functional receptors (Todaro et al., 1977). In addition, normal autocrine mechanisms operating in a cell, such as the action of TGFα on the stimulation of keratinocyte proliferation during wound healing, could be disrupted thereby leading to the malignant conversion of keratinocytes into squamous carcinomas (Coffey et al., 1987).

The next obvious point at which to subvert the normal signalling pathway would be to uncouple the receptor protein-tyrosine kinase activity from the normal negative constraints imposed on it. The mutational activation of the receptor can arise through alterations in the ligand binding domain as a consequence of gene translocation. Truncation of, or point mutations within the external domain might mimic continuous receptor occupancy, thereby causing a constant activation of the protein-tyrosine kinase. Alternatively, oncogenic activation could arise due to the loss of the stearic restraints imposed on the kinase domain by the carboxyl terminus. Both of these mechanisms seem to be responsible for the oncogenic activation of the products of v-erbB (Downward et al., 1984), v-kit (Yarden et al., 1987) and v-fms (Coussens et al., 1986; Roussel et al., 1987). Still at the level of the receptor, overexpression of receptors in the presence of their cognate ligands would lead to an amplification of the resultant mitogenic stimuli. The aberrant overexpression of the EGF receptor and the c-erbB-2 (Slamon et al., 1987) and c-erbB-3 (Kraus et al., 1989) gene products have been implicated in the generation of squamous carcinoma of the breast.

7.3. Towards the understanding of ligand-receptor interactions.

The elucidation of the mechanism of action of growth factors and the identification of all of the components of the signalling cascade is crucial to the understanding of how oncogenic activation of any one of these components can result in cellular transformation. Basic to the determination of the normal mechanisms operating in the growth factor signalling pathway is the process of ligand-receptor interaction. How can the binding of an exogenous ligand with the extracellular domain of its receptor generate a conformational change? This question can be

answered in part, by the determination of the receptor binding face of the ligand and the corresponding ligand-acceptor site of the receptor. The aim of this thesis was to design expression systems for the large-scale production of EGF and TGFα so as to establish their complete three-dimensional structures. The determination and comparison of the receptor-binding sites of EGF and TGFα will generate information as to the ligand-acceptor site of the receptor, which could then be further extended through mutational analysis and co-crystallisation.

As discussed in Chapters 3 and 5, EGF has been purified from several species, the highly conserved nature of its primary structure indicating that the molecule folds into a rigid three-dimensional conformation. The conformation, dictated by the six Cys residues and the other conserved residues, must be rigorously adhered to if receptor binding is to occur (Savage et al., 1973). Since it has been shown that TGFα competes with EGF for binding to the EGF receptor (Todaro and DeLarco, 1977), it can be predicted that the tertiary structures of the two ligands are basically equivalent. Furthermore, the observation that amphiregulin (Shoyab et al., 1988) and several viral growth factors (Blomquist et al., 1984; Chang et al., 1987; Strayer et al., 1987) are able to bind to the EGF receptor led to the suggestion that they too must share tertiary structural motifs in common with EGF. The alignment of the six conserved Cys residues within the amino acid sequences of the EGF family of growth factors reveals that in all, sixteen residues are conserved or conservatively changed. The conserved nature of these residues indicates that they are involved in the determination of the three-dimensional structure of the ligands and in the establishment of the face of the molecule which interacts with the receptor.

^{1}H NMR analysis of EGF and TGFα has led to the proposal of a model for the tertiary structure of those ligands that are capable of binding to the EGF receptor. The model predicts that the backbone of the ligand is arranged in two antiparallel β-sheets with chain reversals occurring at tight β-turns thereby folding the molecule into a compact bilobed structure (Campbell et al., 1989). This model can be used to predict features of the ligand that are responsible for binding to and activating the EGF receptor. More meaningful predictions can be made if the model is used in conjunction with information as to the positioning of the highly conserved residues. The sixteen conserved residues can be divided into two categories, those responsible for folding the molecule into the precise three-dimensional conformation (structural) and those directly responsible for binding to the receptor (functional). Certain amino acids such as Cys and Gly, by their very nature, indicate that they play a structural role. The ^{1}H NMR analysis of EGF and TGFα predict that six of the remaining conserved residues lie on one face of the molecule where they could conceivably be involved in the generation of the receptor-binding site. Mutational analysis of these conserved residues, in association with ^{1}H NMR analysis of the mutant peptides, will help discriminate between those residues that play a structural role and those that are directly involved in receptor binding. Mutation of structural residues will destroy the tertiary structure of the molecule which will be reflected in the dramatic perturbation of the ^{1}H NMR spectrum. Conversley, mutation of functional residues will only affect interactions with adjacent residues resulting in a slight alteration of the ^{1}H NMR spectrum. Ultimately, the complete elucidation of the three-dimensional structures of EGF and TGFα will result from diffractional analysis of crystals.

I have successfully used the baculovirus expression system to produce human EGF and TGFα. Both of the ligands are able to compete with [^{125}I] labelled mouse EGF for binding to the EGF receptor expressed on the surface of NR6 fibroblasts which have been transfected with the EGF receptor cDNA (DiFiore et al., 1987b). Furthermore, the binding of the growth factors causes activation of the intrinsic receptor protein-tyrosine kinase which ultimately leads to DNA replication. Thus, EGF and TGFα produced by the baculovirus system are fully biologically active. Small-scale insect cell culture has led to the expression of EGF and TGFα at yields of approximately 100 ng/ml. However, the yield of the growth factors can be increased significantly by the manipulation of the growing conditions of the insect cells. Insect cells adapt well to growth in suspension culture with the associated advantage of increased cell density and culture volume. When cultured at a cell density of 5.0×10^6 cells/ml in a culture volume of 400 ml in a 1 litre stirring vessel, a yield of 2 mg/ml of the extracellular domain of the EGF receptor has been achieved (C. Greenfield, personal communication). It is anticipated that even greater yields of these less complicated small peptides could be attained.

Preliminary small-scale purification of EGF and TGFα has been hampered by certain components of the growth medium. This material was not removed by filtration through a 1000 dalton filter nor by chromatography through a C_{18} reverse phase Sephak cartridge. Analysis of the constituents of the IPL41 medium implicates the high percentage of lipids and cod liver oil, which are used to supplement the basic medium, in order to protect the cells from the shearing forces created by stirring and aeration. These components could most likely be omitted from the medium for the small-scale monolayer culture of the cells without causing any reduction in the efficiency of cell growth and expression. However, this

approach would not be an option for the subsequent scale-up of the expression systems into the 6 and 60 litre bioreactor facilites. Well-characterised protocols exist for the efficient purification of both EGF (Cohen and Carpenter, 1975) and TGFα (Marquardt et al., 1982). These protocols employ a P_{10} gel permeation chromatography step prior to a C_{18} reverse phase HPLC chromatography step. The P_{10} column is equilibrated in 1M acetic acid and the samples applied in 1M acetic acid. The growth factors are retarded on the column and typically elute in approximately two column volumes as a characteristic peak. It is anticipated that a similar protocol could be employed for the purification of EGF and TGFα from the conditioned media of infected insect cells. However, the absence of five amino acids from the carboxyl terminus of EGF, including two Trp residues, could alter the elution of the peptide from the P_{10} column. It would therefore be necessary to fractionate the column eluate and test aliquots of the fractions for EGF-like activity in a $[^{125}I]$EGF competition assay or $[^{3}H]$Thymidine incorporation assay.

To facilitate the purification of the growth factors from the conditioned medium, alternative medias could be tested. Several alternative medias to IPL41 exist which do not require supplementation with a lipid emulsion. If it is indeed the lipids which are interfering with the purification procedures, employing an alternative medium would remove this problem. The original insect cell medium designed was Graces medium (Gibco BRL). However, there are several disadvantages associated with this medium. Firstly, it must be supplemented with FCS which will complicate the purification procedure in its own right. in addition to elevating the cost of large-scale production dramatically. Secondly, it contains insect haemolymph which may contain growth factor-like activities that would interfere with subsequent mitogenic assays. A more attractive alternative

is Excell (J.R. Scientific USA) which is a low cost synthetic, serum-free medium that does not require lipid supplementation. The insect cells can be readily adapted to culture in Excell medium without loss of expression efficiency.

It was observed that a large amount of EGF and TGFα remains within the cell, potentially as a consequence of the high expression levels exhausting the transport mechanism of the cell. The growth factors are acid stable (Cohen and Carpenter, 1975) and could therefore be extracted from the cells with 1M acetic acid and purified employing the same protocol. Thus, it is anticipated that the large-scale expression of insect cells in Excell medium, coupled with a purification procedure employing a P_{10} gel permeation chromatography step followed by C_{18} reverse phase HPLC chromatography will result in the generation of milligram quantities of pure biologically active human EGF and TGFα. An alternative purification strategy would involve the preperation of an affinity column using specific antibodies which would result in the purification of the peptides directly from the conditioned-medium.

The availability of milligram quantities of EGF and TGFα means that the generation of crystals is realistically within sight. Diffractional analysis of the crystal structures will lead to the complete elucidation of the three-dimensional conformation of these biologically important growth factors and the establishment of the identity of the receptor-binding face. Mutational analysis of the conserved residues in the EGF family of growth factors has already implicated Leu[47] of EGF (Leu[48] of TGFα) in receptor binding (Ray et al., 1988; Lazar et al., 1988). Subsequent mutations can be expressed in the baculovirus system and the resultant peptides evaluated by ^{1}H NMR. It is anticipated that mutational analysis will discriminate between interactions involved in receptor binding and those responsible

for the activation of the receptor protein-tyrosine kinase, leading to the design of a receptor antagonists. Such a peptide would be able to bind to the receptor without illiciting a biological response, thereby masking the receptor from the effects of EGF and TGFα. The design of such an antagonist would have profound clinical implications for the treatment of certain malignancies known to express elevated levels of the EGF receptor and to antagonise the TGFα autocrine loop which is thought to operate in many tumours. Co-crystallisation of the ligands with the extracelluar domain of the EGF receptor, also produced in the baculovirus system (C. Greenfield, personal communication), will generate information as to the active ligand-binding conformation of the receptor which can be tested by mutational analysis.

7.4. The mechanism of transmembrane signalling.

Recent biophysical investigations in our laboratory have confirmed the original suggestion that the binding of ligand to the EGF receptor induces a conformational change in the extracellular domain (Greenfield et al., 1989). However, it is still unclear how this structural perturbation is translated into the activation of the intracellular protein-tyrosine kinase. Several models have been proposed to explain the mechanism of signal transduction. Yarden and Schlessinger (1987) propose that binding of ligand to inactive receptor monomers promotes their aggregation into dimers which exhibit increased protein-tyrosine kinase activity. In contrast, the 'flush chain" model (Biswas et al., 1985) suggests that it is the dimeric form of the receptor which is inactive and that binding of ligand promotes dimer dissociation into active receptor monomers.

Mechanisms involving ligand-activated receptor oligomerisation have been proposed to explain the process of signal transduction mediated

by several other growth factor receptors. For example, the insulin receptor displays high affinity for insulin in its non-covalently associated $\alpha_2\beta_2$ tetrameric unit (Boni-Schnetzler et al., 1987). The IL-2 receptor is composed of two subunits with differing affinities for IL-2, which only confer high affinity ligand binding and ligand-responsivness when associated in a dimeric unit (Hatakeyama et al., 1989). Furthermore, the binding of PDGF-BB to purified PDGF receptors has been shown to involve the induction of dimer formation (Heldin et al., 1989). Thus, a precedent exists amongest the receptors for growth factors and cytokines for the role of functional dimers in the transduction of the mitogenic signal.

There seems to be little doubt that EGF receptor dimers actually exist, although the interpretation of how ligand binding influences receptor-receptor interaction and leads to the activation of the protein-tyrosine kinase is strongly debated. A large body of evidence exists in support of both the oligomerisation and 'flush chain' models which therefore complicates the elucidation of the mechanism of signal transduction actually occurring *in vivo*. Recent investigations using EGF receptor mutants have yielded convincing evidence in support of the oligomerisation model. A kinase-deficient mutant receptor was phosphorylated in an EGF-dependent manner by a kinase-active receptor, indicating that the kinase-deficient receptor acted as a substrate for the active kinase as a consequence of dimer formation (Honneger et al., 1989). Furthermore, experiments with site-specific anti-receptor antibodies demonstrated that a mutant receptor, devoid of the epitopes recognised by the antibodies, was co-immunoprecipitated with the wildtype receptor.

The ability of EGF to stimulate the *in vivo* tyrosine phosphorylation of both the EGF receptor and p185^{neu}, even though p185^{neu} is unable to bind EGF (Akiyama et al., 1986), suggested that p185^{neu} may act as a

substrate for the protein-tyrosine kinase activity of the EGF receptor, resulting in the activation of the $p185^{neu}$ kinase (Stern and Kamps, 1988). A potential explanation for these observations is that EGF promotes the formation of EGF receptor-$p185^{neu}$ heterodimers and therefore activates the $p185^{neu}$ protein-tyrosine kinase. It is conceivable therefore, that $p185^{neu}$ functions as a growth factor receptor and that dimerisation serves to activate the protein-tyrosine kinase in an analogous fashion to that proposed for the EGF receptor. In support of this proposal, it has recently been demonstrated that the activated form of $p185^{neu}$ is organised at the cell membrane in an aggregated form, whereas normal $p185^{neu}$ is not (Weiner et al., 1989).

The isolation of a recombinant baculovirus directing the expression of $p185^{c-erbB-2}$ allowed the oligomerisation theory for signal transduction to be evaluated in an *in vivo* system. The expression levels of both proteins, achieved by coinfection of insect cells, was high enough to allow the immunoprecipitation of EGF receptor-$p185^{c-erbB-2}$ heterodimers and both types of homodimers. The EGF-dependency of dimer formation can now be evaluated by immunoprecipitation from cells pretreated with or without EGF. Initial investigations were hampered when it was discovered that the EGF receptor protein-tyrosine kinase activity became independent of EGF during the post infection period. It was proposed that the constitutive protein-tyrosine kinase activity measured at 72 h post infection was due to the high concentration of the EGF receptor promoting dimer formation, and hence kinase activation, in the absence of ligand. However, the protein-tyrosine kinase activity is still dependent on EGF at 48 h post infection, when the concentration of the EGF receptor is still low enough to require ligand-induced dimerisation. Thus, the ability of EGF to promote EGF receptor-$p185^{c-erbB-2}$ heterodimer formation can be assessed

at 48 h post infection. Furthermore, not only will it be possible to ascertain whether dimer formation is EGF-inducible, but also whether the protein-tyrosine kinase activity of the receptors is activated in the dimeric form. The use of such an expression system should reveal whether the protein-tyrosine kinase activity of the EGF receptor is EGF-inducible in its monomeric or dimeric form.

The availability of an insect cell expression system for the production of p185$^{c-erbB-2}$ will allow the evaluation of a putative growth factor receptor which is highly homologous to the EGF receptor, thereby allowing comparative analysis of the mechanism of growth factor-induced signal transduction. It will be useful to design an expression system for the production of c-erbB-3 (Kraus et al., 1989; Plowman et al., submitted) in insect cells, enabling the mechanism of action of a second EGF receptor-related protein to be investigated. It will be interesting to discover whether the observed lack of sequence homology in the carboxyl terminus of the c-erbB-3 gene product reflects a difference in the mechanism of regulation of the protein-tyrosine kinase activity (Kraus et al., 1989). Furthermore it can be determined whether the region of 29 amino acids, carboxyl-terminal to the ATP binding site in c-erbB-3, which does not display any sequence identity with the same region of the EGF receptor and p185$^{neu/c-erbB-2}$ confers some sort of substrate specificity on c-erbB-3 (Kraus et al., 1989). The generation of chimeric receptors consisting of different domains of the three EGF receptor family members will help define the signal transduction pathways utilised by these putative receptors.

7.5. THE IDENTIFICATION OF POTENTIAL INTRACELLULAR SUBSTRATES FOR GROWTH FACTOR RECEPTORS.

7.5.1. Phospholipase $C\gamma$.

The ligand-induced activation of the receptor protein-tyrosine kinase results in the stimulation of numerous early and delayed responses which eventually culminate in DNA synthesis and cell division. The early events induced by EGF and other growth factors include the stimulation of the Na^+/H^+ antiporter, elevation in intracellular Ca^{2+} concentration, stimulation of phospholipase C (PLC) and activation of amino acid and glucose transport pathways. The hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP_2) by $PLC\gamma$ produces inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which are responsible for the release of Ca^{2+} from intracellular stores (Berridge and Irvine, 1984) and the activation of protein kinase C (Nishizuka, 1984) respectively. Each of these hydrolysis products may subsequently act to initiate other cascades of phosphorylation.

The biological role of these signalling events is poorly understood and until recently, the link between the activation of the EGF receptor and signalling mechanisms operating within the cell was unknown, but anticipated to be of paramount importance for the transduction of the mitogenic signal. A clue to the biochemical association between the EGF receptor and the phosphoinositide signalling pathway was revealed by the inability of kinase-defective EGF receptor mutants to enhance $PLC\gamma$ activity and Ca^{2+} release (Moolenaar et al., 1988). Subsequent experiments demonstrated that EGF treatment of A431 cells increased the recovery of $PLC\gamma$ with antiphosphotyrosine-specific antibodies (Wahl et al., 1988). Margolis et al. (1989, 1990) went on to establish that in A431 cells, EGF induces tyrosine phosphorylation of $PLC\gamma$ *in vivo*. Furthermore, a direct association between the EGF receptor and $PLC\gamma$ was demonstrated by the co-

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immunoprecipitation of the EGF receptor with anti-PLCγ antibodies. These results were in direct agreement with those of Meisenheider et al. (1989) who demonstrated the *in vivo* and *in vitro* tyrosine phosphorylation of PLCγ by EGF and PDGF treatment of A431 cells. Margolis et al. (1989, 1990) and Meisenhelder et al. (1989) propose that in A431 cells, EGF-induced tyrosine phosphorylation of PLCγ enhances its enzymatic activity, thereby linking the protein-tyrosine kinase activity of the EGF receptor to the PIP_2 hydrolysis signalling pathway.

It is well established that certain neurohormones such as bombesin and bradykinin, which act as mitogens for certain cell types, mediate their biological responses by activating the PIP_2 hydrolysis signalling pathway (Nishizuka, 1984). This is thought to be achieved through the activation of a G protein which couples the activated receptors to PLC. However, it is proposed that the tyrosine phosphorylation of PLCγ in response to EGF or PDGF, arises as a consequence of direct phosphorylation by the receptor protein-tyrosine kinase (Margolis et al. 1989, 1990; Meisenhelder et al. 1989). It is feasible that two pathways exist for the activation of the different PLC isozymes, one involving G proteins, as employed by the neurotransmitters. and the other involving direct stimulation of tyrosine phosphorylation via certain growth factor receptors.

The identification of the tyrosine phosphorylation sites in PLCγ is essential to the elucidation of the functional role of tyrosine phosphorylation in enzymatic activation. It has yet to be determined how this process actually modulates the enzymatic activity of PLCγ. It is possible that tyrosine phosphorylation directly induces the enzymatic activity of PLCγ. Alternatively, it could release some form of negative regulator without actually altering the intrinsic activity per se. Interestingly, Meisenhelder et al. (1989) demonstrated that two other isozymes of PLC

(PLCδ and PLCβ) are not significantly phosphorylated in response to EGF or PDGF. Only PLCγ shares sequence homology with the src protein-tyrosine kinase family within the SH2 and SH3 regions, which have been proposed to perform a regulatory function (Pawson, 1988). There are several potential tyrosine phosphorylation sites within the SH2 and SH3 domains and it is feasible that it is these sites that are phosphorylated in response to EGF or PDGF. The phosphorylation of these sites would explain the observed pattern of phosphorylation of the PLC isozymes. Margolis et al. (1989) demonstrated that p185$^{c-erbB-2/neu}$ is also able to phosphorylate and associate with PLCγ. However, neither insulin (Meisenhelder et al., 1989) nor CSF-1 (Downing et al., unpublished data) were able to promote PLCγ phosphorylation, suggesting that not all growth factor receptors are able to couple to the PI hydrolysis pathway through the activation of PLCγ.

The isolation of a recombinant baculovirus directing the expression of PLCγ in insect cells would enable the *in vivo* reconstruction of the EGF receptor-PLCγ system. The tyrosine phosphorylation sites involved in the regulation of PLCγ activity by EGF could be determined by mutation and coexpression of the resultant polypeptide with the EGF receptor. The availability of a recombinant baculovirus directing the expression of p185$^{c-erbB-2}$ will enable the initial observation of the phosphorylation of PLCγ by p185$^{c-erbB-2}$ to be confirmed. It will be interesting to discover whether these highly homologous receptors induce an identical pattern of tyrosine phosphorylation.

7.5.2. PI kinases.

EGF is able to stimulate the metabolism of PI via a second pathway, whereby a PI-4 kinase phosphorylates the D-4 position of the inositol ring to generate PI(4)P (Pike and Eakes, 1987). This PI-4 kinase represents the

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most abundant PI kinase in fibroblasts, its relative abundance and the ability of EGF to regulate its activity suggesting it plays an important role in PI metabolism. One metabolic role for PI-4 kinase is likely to be the production of PI(4)P as a precursor for PI(4,5)P_2, the substrate for the PLCγ -mediated PI hydrolysis pathway.

Kaplan et al. (1987) and Whitman et al. (1987) identified a unique PI kinase activity associated with certain activated protein-tyrosine kinases. This PI kinase, called PI-3 kinase, physically associates with pp60^{v-src} and polyoma middle T/pp60^{c-src} complexes (Courtneidge and Heber, 1987) and with the PDGF receptor in quiescent fibroblasts and smooth muscle cells in response to PDGF (Kaplan et al., 1987). PI-3 kinase specifically phosphorylates the inositol ring at the D-3 position, generating a novel phospholipid, phosphatidylinositol-3-phosphate (PI(3)P) (Whitman et al., 1988). In addition to phosphorylation of PI, PI-3 kinase phosphorylates PI(4)P and PI(4,5)P_2 to form PI(3,4)P_2 and PI(3,4,5)P_3 respectively. These polyphosphoinositides are not substrates for PLCγ, suggesting that they provide signals independently of the conventional PI turnover response.

Evidence suggesting an important role for PI-3 kinase in cellular growth and transformation was provided by studies of mutants of middle T and the PDGF receptor. In middle T mutants defective for transformation, the middle T/pp60^{c-src} complex fails to associate with the PI-3 kinase (Courtneidge and Heber, 1987). A mutant PDGF receptor, displaying a deletion in the kinase insert, retained PDGF-stimulated protein-tyrosine kinase activity and stimulated PI turnover and a rise in intracellular [Ca^{2+}], but failed to associate with PI-3 kinase and to mediate PDGF-dependent DNA synthesis (Coughlin et al., 1989). These results indicate that PI-3 kinase could be an essential link for the transduction of PDGF-induced mitogenic and oncogenic signals. Subsequently, the *in vivo* ligand-stimulatable

association of the PI-3 kinase with the CSF-1 receptor (Varticovski et al., 1989) and the insulin receptor (Ruderman et al., 1990) have been demonstrated. Up to the present time, there has been no indication that the EGF receptor associates with PI-3 kinase, indicating that EGF treatment may stimulate the association between the EGF receptor and another as yet undiscovered PI kinase. It may be that the different subclasses of the receptor protein-tyrosine kinase family transduce their ligand-induced mitogenic signals through different pathways or through differential activation of the same pathways.

The isolation of the cDNA sequences for PI-3 kinase will allow the generation of a recombinant baculovirus directing the expression of PI-3 kinase in insect cells. Coinfection of insect cells with the EGF receptor and PI-3 kinase viruses will allow the production of the EGF receptor and PI-3 kinase within the same cell. Thus, it will be possible to demonstrate whether EGF can promote the formation of a EGF receptor PI-3 kinase complex *in vivo*. Alternatively, the system could be used to demonstrate the association of the EGF receptor with an as yet undiscovered PI kinase. It could be determined whether the association with the PI kinase and its activation was as a result of specific tyrosine phosphorylation and if so, allow the determination of such tyrosine phosphorylation sites by mutational analysis. In addition, the potential association between p185^{c-}erbB-2 and such a putative PI kinase could be investigated to determine whether closely related members of the same subclass of the receptor protein-tyrosine kinase family functionally interact with the same cellular substrates.

7.6. The baculovirus expression system as a tool for the reconstruction of *in vivo* signalling pathways.

As can be seen, the baculovirus expression system represents a valuable tool for the *in vivo* reconstruction of components of the growth factor signal transduction pathway. These components can be studied in isolation or in groups through coexpression, thereby mimicking the events occurring in the genuine situation. Such a system has been used successfully to demonstrate the PDGF-induced association of the PDGFβ receptor and Raf-1 (Morrison et al., 1989).

Intracellular substrates of the EGF receptor could be identified by the use of the baculovirus expressed protein to 'probe' the lysates of cells treated with EGF. The incubation of the immunoprecipitated EGF receptor with the lysates of EGF-treated fibroblasts will allow the association of the receptor with its intracellular substrates resulting in their phosphorylation. These substrates could be analysed by *in vitro* kinase assays performed before and after incubation of the EGF receptor with the cell lysates

7.7. The recent identification of several putative receptor protein-tyrosine kinases.

Over the past year, several putative receptor protein-tyrosine kinases have been cloned thereby extending the previous list of the family members significantly. These putative receptor protein-tyrosine kinases have been isolated in a number of ways. Genomic libraries have been screened with DNA sequences of known receptor protein-tyrosine kinases, under conditions of reduced stringency, or employing oligonucleotide probes corresponding to regions of known sequence homology. The polymerase chain reaction has been employed to amplify the sequence between two oligonucleotides designed on the basis of sequence homology.

The reduced stringency hybridisation of a human genomic library with a probe corresponding to the protein-tyrosine kinase domain of v-fms revealed a hither to unknown form of the PDGF receptor (Matsui et al., 1989). The two PDGF receptors have since been classified by virtue of their ability to bind the three PDGF isoforms. The original cloned PDGF receptor (Hart et al., 1988) is denoted type B since it binds PDGF-BB with high affinity, PDGF-AB with lower affinity and does not appear to bind PDGF-AA at all. The recently identified PDGF receptor is denoted type α since it binds all three PDGF isoforms.

A putative receptor protein-tyrosine kinase was isolated from a human genomic library by reduced stringency hybridisation with a v-erbB probe (Kraus et al., 1989) and denoted c-erbB-3 as a consequence of its high degree of sequence homology with the EGF receptor and c-erbB-2 gene product.

The putative chicken bFGF receptor has been cloned using an oligonucleotide probe based on the amino acid sequence of the purified protein (Lee et al., 1989). A murine bFGF receptor has also been cloned using the polymerase chain reaction to amplify the sequence between two oligonucleotides based on the sequence of highly conserved motifs within the kinase domain of protein-tyrosine kinases (Reid et al., 1990). The extracellular domain contains three immunoglobulin-like domains and a kinase-insert region and is therefore most closely related to the PDGF and CSF-1 receptors. Interestingly, the kinase-insert is only 14 amino acids long in contrast to 104 and 70 amino acids for the PDGF receptor (Yarden et al., 1986) and CSF-1 receptor (Coussens et al., 1986) respectively.

The comparison of the amino acid sequence of the members of the same subfamily may help assign a function to shared structural motifs,

such as the role of the kinase-insert in the sequences of the receptors for PDGF, CSF-1 and bFGF. Kinase-insert mutants of the PDGF receptor fail to transduce a mitogenic signal in response to PDGF, potentially as a consequence of their inability to activate the PI-3 kinase (Coughlin et al., 1989, Severinsson et al., 1990). These observations suggest an important role for the kinase-insert region in signal transduction, consistent with the high degree of sequence homology of this region between mouse and human PDGF receptors (Whitman et al., 1987). Indeed, the two types of PDGF receptors display only 27% sequence homology in this region which may account in part, for the different functional activities of the two receptors. The deletion of the entire kinase-insert region from the CSF-1 receptor does not effect its ability to transduce a mitogenic signal in response to CSF-1 (Taylor et al., 1989), indicating that this region may interact with different substrates in a receptor-specific manner. It will be interesting to see whether deletion of the kinase-insert region fron the sequence of the bFGF receptor has any effect on the mitogenic response to bFGF.

The external domain of the c-erbB-3 gene product shares 40-50% sequence identity with both the EGF receptor and p185$^{c-erbB-2}$ (Plowman et al., submitted). It shares 60% and 62% sequence identity with the EGF receptor and p185$^{c-erbB-2}$ respectively within the protein-tyrosine kinase domain. C-erbB-3 binds amphiregulin (AR) but fails to bind EGF of TGFα (Plowman et al., submitted). The EGF receptor binds AR but with a lower affinity than EGF or TGFα (Shoyab et al., 1989). The ability of AR to bind to both the EGF receptor and c-erbB-3 may account for some of the differences in biological activity between AR and EGF/TGFα, such as the inability of AR to induce anchorage-independent growth of NRK cells in the presence of TGFβ (Shoyab et al., 1988). However, within a 29 amino acid region, carboxy-terminal to the ATP binding site, the sequence of the c-

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erbB-3 gene product differs from those of the c-erbB-2 and the EGF receptor at 28 and 25 positions respectively (Kraus et al., 1989). Furthermore, the carboxyl terminal region shows no significant sequence homology and is in fact, 30-50% longer. Such regions of high divergence within the cytoplasmic domains may confer functional specificity on these closely-related receptor-like molecules as a consequence of their ability to associate with different substrates.

The observation that c-erbB-3 is able to bind a ligand which is highly homologous to EGF, suggests that growth factor receptors possessing a high degree of sequence homology may bind highly homologous ligands. It would be tempting to speculate that the nature of an unknown ligand could be implied from the sequence homologies existing between its receptor and receptors for other growth factors, indicating that the ligand for p185$^{c-erbB-2/neu}$ may be structurally related to EGF. This idea is supported by the observation that the receptors for insulin and IGF-1, sharing 54% homology within their extracellular domains, are able to bind each others ligands, albeit with a lower affinity for the heterologous ligand (Ullrich et al., 1985). However, the failure of EGF to bind to p185$^{c-erbB-2/neu}$ indicates that it is difficult to speculate on the nature of a ligand based on amino acid sequence homology alone. The availability of the sequences for receptor protein-tyrosine kinases and their cognate ligands means that a whole battery of ligand-receptor systems could be reconstructed using the baculovirus expression system allowing the *in vivo* characterisation and comparison of their mechanisms of action.

7.8. Recent advances in the baculovirus expression system.

The major disadvantage of the baculovirus expression system is the labour-intensive isolation of recombinant viruses. The identification of a

recombinant virus depends on the ability of the worker to recognise occlusion-negative plaques by eye. This is a time-consuming and skillful operation which can often hold up progress dramatically. Obviously if several recombinant viruses are being isolated at the same time, the problem escalates. Recently, a novel screening procedure has been devised which overcomes the need to search for occlusion-negative plaques (D. Bishop, personal communication). The polyhedrin gene in the wildtype AcNPV genome has been mutated leading to the generation of a virus with the occlusion-negative phenotype. The foreign coding sequence is cloned into a novel plasmid under the control of another major late promoter, the P10 promoter (Summers and Smith, 1987). The plasmid also encodes the full-length polyhedrin gene so that the resultant recombinant virus directs the expression of polyhedra. The recombinant virus therefore has the occlusion positive phenotype and produces the readily discernable occlusion-positive plaques upon infection. It is assumed that the recombinant virus will direct the expression of the foreign gene product in addition to polyhedrin. A potential problem exists associated with a cross over event occurring between the two genes, but this can be averted if several recombinants are initially analysed for their expression of the protein of interest. Thus, by employing this new technique, the time-span for the isolation of a recombinant baculovirus is considerably reduced.

7.9. The implications of this research.

The elucidation of the mechanism of action of growth factors and the identification of the components of their intracelluar signalling pathways is crucial to the understanding of how oncogenic activation of any one of these components can result in cellular transformation. Basic to the determination of the normal mechanisms operating in target cells, in response to growth factor activation, is the identification of the

interactions occuring within the ligand-receptor complex. This question can be answered in part by the determination of the receptor binding face of the ligand and the corresponding ligand-acceptor site of the receptor.

The aim of this thesis was to design expression systems for the large-scale production of EGF and TGFα so as to establish their complete three-dimensional structure. I have successfully used the baculovirus expression system to produce biologically active human EGF and TGFα and it is anticipated that, by manipulating the culture conditions, milligram quantities of these biologically important growth factors will be available for crystallisation. The elucidation of the three-dimensional structures of the growth factors by diffractional analysis of the crystals, together with mutational analysis of implicated residues, will establish the nature of the receptor binding face. This information will lead to the design of clinically important EGF and TGFα antagonists with profound implications for the treatment of malignancies known to express elevated levels of the EGF receptor or to operate a TGFα autocrine loop.

These recombinant ligands will also allow the continued biophysical characterisation of the conformational changes occuring in the EGF receptor in response to ligand binding. The Cocrystallisation between the ligands and the extracellular domain of the EGF receptor will ultimately lead to the determination of the ligand binding site of the receptor.

It is anticipated that baculoviral coexpression of growth factor receptors and their cognate ligands, in conjunction with intracellular substrates, will play an important role in the identification of their intracellular signalling pathways. Only through an understanding of these signalling pathways will it be possible to determine how the mitogenic signal initiated in the extracellular domain of the growth factor

receptor is transduced to the nucleus culminating in the transcriptional activation of the genes responsible for DNA synthesis and cell division. The elucidation of the normal cellular mechanisms operating in response to growth factors is a crucial factor in the determination of how the subversion of these pathways can lead to unrestricted cellular proliferation and the genesis of cancer.

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