CHAPTER 1

INTRODUCTION
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1.1 On oncogenes

Normal growth and development of a multicellular organism is the result of fine orchestration of gene expression which is achieved by temporal and spatial regulation of expression of genes involved in control of cell proliferation (Scott and Carrol, 1987). There is tremendous amount of information available regarding various factors that control cell division (Boynton and Leffert, 1985; 1987). Despite the intense research activity in this field only the bare outlines of mechanisms have been discerned so far. Usually the signals inducing a cell to divide are external and in the form of binding of growth factors (Evan, 1986). Viewed as abnormal cell proliferation the phenomenon of transformation lends itself as a system to obtain insights into the problem of control of cell proliferation. However it is only recently that the commonality of the molecular events underlying malignant transformation, development and differentiation have been appreciated (Burgess, 1985). This understanding came owing to the discovery of cellular oncogenes or proto-oncogenes (Bishop, 1983). These proto-oncogenes are thought to be vulnerable to carcinogenic influence of various sorts. Abnormal alleles of these genes can be found in retroviruses in which they were detected to begin with, or in tumours where they often arise without the intervention of viruses. In the latter case they include genes not represented in retroviruses (Varmus, 1984; Bishop, 1985).

Are there reasons to believe that the cellular oncogenes have roles in normal cells? The following observations lend support to such a belief.
The heritable predispositions to cancer encountered among human beings are tissue specific, in other words the oncogenes responsible for these predispositions act at precise points in embryological lineages (Knudson, 1979; 1981).

The oncogenes of retroviruses display tissue specificity which is not a mere consequence of host range. Apparently susceptible cells contain substrates for the activity; the resistant cells do not. Differentiated cells can contain and express an oncogene yet resist its transforming power (Graf et al., 1980; Durban and Boettiger, 1981).

Retroviral oncogenes meddle with differentiation. v-src induces the inappropriate expression of an embryonic gene and suppresses the expression of differentiated properties (Boettiger and Durban, 1979). v-erb and v-abl arrest the progress of hematopoietic cells through erythroid and lymphoid developmental pathways respectively (Graf et al., 1978; Baltimore et al., 1979; Beug et al., 1982).

Cellular oncogenes identified by transfection assays display tissue specificity; such as, c-ras from carcinoma of the bladder (Der et al., 1982; Parada et al., 1982; Santos et al., 1982) Kirsten c-ras from carcinoma of the lung and colon (Der et al., 1982) and N-ras from rat neuroblastoma (Shimuzu et al., 1983).

In addition, the expression of c-src, c-abl, c-ras, c-fos, c-myb and c-sis have been shown to be developmentally regulated (Adamson,
Perhaps what is more compelling is the evidence that many oncogenes are conserved through evolution (Reddy et al., 1988) indicating thereby that they perform some essential function(s). It has also been shown that an activated ras gene causes developmental abnormalities in Drosophila melanogaster (Bishop III and Corces, 1988). In the case of int-1 it has been suggested that it has a role in the development of the central nervous system in mouse embryos, owing to its restricted expression in a subset of neural plate cells (Guerrero, 1987). It has recently been shown that the int-1 gene of Drosophila is homologous to the wingless gene (Rijsewijk et al., 1987).

Given that the oncogenes have a role to perform in the normal course of events and at least some of them are perhaps very similar in their function to that of growth factors, it would be interesting to identify the molecular mechanisms underlying their function. The challenge is not simply trying to identify the genes involved in cell growth. Rather, the efforts to understand the pathways through which external mitogenic signal is transduced to its final destinations and how that transduction might be modulated (Evan, 1986).

The chain of events initiated by the growth factors begins outside the cell when they bind specifically to their receptors. This action leads to DNA synthesis and eventually to cell division (Boynton and Leffert, 1985). Some of the oncogene products are similar to the growth factors and growth factor receptors in their properties and subcellular localization. The initiation of chain of events leading to cell division occurs at the surface of the cell. But before proceeding to take a
detailed look at oncogenes, growth factors and signal transduction mechanisms, perhaps it is worthwhile to consider certain other aspects of cell surface.

1.2 Cell surface: normal vs tumour cells

When a comparison of the surfaces of normal cells is made with those of tumours, several differences are at once clear. Tumour cell growth is not inhibited by density (Stoker and Rubin, 1967; Dulbecco, 1970), they are anchorage independent (Freedman and Shin, 1974; Klinger et al., 1976) their surface is under glycosylated which leads to altered lectin agglutinability (Burger, 1969; Inbar and Sachs, 1969), they express fetal and non-fetal tumour antigens (Hellstrom and Brown, 1979) and they tend to invade and gain the ability to metastasize (Fidler and Kripte, 1977; Fidler, 1978; Poste and Fidler, 1980). All the properties stated above are very useful with respect to the study of properties of tumours. A large number of studies have been oriented towards understanding of the cell surface properties of the tumours (Abercrombie and Ambrose, 1962; Burger 1971; Walborg et al., 1979). However, these studies are less helpful when one is faced with the question of cell proliferation because it is difficult to say whether these properties have any causal role. In all probability they are the effects of a primary event occurring elsewhere. Now that the causal role of oncogenes in tumour formation has been established (Bishop, 1983), perhaps it is wise to consider the elements which play a key role in normal or abnormal cell divisions, namely oncogenes and growth factors.
1.3 Oncogenes

Today about 50 oncogenes have been isolated (Reddy et al., 1988). The protein products and their biochemical functions are not known for several of them yet. But what do we know about those oncogenes whose protein products have been identified? A cursory glance (Table I) would show that they are diverse in many respects: size, biochemical function, subcellular localization and so on. However, some patterns are recognizable in their subcellular localization and biochemical function. The product of c-erb B lies just outside the cells as well spanning the membrane (Carpenter, 1984; Adamson, 1987). The src and ras gene products are located at the cytoplasmic surface of the plasma membrane (Adamson, 1987; Barbacid, 1987). The protein encoded by mos oncogene is cytoplasmic (Papkoff et al., 1982). There are oncogenes whose products are situated in the nucleus, for example those of myc, fos and jun and myb genes (Adamson, 1987; Chiu et al., 1988).

The biochemical properties of several oncogenes are known. There is a family of oncoproteins exemplified by src which comprises protein tyrosine kinases (Hunter and Cooper, 1985). The product of mos oncogene is a serine kinase and that of raf (mil) is a serine/threonine kinase (Sefton, 1985). The proteins which are localized in the nucleus like the products of myc, (Person and Leder, 1984) fos (Curran et al., 1984) and myb (Slaman et al., 1986) are DNA binding proteins. Although the biochemical functions of many oncogenes are now known, the exact mechanism through which they bring about cellular transformation remains to be explored. As Michael Bishop (Bishop, 1985) aptly puts it, "In the end we must learn how the products of oncogenes act before we can know
TABLE 1

Cellular oncogenes and their properties

<table>
<thead>
<tr>
<th>c-onc</th>
<th>Location of the oncogene product</th>
<th>Size of the protein</th>
<th>Biochemical function</th>
<th>Possible roles in the cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>c- abl</td>
<td>Plasma membrane</td>
<td>150 kDa</td>
<td>Tyrosine kinase</td>
<td>B-cell differentiation?</td>
</tr>
<tr>
<td>c- erb B</td>
<td>Transmembrane</td>
<td>170 kDa</td>
<td>Tyrosine kinase</td>
<td>Signal transduction for mitogenesis and differentiation; stimulation of tooth eruption, eye opening, lung development</td>
</tr>
<tr>
<td>c- fes</td>
<td>Plasma membrane</td>
<td>92 kDa</td>
<td>Tyrosine kinase</td>
<td>Macrophage development?</td>
</tr>
<tr>
<td>c- fps</td>
<td>Plasma membrane</td>
<td>98 kDa</td>
<td>Tyrosine kinase</td>
<td></td>
</tr>
<tr>
<td>c- fms</td>
<td>Transmembrane</td>
<td>140 kDa</td>
<td>Tyrosine kinase</td>
<td>Signal transduction for mitogenesis and differentiation</td>
</tr>
<tr>
<td>c- neu</td>
<td>Transmembrane</td>
<td>185 kDa</td>
<td>Tyrosine kinase</td>
<td>Receptor for an unknown ligand?</td>
</tr>
<tr>
<td>c- src</td>
<td>Plasma membrane</td>
<td>60 kDa</td>
<td>Tyrosine kinase</td>
<td>Neurone and muscle development</td>
</tr>
<tr>
<td>c- H-ras-1</td>
<td>Plasma membrane</td>
<td>21 kDa</td>
<td>GTP + GDP binding, GTPase</td>
<td>Regulation of adenylate cyclase (and phospholipase C?)</td>
</tr>
<tr>
<td>c- Ki-ras-2</td>
<td>Plasma membrane</td>
<td>21 kDa</td>
<td>GTP + GDP binding, GTPase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>38 kDa</td>
<td>Serine kinase</td>
<td>Oocyte maturation</td>
</tr>
</tbody>
</table>
### TABLE I (CONTD.)

<table>
<thead>
<tr>
<th>c-onc</th>
<th>Location of the oncogene product</th>
<th>Size of the protein</th>
<th>Biochemical function</th>
<th>Possible roles in the cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-fos</td>
<td>Nucleus</td>
<td>55 kDa</td>
<td>DNA binding association with c-jun</td>
<td>G&lt;sub&gt;2&lt;/sub&gt; to G&lt;sub&gt;1&lt;/sub&gt; transition, differentiation</td>
</tr>
<tr>
<td>c-jun</td>
<td>Nucleus</td>
<td>39 kDa</td>
<td>Structurally identical to transcription factor AP-1</td>
<td>Regulation of transcription?</td>
</tr>
<tr>
<td>c-myc</td>
<td>Nucleus</td>
<td>62 kDa</td>
<td>DNA binding</td>
<td>Proliferation, regulation of DNA synthesis</td>
</tr>
<tr>
<td>L-myc</td>
<td>Nucleus</td>
<td>66 kDa</td>
<td>DNA binding</td>
<td></td>
</tr>
<tr>
<td>N-myc</td>
<td>Nucleus</td>
<td></td>
<td>DNA binding</td>
<td></td>
</tr>
<tr>
<td>c-myb</td>
<td>Nucleus</td>
<td>75 kDa</td>
<td>DNA binding</td>
<td>Differentiation of hematopoietic cells?</td>
</tr>
<tr>
<td>c-erb A</td>
<td>Nucleus, cytoplasm</td>
<td>52 kDa</td>
<td>Binds thyroxine (Throid hormone receptor)</td>
<td>Metabolic regulator?</td>
</tr>
<tr>
<td>c-sis</td>
<td>Secreted</td>
<td>14 kDa</td>
<td>PDGF B chain Binds to PDGF-receptor</td>
<td>Mitogenesis, wound healing; Early embryonic growth factor?</td>
</tr>
</tbody>
</table>

* The list is representative, not exhaustive.

References: Bishop (1985); Sefton (1985); Weinberger et al. (1986); Chiu et al. (1988); Sagata et al. (1988).
the inner life of the cancer cell, before we can hope to parlay the explication of oncogenes into strategies for the treatment and prevention of cancer".

1.4 Anti-oncogenes

There are cases when a recessive genetic lesion causes cancer. It is now believed that in these instances involve a set of genes called tumour-suppressor genes (or anti-oncogenes in common parlance) (Klein, 1988; Hansen and Cavenee, 1988; Weinberg, 1988). The well-characterized examples of recessive mutations involved in oncogenesis are retinoblastoma, osteosarcoma, Wilm's tumour, Beckwith-Wiedemann syndrome (embryonal tumours of the kidney, muscle and adrenal gland) and meningioma (Bishop, 1987). In the case of retinoblastoma the gene has been isolated and well characterized. The protein encoded by the retinoblastoma gene is a 110 kDa nuclear phosphoprotein. Recently it has been shown that the protein product of the retinoblastoma gene can form a complex with adenovirus E1A proteins (Whyte et al., 1988) and SV40 large tumour antigen (De Caprio et al., 1988) and E7 oncoprotein of the human papilloma virus type 16 (Dyson et al., 1989). E1A can immortalize primary cells (Houweling et al., 1980; Ruley, 1983; Cone et al., 1988). It can also cooperate with the adenovirus E1B gene or activated ras gene to transform cells in culture, and these cells will induce tumours in animals (Ruley, 1983; Vander Elsen et al., 1983). In the case of retinoblastoma gene it is the loss of the gene that leads to transformation (Weinberg, 1988). Considering the recent data (Whyte et al., 1988) of complex formation between E1A protein and the product of the retinoblastoma anti-oncogene, one can envisage that this would lead to the same effect as the loss
of the retinoblastoma gene (Whyte et al., 1988). The same mechanism could be extended to other cases where the product of the retinoblastoma oncogene is known to complex with the products of transforming genes.

1.5 Growth factors

Although all of the essential components of culture media could be described as growth factors there are sets of proteins which appear to be responsible for proliferation and differentiation of cells in specific tissues (Burgess, 1985). These growth factors are usually referred to as polypeptide growth factors. They differ from classical polypeptide hormones by virtue of (1) their multifocal sites of synthesis (2) the limited number of identified target cells and (3) mode of delivery. Growth factors presumably diffuse within a short-range through intercellular spaces and act locally; they do not act in an endocrine manner (Goustin et al., 1986; Levi-Montalcini and Calissano, 1986).

Several of the growth factors present in serum are presumed to be derived from platelets and released during the blood clotting process (Childs et al., 1982; Holley and Kiernan 1974; Oka and Orth 1983; Vogel et al., 1978). The presence of growth factors in platelets is thought to facilitate delivery of growth factors to the sites of injury where they may play a major role in wound healing. Besides being found in platelets, growth factors are present in a variety of tissues both adult and embryonic, and are thought to be released by many, if not all, cells in culture (Shields, 1978). Membrane receptors for growth factors are ubiquitous with most cells having receptors for more than one growth factor (Bowen-Pope et al., 1983; Fabricant et al., 1977; Wrann et al., 1980). Growth
factors have differing cell type specificities, some factors such as those of the hematopoietic system (e.g. interleukin 2 or CSF-1) stimulate only one or a few cell types while others such as somatomedin C and EGF stimulate a wide variety of cell types, both epithelial and mesenchymal (Goustin et al., 1986). It has been demonstrated that multiple growth factors are required for maximum stimulation of specific cell types (Pledger et al., 1978; Leof et al., 1982). The requirement of nontransformed cells for more than one growth factor for proliferation is also supported by studies on the growth of cells in defined serum-free media. Unless the cells are neoplastically transformed more than one growth factor supplement is necessary for growth (Barnes and Sato, 1980; Walthall and Ham, 1981). Exposure of a cell to one growth factor can lower the threshold for mitogenicity of a second growth factor (Wharton et al., 1983). Moreover, growth factors operate at different points of the cell cycle (Pledger et al., 1978; Leof et al., 1982). For instance, transient treatment of fibroblasts with PDGF will induce a stable state (competence) whereby cells are made responsive to other circulating plasma derived factors (O'Keefe and Pledger, 1983). The multiplicity of growth factors in various tissues, the varying cell type specificity of growth factors, and the requirement for multiple growth factors for the stimulation of specific cell types presumably provide the fine tuning of relative proliferation rates necessary for coordinated growth of cells to form tissues during development and to maintain tissues in the adult state (Goustin et al., 1986).

Much of the impetus for study of growth factors has come through their presumed involvement in cancer. Evidence for this involvement
dates to early work showing a decreased serum requirement for growth of neoplastically transformed cells (Temin, 1966; Dulbecco, 1970; Paul et al., 1971). With the advent of serum-free culture techniques and the availability of purified growth factors, the altered serum requirement in transformed cells could be translated into a diminished or lack of requirement for specific growth factors (Barnes and Sato, 1980; Kaplan et al., 1982). Loss of requirement for specific growth factors is a common finding in many types of cancer cells (Moses et al., 1978; Kaplan et al., 1982) and could be mediated by (1) the activation of autologous growth factor synthesis (autocrine activation), (2) synthesis of an altered growth factor receptor or (3) activation of a post-receptor pathway that bypasses the growth factor requirement (Goustit et al., 1986).

1.6 Oncogenes and growth factors

Recent work has provided some of the more convincing evidence linking oncogenes and growth factors. One proto-oncogene, c-sis codes for the B chain of PDGF (Doolittle et al., 1983; Waterfield et al., 1983); another (c-erb B) codes for epidermal growth factor receptor (Downward et al., 1984). Similarly, the product of the c-fms oncogene appears very similar to the CSF-1 receptor (Sherr, et al., 1985). Moreover, there is evidence to suggest that several other oncogene products are similar to growth factor receptors in that both have transmembrane and tyrosine kinase domain (Hunter, 1984). Recent data indicate that the p21 ras oncogene protein is involved in transduction of growth factor signal and may be an obligatory intermediate in this pathway (Mulcahy et al., 1985). Growth factors have been shown to increase transcription of certain proto-oncogenes like myc and fos (Kelly et al., 1983; Greenberg and Ziff,
1984; Kruijer et al., 1984; Muller et al., 1984) the products of which may in turn regulate the transcription of other genes necessary for stimulation of cell proliferation. To this list a few exciting discoveries have been added. The c-erb-A gene has been shown to encode a thyroid hormone receptor (Weinberger et al., 1986). The product of oncogene c-jun is indistinguishable from the transcription factor AP-1 (Bohmann et al., 1987; Angel et al., 1988). It has been shown that microinjection of antibodies to phosphatidyl inositol 4,5 biphosphate (PIP$_2$) abolishes both growth factor-induced (Matuoka et al., 1988) and oncogene induced (Fukami et al., 1988) mitogenesis. It is interesting to note that PIP$_2$ is the precursor for second messengers utilized by a recently established (Berridge and Irvine, 1984) signal transduction pathway. These findings suggest that the oncogene products may be involved in the growth factor-receptor-response pathway and indicate points at which alterations may occur leading to the development of neoplastic transformation.

1.7 Oncogenes, anti-oncogenes, growth factors and cellular proliferation: a synthesis

The broad biochemical strategies by which the protein products of oncogenes transduce signals could be classified into two categories. The first one is through phosphorylation which involves oncogenes encoding kinases. These oncogene products phosphorylate proteins and polyphosphoinositides. The second mechanism is through regulation of enzymes which generate second messengers. For example yeast RAS 2 has been clearly shown to be involved in the regulation of cyclic AMP (cAMP) production (Toda et al., 1985). A recent report has implicated the RAS 1 gene in the regulation of turn over of inositol phospholipids (Tamanoi,
1988), the metabolites of which have been established as second messengers that convey the signals to the nucleus for initiation of DNA synthesis and eventual cell division (Berridge and Irvine, 1984).

Even a casual glance at the literature on growth factors would reveal astounding similarities in action with those of oncogenes. The receptors for epidermal growth factor (EGF) and platelet-derived growth factor are tyrosine kinases (Boynton and Leffert, 1985) PDGF utilizes inositol-lipid second messengers for its mitogenic action (Berridge et al., 1984; Hasegawa-Sasaki, 1985; Takuwa et al., 1987). It has also been shown that EGF stimulates the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (Pike and Eakes, 1987). The level of inositol second-messenger goes up when normal N-ras is over expressed in NIH3T3 cells (Wakelam et al., 1986). Cells transformed with v-src have an enhanced inositol lipid turnover (Sugimoto et al., 1984). Also the cells transformed with polyoma virus have elevated levels of inositol 1,4,5-trisphosphate which is thought to arise through the interaction of middle T with pp60c-src (Kaplan et al., 1986). In the case of abl oncogene it has been reported that an increase in inositol lipid turnover is observed along with increased tyrosine kinase activity of the abl protein. A few recent reports lend support to the link between oncogenes and growth factors. Kaplan et al. (1987) observed that a phosphatidyl inositol kinase is a substrate for both the PDGF receptor and polyoma middle T/pp60c-src tyrosine kinase activities. Stahl et al. (1988) showed the sequence similarity of phospholipase C (PLC) with non-catalytic region of src, implying a possible regulation of PLC by tyrosine kinases. A novel viral oncogene called crk has been shown to share a structural similarity
with bovine brain phospholipase C (Mayer et al., 1988).

The connection between oncogenes and growth factors has been established at the nuclear level as well. When growth factors act on a quiescent cell one of the first events that takes place is increased transcription of fos and myc oncogenes (Kelly et al., 1983; Greenberg and Ziff, 1984; Kruijer et al., 1984; Muller et al., 1984). To this scenario the recently discovered anti-oncogenes can be added. These genes are thought to be negative regulatory elements in control of cell proliferation. The loss of anti-oncogenes or the inactivation of their protein products would lead to the same effect on cell proliferation as the inappropriate expression of oncogenes.

Almost in parallel with oncogene research there has been intensive study of genes controlling the cell cycle. Where do these two fields stand in relation to each other today? So far none of the oncogenes have been found among the genes that control cell division. But recent discoveries indicate that these two sets of genes may be able to influence activities of each other. It has been observed that the maturation promoting factor (MPF) which is a kinase is the immediate trigger for cell division in higher organisms. The MPF can phosphorylate the protein product of the src oncogene (Marx, 1989). It might be noted that MPF is the homologue of cdc 2 which is one of the genes controlling cell cycle in yeast Schizosaccharomyces pombe (Norbury and Nurse, 1989). Recently it has been shown that the product of the mos proto-oncogene might be a "candidate initiator" for oocyte maturation. Microinjection of synthetic mos RNA into oocytes activated MPF and initiated germinal
vesicle breakdown. The authors believe that mos oncogene could be an initiator protein of MPF and at least one of the triggers of G2 to M transition in the cell cycle (Sagata et al., 1989). Parallels may also exist between the induction of oocyte maturation by over expression of mos and the expression of the transformed phenotype as a result of constitutive expression of mos in somatic cells (Paules et al., 1988). It has been postulated (Paules et al., 1988; Sagata et al., 1988) that mos oncogene expression in somatic cells may activate directly or indirectly the component of MPF prematurely and that the transformed phenotype may be due to the expression of mitotic phenotypes at non-mitotic stages of the cell cycle (Sagata et al., 1988). Similar arguments have been made for the c-src gene product (Chackalaparampil and Shalloway, 1988). Also recently it has been shown that G1/S transition in normal human T-lymphocytes requires the expression of the nuclear protein encoded by c-myb (Gewirtz et al., 1989). MPF-activation through over expression of the protein product of mos oncogene shows similarities with the regulation of a cell cycle gene cdc 2 in yeast Schizosaccharomyces pombe such as nim 1 (Russell and Nurse, 1987). The protein product of nim 1 is a kinase that suppresses the function of mitotic inhibitor wee 1 and induces entry into mitosis probably via cdc 2 function (Russell and Nurse, 1987).

Regulation of cellular proliferation is a complex process which has to involve signals acting both positively and negatively. Evidence has been obtained for stimulatory and inhibitory regulation of cell division at least in the case of abnormal cell growth, that is of tumour formation. Given that the effect of oncogenes or anti-oncogenes is because
of their normal function going amuck because of some genetic lesion, it is not difficult to envisage a similar scenario for normal control of cell growth and division by polypeptide factors and growth factors. In fact such a model has been proposed at least in the case of a signal transduction pathway which utilizes "second messengers" generated by inositol metabolism. This would involve growth factors, oncogenes and anti-oncogenes which would act as both positive and negative regulatory elements of the pathway (Berridge, 1984). Perhaps the genes controlling cell cycle and the role of oncogenes in control of cell cycle could also be fitted into this general scheme once the relevant information regarding the link between oncogenes and cell cycle becomes available.

An examination of signal-transduction pathways involving growth factors or oncogenes would reveal use of some common functional motifs: (1) phosphorylation (2) guanine-nucleotide-binding proteins and (3) second messengers like inositol lipids and cAMP, the latter only one step removed from phosphorylation events.

1.8 Other factors which influence cellular processes via inositol lipid second messenger

Apart from proliferation the cell has to carry out many processes such as contraction, secretion, metabolism and long-term functions such as growth and information storage in the brain (Berridge, 1987; Berridge and Irvine, 1989). It is becoming clear that the cell uses only a few mechanisms and shuffles them depending on the purpose and the context. A large variety of hormones, neurotransmitters and growth factors mediate their effects by employing a limited repertoire of second messengers.
(Berridge, 1986). Just as the growth factors, the polypeptide factors angiotensin II (Uhing et al., 1986), bombesin, insulin and vasopressin (Heslop et al., 1986) utilize inositol lipid second messengers for their action. Even glucagon which traditionally has been regarded as a typical example of a hormone that acts solely through cyclic AMP has been shown to stimulate inositol lipid metabolism (Wakelam et al., 1986). Even the agents which act on nerve cells, like bradykinin, acetylcholine (via muscarinic receptors), H1-histamine, neurotensin and serotonin have been shown to stimulate inositol lipid metabolism (Hanley et al., 1988). The distinction between growth factors and factors acting on the nervous system seems to be blurring as well. A recent report (Ashkenazi et al., 1989b) shows that acetylcholine acting through a muscarinic receptor could be mitogenic to a neural derived cell line. Also, it has been shown (Julius et al., 1989) that a subclass of serotonin receptor when expressed ectopically in NIH3T3 cells can act as an oncogene thus suggesting similarities between action of oncogenes and these factors. In addition the mas oncogene has been shown to encode an angiotensin receptor (Jackson et al., 1988). It may also be recalled that the src and ras genes are expressed in significant quantities in the brain indicating a non-proliferative function (Cotton and Brugge, 1983; Mannes 1986; Mannes et al., 1986; Tanaka et al., 1986).

1.9 Inositol lipids and modulation of cellular events

The observation that phosphatidylinositol (PI) turnover could be increased by certain external stimuli was made over 30 years ago (Hokin and Hokin, 1953). Subsequently, many mitogens have been shown to elicit this response. However, the potential significance of this increase with
respect to transduction of the mitogenic signals delivered by growth factors has only recently been appreciated as direct links between certain components of the PI cycle and components of other signal systems have been identified (Berridge, 1984a; Hokin 1985; Majerus et al., 1986; Olshaw and Pledger 1988; Whitman and Cantley, 1988; Berridge and Irvine, 1989).

When the growth factor binds to its receptor, the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) produces two second messengers, inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG).

The hydrolysis of PIP$_2$ to IP$_3$ and DAG can be catalysed by any one of what is emerging as a large family of enzymes collectively called phospholipase C (PLC). At least five immunologically distinct forms of this enzyme have been identified in mammals (Rhee et al., 1989). One of the mechanisms by which PLC is regulated in the cell probably involves G proteins. For instance, in some membrane systems PLC activity can be stimulated by GTP$\gamma$S (Cockroft and Gomperts, 1985). In addition, in certain cell types stimulation of PLC activity is sensitive to pertussis toxin (PTX) an inhibitor of specific G proteins. In other cells or with other agonists in the same cell type PTX has no effect. This suggests the existence of multiple G proteins (termed Gp) linking cell surface receptors to PLCs (Cockroft and Stutchfield, 1988; Casey and Gilman, 1988; Ashkenazi et al., 1989a) or else that there are G-protein-independent PLC activation pathways. Different Gp proteins could interact with different forms of PLC to endow a cell's PI response with specificity in terms of amplitude and duration. Based on inhibitor studies G proteins appear to mediate the stimulation of PLC activity by a wide variety of agonists.
including bombesin, vasopressin, bradykinin, thrombin and angiotensin II (Cockroft and Stutchfield, 1988). However this list does not include all agonists of PI turnover, and G proteins need not necessarily be involved in the interaction between all receptors and PLCs. Indeed PDGF-induced Ca\(^{2+}\) flux in Swiss 3T3 cells follows kinetics that differ from those induced by bombesin or vasopressin, suggesting that PDGF operates via a different signal transduction pathway (Lopez-Rivas et al., 1987). One obvious possibility is that this distinct pathway involves the mediation by tyrosine kinase activity of the PDGF receptor. In support of this idea Wahl et al. (1988) have shown recently that the amount of PLC activity recovered from A431 cell lysates bound to anti-phosphotyrosine antibody columns increases when the cells are first treated with EGF. Since then, two groups (Margolis et al., 1989; Meisenhelder et al., 1989) have established that phospholipase C-γ is a substrate for EGF receptor tyrosine kinase. Meisenhelder et al. (1989) have also shown that the phospholipase C-γ is a substrate for PDGF receptor protein tyrosine kinase.

The IP\(_3\) molecule generated by the hydrolysis of PIP\(_2\) diffuses into the cytoplasm and DAG remains in the plasma membrane. IP\(_3\) releases calcium from the endoplasmic reticulum. This leads to an increase in internal calcium level.

The changes in the intracellular level of calcium have long been recognized as one of the important signals for cell proliferation (Boynton et al., 1974; Berridge, 1975; Whitfield et al., 1976; Metcalfe et al., 1980; Whitfield et al., 1981; Durham and Walton, 1982; Jaffe, 1982; Berridge and
Irvine, 1989). In the case of fibroblasts, growth factors appear to act by stimulating the release of calcium stored within the intracellular membranes (Lopez-Rivas and Rozengurt, 1983). Direct measurements with quin 2 have also revealed that growth factors can induce an increase in the intracellular level of calcium even when cells are bathed in a calcium-free medium (Moolenaar et al., 1984). The link between these surface receptors and the internal reservoirs is thought to be IP₃ which has been proposed as a second messenger for calcium mobilization (Berridge 1983; Streb et al., 1983). When tested on permeabilized Swiss 3T3 cells IP₃ induced a large and rapid release of calcium from intracellular stores (Berridge et al., 1984). The nature of IP₃ sensitive mechanism responsible for this release of calcium is unknown but would clearly represent an important locus that might be altered by oncogenes (Berridge, 1984).

Oncogenes may act to raise the intracellular level of calcium either by amplifying the signal pathway responsible for release or by inhibiting the mechanisms that remove calcium from the cytosol (Berridge, 1984b). Such an elevation in the intracellular level of calcium in transformed cells is certainly consistent with the observation that such cells are less sensitive than normal cells to the effects of reduction in the level of calcium in the bathing medium (Balk et al., 1973; Boynton et al., 1974; Swierenga et al., 1978; Lechner and Kaighn, 1979). Likewise the proliferation of normal cells is considerably enhanced in low calcium concentrations if they are treated with a growth factor such as EGF (McKeehan and McKeehan, 1979).
The other branch of the signal pathway is controlled by DAG which acts by stimulating protein kinase C (PKC). The enzyme PKC is a calcium-activated, phospholipid-dependent enzyme and it is a receptor for tumour-promoting phorbol esters (Castagna et al., 1982). One consequence of PKC-mediated protein phosphorylation is the activation of Na\(^+\)/H\(^+\) antiport which extrudes protons in exchange for sodium ions. The resulting fall in the intracellular level of hydrogen ions and the increase in sodium appear to be important steps in initiating cellular proliferation (Rozengurt, 1980; Rozengurt and Mendoza, 1980; Moolenaar et al., 1982; Moolenaar et al., 1983). When cells are stimulated with growth factors there is a rapid increase in pH as protons are extruded from the cell (Moolenaar et al., 1981; 1982; 1983). A link between PKC and the Na\(^+\)/H\(^+\) antiport was obtained by the finding that phorbol esters are capable of stimulating a similar alkalinization of the cytoplasm (Burns and Rozengurt, 1983). It has been reported that when ras p21 was microinjected into quiescent mouse NIH3T3 cells a rapid and sustained rise in intracellular pH was observed which has been shown to involve the Na\(^+\)/H\(^+\) antiporter (Hagag et al., 1987). In addition to the role of PKC in cell proliferation and gene expression many other roles for the enzyme such as, modulation of ion conductance, regulation of receptor interaction with components of the signal transduction apparatus, and regeneration of contraction of smooth muscle have been suggested (Nishizuka, 1988).

### 1.10 Phosphorylation

Since the late nineteenth century it has been known that phosphate could be tightly associated with proteins. The hints about the phosphate moiety being covalently linked were gleaning in 1906 but the first phos-
phoaminoacid was isolated only in 1933. Since then a variety of amino-acids have been found to be phosphorylated. The most common types of phosphorylation involved esterification of phosphate to serine and threonine, with smaller amounts being linked to lysine, arginine, histidine, aspartic acid glutamic acid and cysteine (Hunter and Cooper, 1985). Reports of orthophosphate esterified to tyrosine emerged in 1979 (Eckhart et al., 1979). Since then there has been a virtual explosion of information on the involvement of tyrosine phosphorylation in both transformed and normal cells (Hunter and Cooper, 1985). Protein phosphorylation was identified as a regulatory mechanism in the mid 1950s by Fischer, Krebs and Sutherland during their studies of the control of glycogen metabolism. Until the late 1960s the only proteins known to be regulated in this manner, were three enzymes involved in the degradation and synthesis of glycogen (Krebs and Fischer, 1956; Wosilait and Sutherland, 1956; Krebs et al., 1959; Friedman and Larner, 1963). The mitochondrial pyruvate dehydrogenase complex was shown to be controlled by phosphorylation in 1969 (Linn et al., 1969) extending the concept to another pathway and another organelle and during the 1970s it became obvious that this was a major mechanism by which many metabolic pathways responded to neuronal and hormonal stimuli. A further development was the finding that proteins devoid of enzyme activity such as histone H1 (Langan, 1969) and cardiac troponin-I (England, 1975; 1982) were also regulated in this manner leading to the realization in the 1980s that protein phosphorylation is a principal mechanism by which all cell functions in eukaryotic cells are controlled by extracellular signals (Cohen, 1982; 1985). Processes as diverse as metabolism, membrane transport and permeability, secretion, contractility, transcription and translation of genes, fertilization and
cell division, neurotransmission and even memory are all regulated by this versatile post-translational modification (Hunter and Cooper, 1985; Nairn et al., 1985; Edelman et al., 1987; Cohen, 1988b).

Many key regulatory proteins exist in cells as either a phosphorylated or dephosphorylated form, their steady-state levels of phosphorylation reflecting the relative activities of the protein kinases and protein phosphatases that catalyse the interconversion process. Phosphorylation of various amino acid residues triggers small conformational changes in these proteins that alter their biological properties. Growth factors, hormones, neurotransmitters and other extracellular signals transmit information to the interior of the cell by activating transmembrane signalling systems that control the production of relatively small number of chemical mediators termed "second messengers". These substances regulate the activities of protein kinases and phosphatases and so alter the phosphorylation states of many intracellular proteins accounting for the diversity of action of the various factors which generate these second messengers (Cohen, 1988b).

Many of the phosphorylation and dephosphorylation reactions that take place in vivo appear to be catalyzed by relatively few protein kinases and protein phosphatases with pleiotropic actions. Thus cAMP dependent protein kinase (cAMP-PK), the CaM-PK (calmodulin dependent multiprotein kinase) and protein kinase C mediate many of the actions of signals that work through cyclic AMP, Ca$^{2+}$ or metabolites of PIP$_2$. The importance of protein tyrosine kinases in normal and malignant cells is only too well known. Several additional protein kinases are also
important in cellular control (For example glycogen synthase kinase-3, acetyl CoA carboxylase kinase, tyrosine hydroxylase kinase and casein kinase-2) which are themselves controlled by allosteric effectors, phosphorylation, insulin and other growth factors, or by regulators that are yet to be identified (Cohen, 1988b).

A large number of hormones and other extracellular signals exert their effects by increasing or decreasing the intracellular concentration of cyclic AMP. The transmembrane signalling systems involved in the formation of this second messenger are understood in considerable molecular detail (Birnbaumer et al., 1985). In the cell cyclic AMP activates the enzyme cAMP-PK. Many cellular processes are mediated by phosphorylation of proteins by cAMP-K. Phosphorylation of several of the substrates is physiologically significant. Among the substrates of such physiologically significant phosphorylation reactions are: glycogen synthase (1A), protein phosphatase - 1, pyravate kinase, tyrosine hydroxylase, phosphorylase kinase B, Histone H1 and hormone sensitive lipase (Cohen, 1988b).

Many extracellular signals regulate intracellular events by initiating a transient rise in the cytosolic concentration of \( \text{Ca}^{2+} \) from less than 0.1\( \mu \text{M} \) in the resting state to 1-10\( \mu \text{M} \) in the stimulated state. This increase results from either influx of calcium from outside or from the intracellular pool. Calcium ions regulate cellular processes by binding to a family of structurally related \( \text{Ca}^{2+} \)-binding proteins of which calmodulin is the most important member. The binding of \( \text{Ca}^{2+} \) to calmodulin triggers a change in its conformation allowing this remarkable multifunc-
tional protein to interact with a wide variety of enzymes. The principal action of the Ca\(^{2+}\)-calmodulin complex is to alter the phosphorylation states of intracellular proteins. This can be achieved by either indirect or direct mechanisms. The first two calmodulin-dependent enzymes to be identified were an isozyme of cyclic nucleotide phosphodiesterase (Cheung, 1970; Kaikuchi and Yamazaki, 1970; Teo and Wang, 1973) and an adenylyl cyclase (Brostrom et al., 1978). In cells where these calmodulin-dependent enzymes are present, the Ca\(^{2+}\)-calmodulin complex can therefore modulate the phosphorylation states of proteins indirectly, by altering the activities of cyclic AMP or cyclic GMP-dependent protein kinases. (Cohen 1988b). The calcium-calmodulin complex regulates phosphorylation directly by activating a number of protein kinases such as myosin kinase (Dabrowska et al., 1977; Yazawa and Yagi, 1977) and phosphorylase kinase (Cohen et al. 1978). Many of the calmodulin-dependent protein kinase activities are now known to be characterized by a single enzyme that has been termed the calmodulin-dependent multiprotein kinase (CaM-MPK) (McGuinnes et al., 1983). Some of its best characterized substrates to date are: synapsin 1, tyrosine hydroxylase, microtubule associated protein-2, glycogen synthase and phospholambin (Cohen, 1988a).

Phosphorylation of cellular proteins is regulated by the products of polyphosphoinositides. Both the metabolites IP\(_3\) and DAG (from the hydrolysis of PIP\(_2\)) influence phosphorylation. IP\(_3\) which is released into the cytoplasm mobilizes calcium which in turn regulates phosphorylation. The other product DAG stimulates protein kinase C (PKC). Protein kinase C can directly phosphorylate some key proteins in the cell. It has been shown for example that protein kinase C can mediate
down-regulation of the epidermal growth factor receptor, the adrenergic receptor and the transferrin receptor (Sibley et al., 1987). PKC can also regulate protein phosphorylation by virtue of its effect on intracellular pH by activation of Na\textsuperscript{+}/H\textsuperscript{+} antiporter. The decrease in hydrogen ions within the cell may also lead to an increase in protein phosphorylation (Berridge, 1984). Within minutes of stimulating the 3T3 cells with PDGF there is an increase in the phosphorylation of the ribosomal protein S6 associated with enhanced polysome formation and protein synthetic activity (Wettenhall et al., 1983). Phosphorylation of S6 was blocked by amiloride suggesting that the protein kinase might be sensitive to changes in intracellular pH (Pouyssegur et al., 1982).

In addition to the usual phosphorylations on serine, threonine and tyrosine residues there also occur in the cell, albeit less commonly, a number of phosphorylations reactions involving arginine, histidine, lysine, which are called phosphoramidates and aspartic acid and glutamic acid which are termed acyl phosphates (Fujitaki and Smith, 1984). Phosphorylation is also known to occur on cysteines (Pigiet and Conley, 1978). These phosphoamino acids and their role in the cellular processes are not very well understood.

1.11 G proteins

A family of proteins which bind guanine-nucleotides and function as intermediaries in transmembrane signalling pathway are termed 'G proteins' (Gilman, 1987). The pathways which transduce signals across the plasma membrane consist of three proteins: receptors, G proteins and effectors. The receptors that participate in such pathways are legion.
They include those for a large numbers of biogenic amines, proteins, polypeptide hormones, autacoids and neurotransmitters. The best characterized of these receptors are those for epinephrine which are termed β-adrenergic receptors (Ross and Gilman, 1980), serotonin (Siegelbaum et al., 1982), IgE-antigen complex which bind mast cell IgE receptor (Nakamura and Ui, 1985; Smith et al., 1985) f-met peptide (Krause et al., 1985) and acetylcholine (Breitwieser and Szabo., 1985; Pfaffinger et al., 1985). Rhodopsin too is a G protein linked receptor for which light is the stimulus (Stryer, 1986). Of late it has been clear that the receptors for odorants are coupled to a G protein and a G-protein specific to olfactory neurons has been cloned (Jones and Reed, 1989). The number of effector molecules known to be controlled by G proteins is still small: interactions of adenylyl cyclase and a retinal cyclic GMP-specific phosphodiesterase with G proteins are rather well understood. The regulation of activity of a phosphoinositide phosphodiesterase (phospholipase C) by a G protein termed Gp has been demonstrated by circumstantial evidence (Cockroft and Gomperts, 1985; Cockroft, 1987); however, the identity of this protein is yet to be unequivocally established. There is also recent evidence available for the direct action of G proteins on potassium and calcium channels (Brown and Birnbaumer, 1988).

The G proteins have a common design. They are heterotrimers consisting of a guanine-nucleotide-binding α chain (39-52 kDa), a β chain (35-36 kDa) and a γ chain (8 kDa). When GDP is bound, the α chain associates with β and γ to form a Gαβγ complex (G-GDP) that is membrane-bound. When GTP is bound the α chain (Gα-GTP) disassociates from the β and γ chains (Gβγ). Conversion from the GDP to the GTP
state is slow in the absence of excited receptors (e.g. a hormone-receptor complex or photoexcited rhodopsin). The role of the excited receptors is to catalyse the activation of the G protein by markedly accelerating the rate of exchange of GTP for bound GDP. Ga-GTP released from \( G_{\beta\gamma} \) then alters the activity of the target enzyme or channel. The \( \alpha \) chain also has a built-in hydrolytic activity that converts bound GTP to GDP to terminate activation. The role of \( G_{\beta\gamma} \) is to present Ga-GDP to the activated receptor. All G proteins are activated by receptors that are integral membrane glycoproteins. When a ligand specifically binds these receptors, they undergo a conformational change. In this conformation they activate the G proteins which is coupled to it. The latter activation occurs at the cytosolic face of the plasma membrane (Gilman, 1984; Schram and Selinger, 1984; Smigel et al., 1984; Levitzki, 1986; Stryer, 1986; Stryer and Bourne, 1986; Gilman, 1987). In addition to the properties described above, G proteins have other unusual characteristics. For example, they are activated by fluoride plus aluminium; the actual ligand probably being \( \text{AlF}_4^- \) (Sternweis and Gilman, 1982). Recently it has been shown in the case of transducin that \( \text{AlF}_4^- \) acts as the \( \gamma \)-phosphate when complexed with GDP bound to the G protein (Bigay et al., 1987). In addition to the properties described above the \( \alpha \) subunits of G proteins can be substrates for ADP-ribosylation by bacterial toxins. The toxins from Vibrio cholerae and Bordetella pertussis (Moss and Vaughan, 1977; Cassel and Pfeuffer, 1978; Gill and Meren, 1978; Katada and Ui, 1982) catalyze the transfer of ADP-ribose from NADP to these proteins.

Based on past research a few well defined characteristics of G proteins have emerged which can be used as criteria for the involve-
ment of G proteins in a newly identified biological phenomenon. Gilman (1987) has laid them out quite clearly. They are (i) an appropriate ligand for the receptor of interest and GTP are both required to initiate the response in question; (ii) the response can be provoked independently of receptor by inclusion of non-hydrolyzable analogues of GTP (GTP\_\_s or GppNHp) or F\_ plus Al\^{3+}; (iii) there is a negative heterotropic interaction between the binding of guanine nucleotide to a G protein and the binding of agonist to a G-protein-linked receptor; (iv) cholera toxin and pertussis toxin have characteristic effects on the function of known G proteins and they can be utilized with either intact cells or purified components; and (v) a mutant of murine S49 lymphoma which lacks the G protein but has a functional catalytic subunit of adenylyl cyclase could be used for complementation when a newly discovered G protein is obtainable in pure form. Although the criteria mentioned above, if satisfied, could provide strong evidence for the involvement of G proteins in a biological context, the ultimate confirmation can only be made when the individual components are purified and reconstituted and shown to be active. This has been achieved with the adenylyl cyclase complex and the retinal phosphodiesterase system (Gilman, 1987).

G proteins transduce signals for various kinds of processes in the cell including those for cell proliferation. It may be recalled that there is strong evidence for the involvement of G proteins in a pathway which uses inositol lipid metabolites as second messengers for transducing signals for cell division. It has been shown that in a subset of pitutary cells cyclic AMP is the second messenger for cellular proliferation. The generation of cAMP is mediated by G\_s. Altered G\_s and adenylyl cyclase
activity was found in GH-secreting human pituitary adenomas (Vallar 
et al., 1987). If G proteins are involved in transduction of signals in 
normal proliferation one would expect mutations in the genes coding for 
G proteins at least in some of the tumours (Bourne, 1987). Indeed it has 
been shown that GTPase inhibiting mutants activate the \( \alpha \) chain of \( G_s \) 
and stimulate adenylyl cyclase in human pituitary tumours (Landis et al., 
1989). Although the tumourigenicity criteria has not been satisfied, in 
anticipation that it would be, the authors have designated this putative 
oncogene as \( gsp \) (for \( G_s \)-protein, Landis et al., 1989).

1.12 ras proteins

Among the oncogenes ras genes are the most extensively and inten-
ensively studied; hence the literature abounds with information regarding 
ras (Barbacid, 1987). The name ras is an acronym derived from the words 
rat sarcoma since these genes were first identified as the transforming 
principle of the Harvey and Kirsten strains of rat sarcoma viruses (Harvey, 
1964; Kirsten and Mayer, 1967), two acutely transforming retroviruses 
generated by transduction of the rat H-ras-1 and K-ras-2 cellular genes 
respectively (DeFeo et al., 1981; Ellis et al., 1981). ras genes were "re-
discovered" when investigators using gene transfer assays established 
the existence of dominant oncogenes in human (Krontiris and Cooper, 1981; 
Perucho et al., 1981; Shih et al., 1981; Pulciani et al., 1982) and carcinogen-
induced animal tumours (Balmain and Pragnell, 1983; Eva and Aaronson, 
1983; Sukumar et al., 1983; Guerrero et al., 1984).

Todate, three ras genes have been identified in the mammalian 
genome (De Feo et al., 1981; Ellis et al., 1981; Der et al., 1982, Parada
et al., 1982; Santos et al., 1982; Hall et al., 1983; Shimizu et al., 1983). They have been designated H-ras-1, K-ras-2 and N-ras. These ras genes are highly conserved during evolution (Shilo and Weinberg, 1981). They have been identified in chicken (H-ras gene; Westaway et al., 1986), Drosophila melanogaster (Dras 1, Dras 2/64B and Dras 3 genes; Neuman-Silberberg et al., 1984; Schejter and Shila, 1985; Mozer et al., 1985), Aplysia (Apl-ras gene, Swanson et al., 1986), Dictyostelium discoideum (Ddras gene, Reymond et al., 1984); plants (Allium cepa, Barbacid, 1987) and yeasts (Saccharomyces cerevisiae RAS 1 and RAS 2 genes and Schizosaccharomyces pombe SPRAs gene, De-Feo-Jones et al., 1983, Powers et al., 1984; Fukui and Kaziro., 1985). Recently it has been shown that a ras-like protein (Era) is also expressed in Escherichia coli (March et al., 1988).

ras genes code for a protein of 21,000 molecular weight. The ras proteins, independently of their phylogenetic origin have been shown to bind guanine nucleotides GTP and GDP (Scolnick et al., 1979; Shih et al., 1980; Tamanoi et al., 1984; Temeles et al., 1985) and possess intrinsic GTPase activity (Gibbs et al., 1984b; McGrath et al., 1984; Sweet et al., 1984; Manne et al., 1985; Temeles et al., 1985). The relevance of these activities to the biological function of ras proteins has been demonstrated by three independent lines of evidence. (1) microinjection of anti-ras antibodies that inhibit guanine-nucleotide binding (Clark et al., 1985) reverses the malignant phenotype of NIH3T3 cells transformed by ras oncogenes (Feramisco et al., 1985); (2) ras mutants that have lost their ability to bind guanine nucleotides do not transform NIH3T3 cells (Lacal et al., 1986a; Williamsen et al., 1986) and (3) the GTPase activity of ras genes is severely impaired in their transforming alleles (Gibbs et al.,
1984b; McGrath et al., 1984; Sweet et al., 1984; Manne et al., 1985; Temeles et al., 1985). Recently it has been argued that activation of efficient transforming properties of ras p21 proteins can occur by mechanisms independent of changes in GTP binding and GTPase activity (Finkel et al., 1989; Der et al., 1986; Lacal et al., 1986b). The GTPase activity of normal ras, but not oncogenically activated ras has been shown to be regulated by a GTPase activating protein (GAP) which is localized in the cytosol (McCormick, 1989).

ras genes attain transforming potential when a mutation occurs at codons 12, 13, 59 or 61 (Tabin et al., 1982; Yuasa et al., 1983; Santos et al., 1984; Bos et al., 1985; Pincus et al., 1987). Even after the solution of the crystal structure of normal and oncogenically activated ras proteins (DeVos et al., 1988; Pai et al., 1989; Tong et al., 1989a,b) the functional difference between the two versions is far from clear. The mutational analysis of mammalian ras genes has led to the definition of five noncontiguous domains (residues 5-63, 77-92, 109-123, 139-165 and the carboxyl terminal sequences Cys$^{186}$-A-A-X-COOH) that are absolutely required for ras function (Willumsen et al., 1986). The last domain is important for attachment of ras to the membrane. This involves a post translational modification (acylation) of Cys$^{186}$ by palmitic acid (Sefton et al., 1982; Chen et al., 1985; Buss and Sefton, 1986; Fujiyama and Tamanoi, 1986).

The biochemical properties of ras proteins closely resemble those of the G proteins involved in the modulation of signal transduction through transmembrane signalling systems (Gilman, 1984). In fact, certain domains of ras proteins exhibit significant sequence homology with the $\alpha$ subunit
of G proteins such as Gs, a protein which activates adenylyl cyclase in response to adrenergic stimuli; G\textsubscript{i}, which inhibits this enzyme; G\textsubscript{o}, a protein of as yet unknown function and transducin, a protein that regulates cGMP phosphodiesterase activity in the visual signal transduction (Hurley et al., 1984; Lochrie et al., 1985; Tanabe et al., 1985; Itoh et al., 1986). In addition to G proteins, other nucleotide-binding proteins such as the bacterial elongation factor Tu (EF-Tu), the subunits of ATP-synthetase, adenylate kinase, phosphofructokinase and tubulin also exhibit certain sequence homology with ras proteins (Gay and Walker, 1983; Leberman and Egner, 1984; Jurnak, 1985). Recently it has been shown that one of the genes of human immunodeficiency virus (HIV1) codes for a protein named nef-HIV1 which is very similar to ras in sequence and it also has GTP binding and GTPase activity (Guy et al., 1987).

The search for biological function of ras has yielded some results in the case of yeast. It has been found that RAS 2 gene has been shown to regulate adenylyl cyclase in yeast (Toda et al., 1985; Tamanoi 1988). However, attempts to show this in mammalian cells have yielded negative results (Beckner et al., 1985; Broek et al., 1985) consistent with the earlier finding that cyclic AMP levels in ras transformed cells are generally depressed or unchanged (Anderson et al., 1974; Carchman et al., 1974). Many recent reports suggest that ras proteins perhaps regulate phospholipase C since ras proteins at least in intact cells seem to stimulate the metabolism of phosphatidylinositol 4-5 bisphosphate (PIP\textsubscript{2}) (Fleischman, et al., 1986; Wakelam et al., 1986; Morris et al., 1989). There have been also some reports showing regulation of phospholipase A\textsubscript{2} (PLA\textsubscript{2}) activity by ras resulting in the induction of membrane ruffling and
pinocytosis (Bar-Sagi and Feramisco, 1986). Even a protease inhibitory function for ras p21 proteins has recently been reported (Hiwasa et al., 1987a, b).

Studies on the expression of ras genes in mammalian cells indicate that they are expressed at low levels in most, if not all cell lineages. Unlike other proto-oncogenes, ras genes are consistently expressed throughout development of the mouse embryo (Muller et al., 1983; Slamon and Cline, 1984). Increased expression (upto eight fold) of ras genes has been reported in actively proliferating tissues such as regenerating rat liver (Goyette et al., 1983). Increased levels of ras expression do not always correlate with cellular proliferation. Studies aimed at determining the levels of ras proteins in different rat organs have found highest p21 ras expression levels in the brain, whereas proliferating tissues show only limited expression (Tanaka et al., 1986). A similar study in mouse revealed the highest levels of expression in heart, another non-dividing tissue (Spandidos and Dimitrov and, 1985). However microinjection of p21 ras into quiescent cells results in transient stimulation of proliferation (Feramisco et al., 1984; Stacey and Kung, 1984). Also, it has been shown that microinjection of ras proteins into PC12 cells induces morphological differentiation (Bar-Sagi and Feramisco, 1985).

Although the connection between many oncogene products and phosphorylation has now been established such a link is not clear in the case of protein products of ras genes. However, there are a few reports which show that phosphorylation might be a mechanism which might modulate ras function. The ras protein can be phosphorylated if it has
a threonine (as in the case of viral Harvey ras) in the 59th position (Shih et al., 1980; 1982). This occurs owing to an autokinase activity (Gibbs et al., 1984a). Recently it has been shown that the yeast RAS 1 and RAS 2 proteins are phosphorylated in vivo on serine residues (Cobitz et al., 1989). No transphosphorylating activity has been detected with any ras proteins including those carrying Thr-59 (Barbacid, 1987). One can also conceive effects of ras proteins on phosphorylation of cellular proteins; for it is known that at least in the case of yeast (Tamanoi, 1988) ras proteins regulate generation of cyclic AMP which acts on cellular targets through phosphorylation.

1.13 Rationale for the present investigation

The surface of the cell or the plasma membrane is not just a delimiting zone or barrier for the cell; it is an interphase between the cell and its environment. The cell receives signals, sends its signals to its immediate environment and also to targets at a distance via the plasma membrane. The attachment of the cell to the substratum, adhesion to other cells and a host of other processes like nutrient transport and influx or efflux of ions occurs through the plasma membrane (Haber, 1984). Of late the importance of the plasma membrane in the transduction of signals to mediate various cellular processes like cell proliferation, metabolism, secretion, contraction, transport of substances and membrane permeability, fertilization, neurotransmission and even memory, has been recognized (Haber, 1984). The molecules which are pivotal in signal-transducing cellular machinery are situated in the plasma membrane. They include several receptor systems, G proteins and other regulatory molecules (Gilman, 1987). It is of interest to note that in every one of
the cellular processes enumerated above phosphorylation plays an important regulatory role (Cohen, 1988b, Nishizuka, 1988).

Phosphorylation appears to be of importance in abnormal cellular proliferation as well. It should be noted that a large number of oncogenes are protein kinases (Sefton 1985). There are oncogenes like the ones in the ras family which are G protein like molecules. Is there any connection between ras proteins and phosphorylation? Although ras proteins are known to be phosphorylated at Thr-59 by an autokinase activity (Gibbs et al., 1984a) no phosphorylation of any protein by ras has been reported in the past. In yeast ras proteins regulate the generation of cAMP which affects cellular processes through phosphorylation. So far, no such evidence is available for mammalian cells. However, there have been reports of involvement of the ras protein in the modulation of phosphorylation of certain mitochondrial membrane proteins (Backer and Weinstein, 1986a, b). Although this represented an artificial situation, for ras proteins are not expressed in the mitochondria, it indicated that ras proteins possessed an ability to modulate phosphorylation reactions. Evidence obtained by electron microscopy and subcellular fractionation has shown that both normal and transforming ras proteins are present at the inner surface of the plasma membrane (Willingham et al., 1980; Furth et al., 1982). Although a vast amount of information is available on ras proteins (Barbacid, 1987) and they are known to bind GTP and GDP, have GTPase activity and are subjected to regulation by GTPase activating proteins (McGrath et al., 1984; Bourne, 1985; McCormick, 1989) the precise function of these proteins and their role in malignant transformation is not yet fully understood. If ras proteins have biological
functions in addition to, or apart from their GTP-binding properties, the target proteins are likely to be in the plasma membrane, the natural location of p21 proteins. Since phosphorylation is one of the most common and important post-translational modifications it was logical to look at the possible effects of ras proteins on the phosphorylation of membrane proteins.

Rat liver offers a good model system to study signal transduction and cellular proliferation. First and foremost, it can be obtained in fully differentiated and non-dividing state (in the adult) and also in actively dividing state (regenerating and fetal livers). In addition, abnormal liver cells in the forms of liver tumours are not difficult to get hold of. Liver cells can be good models for the study of ras also since it has been documented that in regenerating rat liver increased expression of ras is found (Goyette et al., 1983). Many a time ras genes are encountered in their oncogenically activated forms in liver tumours induced by carcinogens (Barbacid, 1987; Huber and Cordingly, 1988). Liver cells are known to utilize major signalling pathways like hormone sensitive adenylyl cyclase pathway (Ross and Gilman, 1980) and the recently discovered pathway which uses second messengers generated by metabolism of inositol lipids for signal transduction. Both of these pathways are initiated by polypeptide factors (Williamson et al., 1985; Johnson and Garrison, 1987).

On the basis of the background described above, we examined whether ras proteins play any role in phosphorylation reactions using plasma membrane proteins from rat liver cells as substrates. These
experiments revealed a dramatic increase in the phosphorylation of a 38 kDa protein (p38) in the liver plasma membrane. There was no such effect when plasma membrane preparations from a liver tumour, the Zajdela Ascitic Hepatoma, a chemically induced tumour, were used in the phosphorylation experiments. The nature of the phosphorylation was also found to be unusual. Results of a detailed investigation beginning from this observation are presented in the following chapters.