DISCUSSION

Some of the major questions in biology, which still remain unanswered have to do with how cell proliferation is controlled and what goes wrong when a cell becomes malignant. Signals received at the cell-surface are transmitted into the cell. This process involves many components including second messengers. Second messengers are a heterologous group of molecules that include phosphoproteins, Na⁺, Ca²⁺, H⁺, DAG, inositol phosphates, cyclic nucleotides, prostaglandins, polyamines and many others, whose concentrations change as a result of activation of a cell by growth factors (Pardee, 1989). These second messengers are involved in several processes in the cell including oncogene activation and cell proliferation (Berridge, 1984a).

Major changes occur in the signal transducing machinery of a cell upon transformation. G proteins are very important components in the transduction of many extracellular signals to intracellular effector molecules. However, there have been very few studies to examine the role of G protein subunits and in turn, modification of the process of signal transduction during transformation. With a view to examine the role of G protein subunits in this process, we have chosen the Zajdela Ascitic Hepatoma, a rat hepatoma induced by a chemical carcinogen, dimethylaminoazobenzene (DAB) as a model system.

As a first step in understanding the role of G protein subunits, we have isolated a partial cDNA clone for the rat Gβ2 subunit and studied its expression in various rat tissues as well as in regenerating rat liver. We have examined the possible changes in the expression and organization of the G protein subunit (Ga and Gβ) genes in ZAH in comparison to its normal counterpart, rat liver.

The importance of G proteins in transformation has been strengthened by the observation that activating mutations in the G protein alpha subunits have been observed in naturally occurring human endocrine tumors. Consequently, we have carried out experiments to determine possible activating mutation(s) in the G protein alpha subunit, Gas, in ZAH.
The possible significance of the findings of the present study is discussed in this section.

4.1 Isolation of a cDNA clone of rat Gβ2

A partial cDNA clone for rat Gβ2 was isolated from rat liver and sequenced. The EMBL nucleotide sequence database was screened for the homologous sequences. It was observed that at the nucleotide level, rat Gβ2 is 91.2% homologous with human Gβ2. At the protein level, rat Gβ2 was found to be identical with human Gβ2 (see fig. 11). This is in agreement with previous reports which show that human and bovine Gβ1 are identical at the protein level, as are human and bovine Gβ2. Rat Gβ2 was found to be 90.2%, 81.8% and 89.3% homologous with human Gβ1, human Gβ3 and mouse Gβ4, respectively (see fig. 12-14). These findings indicate that Gβ2 subunits have been very well conserved through evolution and suggest important roles played by them in the cell.

4.2 Expression and membrane targeting of beta and gamma subunits of G proteins (Gβγ)

G proteins consist of α, β and the γ subunits, which upon activation, dissociate into Ga and Gβγ. The β and γ subunits are not covalently linked to each other. However, they are very tightly associated under physiological conditions and can be dissociated only by the use of denaturants. Consequent to this, β and γ subunits form a single functional unit in the cell. However, there are two reports in the literature where the presence of free β and γ subunits has been demonstrated under non-denaturing conditions (Yamazaki et al., 1987; Morishita et al., 1994). The significance of these observations is not known. Studies of separated β and γ subunits have been difficult because βγ purified from tissues can only be dissociated by irreversible denaturation. Therefore, it is not entirely clear whether all the functions studied using Gβγ complex can be attributed only to the Gβγ complex or to the subunits separately.
The primary sequences of each of the G protein subunits lack hydrophobic, membrane spanning domains (Spiegel et al., 1991). Recently, Simonds et al. (1991) have examined the expression and membrane targeting of β and γ subunits in a mammalian cell line. They observed that the expression of either subunit in COS-7 cells required cotransfection with both β and γ cDNAs. They also showed that the lack of protein expression in cells transfected separately with β or γ cDNA occurred despite the synthesis of relevant RNA transcripts. Mutation of the carboxy-terminal cysteine residue of γ (shown to undergo isoprenylation and carboxy methyl-esterification) preserved βγ expression but blocked isoprenylation and appreciable membrane attachment. Similar results were obtained by Crespo et al. (1994). In other studies also, it was observed that isoprenylation of the γ subunit is essential for membrane attachment of Gβγ (Iniguez-Lluhi et al. 1992). It appears that the requirement for β and γ coexpression may reflect rescue of individual subunits from a rapid degradation pathway as seen in the assembly of other multisubunit membrane proteins (Klausner and Sitia, 1990).

Thus, the assembly of βγ heterodimer confers stability. The carboxyl-terminal processing of the γ subunit is not required for association of γ with β subunits but is critical for membrane attachment and the β subunit by itself is insufficient to ensure membrane attachment.

Taking the above observations into account and considering our observation of high steady state levels of Gβ in ZAH (about four-fold more in ZAH as compared to rat liver cells) at the protein level, together with the observation that overexpressed Gβ is associated with the plasma membrane (see fig. 17 and 18) indicates that Gγ may also be correspondingly overexpressed and isoprenylated in ZAH. The functions of Gβγ have been studied using βγ as a complex. Hence, the functional significance of the findings of this study has been discussed assuming that Gβ and Gγ may both be overexpressed in ZAH. Thus, it is possible that Gβγ complex may be about four-fold more in ZAH as compared to liver.
4.3 Expression of Gβ2 in different tissues of the rat

The knowledge of the expression pattern of a protein in different tissues has been useful in elucidating its functions. G proteins are present in almost all tissues, indicating their involvement in basic functions of a cell.

We decided to examine the expression level of G protein β2 subunit at the RNA level in different rat tissues. Our results demonstrate that Gβ2 is expressed in all the tissues examined and that it is expressed at very low levels in the skeletal muscle (see fig. 15). This is in accordance with the reports of Hinsch et al. (1989), who also observed very little expression of Gβ2 in the rat skeletal muscle (at the protein level). It is worth mentioning here that Hinsch et al. (1989) observed very low levels of Gβ2 subunit at the protein level in rat heart whereas, we have observed good expression at the RNA level. This discrepancy may arise due to the difference in the stability of the protein and/or due to the differential translational efficiency of the mRNAs in different tissues. A discrepancy in the relative distribution of G protein α subunit mRNA compared to the abundance of α subunits as estimated by immunoblotting has been observed by Brann et al. (1987) also.

4.4 Expression of Gβ2 during liver regeneration

The liver exhibits a remarkable capacity to regenerate following partial hepatectomy or after injury. In normal liver, hepatic cells are quiescent, but within hours after liver injury or following a partial removal of liver tissue, they rapidly progress into mitosis (Fausto and Mead, 1989; Michalopoulos, 1990). Based on studies with hepatocyte cultures and gene expression in regenerating liver EGF, TGFα, HBGF-1, HGF and HPTB have been defined as complete mitogens for hepatocytes and implicated in the control of liver growth. The plasticity of growth responses seen in liver may be controlled by these factors as well as others known as comitogenic substances (incomplete mitogens or growth triggers) such as norepinephrine, vasopressin, angiotensin II and III, estrogens, as well as by mitogenic inhibitors such as TGFβ. Though a number of mitogens have been identified that activate hepatic cellular
division, the mechanisms underlying initiation of the liver growth program remain unclear and may involve more than one pathway (Fausto and Mead, 1989; Michalopoulos, 1990). HGF and TGF-α levels have been shown to increase at times slightly preceding the first wave of regenerative cell division. Phenomena described as occurring very early after 2/3 PH include membrane hyperpolarization, glycogenolysis and an increase in the level of diacylglycerol (Houck and Michalopoulos, 1989).

We have observed increase in the expression of Gβ2 at about 12 h after 2/3 partial hepatectomy (see fig. 16). Considering the stimulatory effect of Gβγ on phospholipase C, it is possible that increased expression of Gβ(γ) may be involved in the liver regeneration by stimulation of PLC to produce second messengers, IP3 and DAG. In this regard, it is very interesting to note that many of the co-mitogens such as vasopressin and angiotensin III as well as complete mitogen such as EGF, are linked to the PI metabolism and increased level of DAG is seen very early after 2/3 PH. It is possible that the second messengers - DAG and IP3 produced from the hydrolysis of PIP2 by phospholipase C, could play a role in the liver regeneration.

4.5 Expression of Gβ2 in ZAH is regulated at both transcriptional as well as post-transcriptional level

We have observed that steady state level of Gβ2 mRNA in ZAH is about six-fold more than that in the liver. In order to find out if this was a consequence of high rate of its mRNA synthesis, we carried out nuclear run-off transcription on the liver and ZAH C nuclei. The results showed a 2.7 fold increase in its mRNA synthesis in ZAH compared to liver. Thus, indicating that the higher expression of Gβ2 in ZAH is to some extent because of the high rate of its mRNA synthesis.

4.6 Hepatocyte transformation and phosphatidylinositol turnover

The growth regulatory signals involved in the development of neoplasia, regeneration, cell proliferation and hyperplastic growth in hepatocarcinogenesis have received attention for many years. Many hormones, mitogenic growth factors, and
autocrine stimuli are known to induce hepatocyte proliferation and DNA synthesis in vitro and in vivo (Bucher et al., 1978; Cruise et al., 1986; Luetteke and Michalopoulos, 1987). However, the exact nature of stimulus in chemically induced hepatocellular carcinomas is not known. It has long been postulated that phosphoinositide turnover may be critical for cellular proliferation (Berridge, 1987c). Many reports have demonstrated that the hepatocytes, through receptor mediated pathways, respond to external stimuli such as epidermal growth factor (EGF), angiotensin II (Johnson and Garrison, 1987) and epinephrine (Cruise et al., 1986, 1989) to rapidly turnover phosphatidylinositol to form the second messengers, IP_3 and DAG.

We have attempted to study the role of G proteins in a hepatoma, the ZAH. We have shown that Gβ2 is overexpressed about four-fold and Gaα3 about two-fold more as compared to its expression at 3 h after 2/3 hepatectomy. Since the activation of G proteins leads to dissociation of Ga and Gβγ subunits which are free to interact with the effector molecules, the possible role of these subunits in transformation has been discussed separately.

4.7 Significance of overexpression of G protein β(γ) subunits in ZAH.

We have observed that Gβ is overexpressed about four-fold more in ZAH as compared to its normal counterpart, liver. This enhanced expression was seen at both protein and mRNA level (see fig.17-19). Based on the known functions of Gβγ, it appears that overexpression of Gβγ in ZAH may provide a proliferative advantage to the cell by one or more mechanisms discussed below. In each of the following sub-section, we have tried to provide important background information followed by possible effects of Gβγ.

4.7.1 Stimulatory effect of Gβγ on phospholipase C

The action of phospholipase C on PIP_2 generates two second messengers, IP_3 and DAG. Phospholipase Cs are divided into 3 classes designated as PLCβ, PLCγ and
PLCβ isoforms are regulated by G proteins whereas, PLCγ is regulated by the receptor tyrosine kinases. Gβγ subunits stimulate the β isoform of PLCs.

Several studies have demonstrated that generation of the second messengers and phosphoinositide turnover are critical events in the stimulation of cell-proliferation (Habenicht et al., 1981; Thomas et al., 1983; McPhee et al., 1984; Berridge et al., 1984; Taylor et al., 1984). IP₃ releases Ca²⁺ from the intracellular stores and DAG is involved in the activation of PKC. It is now widely accepted that calcium acts as a critical regulator of many cellular functions including cell growth. Tumor-promoting phorbol esters are potent activators of protein kinase C (Castagna et al., 1982). Also, protein kinase C stimulates the Na⁺-H⁺ exchanger to bring about the increase in the cytoplasmic pH, which is a key event for the onset of DNA synthesis (Rozengurt, 1986).

The effect of norepinephrine at α1-adrenergic receptors involves phosphatidylinositol hydrolysis and the stimulation of Ca²⁺ mobilization (Exton, 1980; Tolbert et al., 1980). Epinephrine and vasopressin have also been shown to activate PIP₂ hydrolysis by phospholipase C in isolated hepatocyte plasma membranes (Thakker et al., 1989). It has been suggested that α1-adrenergic receptors may modulate the initial proliferative response in liver regeneration or contribute to the increased growth during hepatocarcinogenesis (Cruise et al., 1985, 1986).

From various studies it is clear that there is a very strong correlation between the growth stimuli associated phosphoinositide turnover and cell-proliferation. We have shown that Gβ2 is overexpressed about four-fold more in ZAH as compared to rat liver and the expression of Gβ2 mRNA is regulated at the transcriptional as well as post-transcriptional level. Gβγ complexes have been shown to regulate the activity of many second messenger systems, including phospholipase C. Since, (a) many growth factors and oncogenes affect phospholipid metabolism (Jackowski et al., 1986; Maly et al., 1989; Johnson et al., 1989); (b) inositol phospholipid turnover is involved in
cellular proliferation (Rozengurt, 1986; Berridge, 1987b; Nishizuka, 1988; Matsuoka et al., 1988; Smith et al., 1989); (c) phospholipid metabolism has been shown to be altered in transformed cells (Sugimoto et al., 1984; Fry et al., 1985); (d) Gβγ activate phospholipase C (PLC), the key enzyme in the phospholipid metabolism to generate second messengers: inositol trisphosphate (IP3) and Diacylglycerol (DAG); (e) mitogens for liver affect PI metabolism, it is reasonable to think that higher levels of Gβγ in ZAH may provide proliferative advantage to the cells by its action on the PLCs and consequent increase in the level of IP3 and DAG. This proposal is in agreement with the studies by Lalwani et al. (1991), who have demonstrated that the chemically induced hepatic tumors possess elevated levels of second messengers, IP3 and DAG. Thus, it is possible that the altered state of growth exhibited by ZAH, a liver tumor, may at least in part be mediated by sustained increased levels of these second messengers.

4.7.2 MAP kinase activation by Gβγ

MAP kinases or ERKs are a family of protein serine/threonine kinases that are activated in response to various extracellular stimuli such as growth factors, hormones and neurotransmitters (Blenis, 1993; Crews and Erikson, 1993). ras and heterotrimeric guanine nucleotide proteins also regulate ERK network (Robbins et al., 1994). In turn, ERKs regulate key intracellular enzymes and transcription factors involved in the control of cell-proliferation (Pelech, 1993; Davis, 1993).

In mammalian cells, mitogens that use G protein coupled-receptors activate the MAP kinase cascade through ras-dependent and independent pathways. It now appears that in mammalian cells, the MAP kinase cascade may be regulated by both α-subunits and βγ dimers of G proteins. Crespo et al. (1994) have demonstrated that ERK activation in COS-7 cells is mediated by Gβγ subunits acting on a ras-dependent pathway. There are two ways by which Gβγ may be involved in the activation of MAP kinases.

(i) Through interaction with pleckstrin homology domains (PH domains) of ras-regulatory proteins.
(ii) By stimulation of phospholipase C (PLC), which produces inositol trisphosphate and DAG. Faure et al. (1994) have shown that phorbol ester (a PKC activator) stimulates MAP kinase activity.

Thus, it is possible that Gβγ released after the receptor stimulation may affect MAP kinase activity and thus proliferation of the cells by two different routes. In the light of the above observations, we think that overexpression of Gβ(γ) may contribute to the proliferation of ZAH cells by activating the MAP kinase activity. This is in agreement with the observations by Faure et al. (1994), who have shown that Gβγ overexpressed together activated MAP kinase activity.

4.7.3 Interaction of Gβγ with PH domain-containing proteins

The pleckstrin homology domain (PH domain) was originally identified in the protein, pleckstrin. Subsequently, it was found in many different proteins involved in cellular signal transduction. Recently, Touhara et al. (1994) have demonstrated that glutathione S-transferase-fusion proteins, containing sequences encompassing the PH domains of nine different proteins bind Gβγ. It is thought that protein-protein interactions between Gβγ and the PH domain containing proteins may play a very significant role in cellular signaling, analogous to that previously demonstrated for Src homology domains 2 and 3.

It is interesting to note that relatively strong interaction of Gβγ with PH domain of PLCγ was observed. However, so far no interaction of Gβγ to PLCγ isozyme has been demonstrated. In view of the fact that PLCγ contains PH domain and Gβγ, at least in vitro, binds to the PH domain of PLCγ (Touhara et al., 1994), it is possible that this interaction of Gβγ and PLCγ may play a significant, but currently unappreciated role in the regulation of cellular signal transduction.

Thus, it appears that overexpression of Gβ(γ) in ZAH could have important roles in the signal transduction mechanisms involving PLCγ, (which is normally regulated by
receptor tyrosine kinases) and provide proliferative advantage to the cells. This may be a possible link between the two predominant signal transduction pathways in the cell, the receptor tyrosine kinase pathway and the G protein pathway.

4.7.4 Stimulatory effect of \( G\beta y \) on PI3 Kinase

The phosphoinositide 3 kinase (PI3 K) is a key signaling enzyme implicated in many cellular processes including receptor stimulated mitogenesis. The function of this enzyme is to attach a phosphate group to the 3′-hydroxyl group of the inositol ring of PI and/or its phosphorylated derivatives \( \text{PI(4)P} \) and \( \text{PI(4,5)P}_2 \) to produce \( \text{PI(3)P} \), \( \text{PI(3,4)P}_2 \) and \( \text{PI(3,4,5)P}_3 \).

A very strong correlation between PI3 kinase activity and transformation has been shown in a study in which a mutant of the oncogenic Abl protein tyrosine kinase, that does not become myristoylated and therefore does not associate with the plasma membrane, is unable to activate PI3-kinase and to transform cells (Varticovski et al., 1991).

Recently, Stephens et al. (1994) have shown that a novel PI3 kinase activity in myeloid-derived cells is activated by G protein \( \beta y \) subunits. In the light of the postulated roles of \( \text{PIP}_2 \) metabolites (Berridge et al., 1984) and PI3 kinase in growth regulation (Cantley et al., 1991; Downes and Carter, 1991; Panayotou and Waterfield, 1992) and its activation by \( G\beta y \) (Stephens et al., 1994), it is possible that overexpression of G protein \( \beta(y) \) subunits in ZAH could provide growth advantage to the cells.

4.7.5 Effect of \( G\beta y \) on phospholipase A\(_2\)

Phospholipase A\(_2\) (PLA\(_2\)) acts on phosphatidylcholine to produce arachidonic acid (AA) and lysophosphatidylcholine. Eicosanoids produced by the metabolism of arachidonic acid have a wide range of biological activities, including effects on cell growth (Needleman et al., 1986). It has been observed that treatment of cells with
growth factor causes an increase in the formation of arachidonic acid, which is the rate-limiting precursor for eicosanoid biosynthesis (Davis, 1993).

In addition, both free arachidonate and its metabolites have been shown to modulate other second messenger systems such as cAMP, cGMP, PKC, PLC and Ca²⁺ (Axelrod et al., 1988) Arachidonic acid and its metabolites, in addition to acting intracellularly as second messengers, may also exit from the cell to act as primary messengers.

In the light of the effects of Gβγ on PLA₂ (Jelsema and Axelrod, 1987) and the importance of eicosanoid pathway in cell-growth, we think that overexpression of Gβγ in ZAH could affect the cell proliferation through stimulation of PLA₂, thus leading to increased eicosanoid production. In this regard, it is important to note that Xu et al. (1993) have recently demonstrated that overexpression of wild-type α12 in NIH 3T3 cells is itself weakly transforming, but activated α12 (α12 Q229L) behaves as a very potent oncogene. Transformation by α12 correlates with alterations in the eicosanoid pathway. This suggests strong link between eicosanoid pathway and transformation.

In the above sections, we have discussed the possible significance of overexpression of Gβγ in ZAH. An overall examination of all the above-mentioned pathways would reveal that broadly, Gβγ may affect the tumorigenic property of ZAH cells in two ways.

A. By its effect on phospholipid metabolism. This would include the activation of PLC, PLA₂, stimulation of PI3 kinase and interaction with PLCγ.

B. By its involvement in the ras dependent activation of MAP kinase and also its interaction with the PH domain containing ras regulatory proteins.

Earlier studies in our group on the role of ras in ZAH has demonstrated that it is neither overexpressed nor activated by mutations at the known positions. Although these observations do not rule out the involvement of ras (and hence, involvement of
Gβ(γ) in its action) in ZAH, they lend more credence to the first mechanism i.e. effect of Gβ(γ) on phospholipid metabolism. It is interesting to note that alteration in the phospholipid metabolism has frequently been observed during growth and transformation. With respect to hepatocyte proliferation and transformation, it has been shown that many of the mitogens for liver cells affect phospholipid metabolism. In this context, it is interesting to note that Lalwani et al. (1991) have observed high steady state levels of second messengers IP₃ and DAG in chemically induced liver tumors.

4.8 Significance of overexpression of Gaι3 in ZAH

Gia was originally defined as the α subunit of the G protein mediating inhibition of adenylyl cyclase. It consists of a family of closely related 3 subtypes, Gaι1, Gaι2 and Gaι3. Apart from mediating inhibition of adenylyl cyclase, the subtypes of Gia also control various other cellular effector systems such as K⁺ channels (Birnbaumer et al., 1990), neuronal and dihydropyridine-sensitive Ca²⁺ channels (Linder et al. 1990; Schmidt et al., 1991) and perhaps phospholipase D (MacNulty et al., 1992).

The significance of G proteins in mitosis is strengthened by the observation that induction of mitosis by certain growth factors is inhibited by pertussis toxin (Gilman 1987; Spiegel, 1987; Murayama and Ui 1987; Chambard et al., 1987). Crouch (1991) has demonstrated a connection between the receptor tyrosine kinases and guanine nucleotide binding proteins and shown a direct involvement of Gaι in cell division. Yang et al. (1993) have shown that EGF activates PLC-γ through Gi. Kikuchi et al. (1986) have shown that rat brain Gi and Go have the potency to couple functionally the fMLP receptor to phospholipase C mediated polyphosphoinositide hydrolysis in HL-60 cells.

We have observed that the steady state level of Gaι3 mRNA in ZAH is about two-fold more than that in the rat liver (see fig. 25). The possible significance of this observation is discussed in the light of the known functions of Gaι, particularly Gaι3.
There are at least two ways by which overexpression of Ga\textsubscript{i3} in ZAH could affect the proliferation of the cells.

4.8.1 Effect of Ga\textsubscript{i3} on phospholipid metabolism and other second messenger systems

Fargin et al. (1991) have shown that the Gi proteins but preferentially Gi3, mediates the effects of 5-hydroxytryptamine (5-HT) both to inhibit adenylyl cyclase and to stimulate phospholipase C, when 5-HT\textsubscript{1A} receptor is stably expressed in HeLa cells.

Yatani et al. (1988) have shown that recombinant Gi subtypes - Gia1, Gia2 and Gia3 stimulate the atrial muscarinic K\textsuperscript{+} channels. Schwiebert et al. (1990) have shown that Gai3 stimulates a Cl-channel in the apical membrane of rabbit kidney CCD cells. The Gai3 subunit regulates a cation channel in the apical membrane of renal inner medullary collecting duct cells (Light et al., 1989) and a sodium channel in A6 cells (Cantiello et al., 1989). Thus, it is clear that Gai3 affects many processes including hydrolysis of phosphatidylinositol. Alterations in the metabolism of phosphoinositides have frequently been implicated in cellular transformation (Fleischman et al., 1986; Hancock et al., 1988) Hence, it is possible that overexpression of Gai3 in ZAH could promote cell-proliferation by affecting the hydrolysis of phosphatidylinositol to generate IP\textsubscript{3} and DAG or other above-mentioned second messenger systems.

4.8.2 Effect of Gai3 on cAMP level and MAP kinase activity

Recently, Graves et al. (1993) have shown that the compounds that increase cAMP and activate protein kinase A (PKA), inhibit the PDGF-B-B-induced activation of MAPKK and MAPK in arterial smooth muscle cells. Sevetson et al. (1993) have suggested that activation of cAMP-dependent protein kinase may represent a general counter-regulatory mechanism for opposing MAP kinase activation. This demonstrates a cross-talk between two important signaling systems - the PKA pathway and the growth factor activated MAP kinase cascade. In addition, it has been shown earlier that PKA can phosphorylate and partially inhibit the autophosphorylation of the insulin and
EGF receptors (Tanti et al., 1987; Stadtmauer and Rosen, 1986; Ghosh-Dastidar and Fox, 1984). It should be noted that EGF is a mitogen for hepatocytes.

It is pertinent to consider what consequences cAMP inhibition of MAP kinase signaling might have on cell proliferation. MAP kinase is believed to be a key participant in cell's response to growth factors. Given the central role of MAP kinase in mediating the action of numerous hormones, growth factors and mitogens, cAMP dependent inhibition of the enzyme may be of broad physiological significance. In addition to many other functions, MAP kinase is also involved in the regulation of PLA2 (Davis 1993). Thus, cAMP level may be reflected on arachidonic acid production also.

In principle, the increased expression of Gia3 in ZAH could lead to decrease in the adenylate cyclase activity, in turn leading to reduced cAMP level in the cell and relieving the MAP kinase inhibition due to cAMP, thus promoting cell-proliferation in response to the growth factors.

4.9 Possible role of enhanced expression of G protein subunits in ZAH

Heterotrimeric G proteins are essential for transferring many extracellular signals from cell surface receptors to intracellular effectors. Since G proteins are involved in generation of intracellular effectors (many of which are involved in cell growth), role of G proteins in transformation assumes considerable significance. In the present study, we have observed enhanced expression of G protein subunits Gα3 and Gβ2 in ZAH, a rat hepatoma. Considering the effects of G protein subunits on various signal transduction pathways and a potential role of Gi in mitogenic signal transduction, it is reasonable to assume that the overexpression of Gα3 in ZAH could have contributed to cellular proliferation, through the MAP kinase cascade, by the effect of Gα3 on phospholipid metabolism or by affecting other second messenger systems. Similarly, overexpression of Gβ(γ) in ZAH may provide proliferative advantage through its effect on phospholipase C, MAP kinase cascade, PI3 kinase, phospholipase A2 or by interacting with the PH domain containing ras regulatory components. It is interesting
to note that many of these pathways have been shown to be altered in transformed cells.

4.10 Genomic organization of Gai3 and Gβ2 genes in ZAH

Deregulated expression of a gene in a tumor cell is quite often associated with its translocation. The Southern analysis of Gai3 and Gβ2 genes did not show any apparent change in their organization as compared to rat liver, indicating that the overexpression of these genes may not be because of their translocation.

4.11 Search for activating mutation(s) in the α subunit of Gs (Gas) in ZAH

Oncogenic mutations in G proteins have been identified in many human endocrine disorders. Mutation in the α subunit of Gs (G protein involved in the activation of adenyl cyclase) have been demonstrated in 40% of humans growth hormone secreting pituitary adenomas and 10% of thyroid adenomas. Landis et al. (1989) showed that in pituitary tumors which showed enhanced level of cAMP and growth hormone production, Gs was activated by somatic point mutation.

In these cases, single amino acid substitutions replacing Arg 201 with either Cys or His or Gln 227 with Arg was found to be responsible for constitutive activation of adenyl cyclase. Arg 201 in Gsa is the site for ADP-ribosylation by cholera toxin and Gln 227 is equivalent to Gln 61 of the p21ras. The result of both mutations leads to reduced GTPase activity of the protein, leaving it in the constitutively active form. This leads to persistent activation of adenylate cyclase, thus bypassing the need for the stimulatory ligand. Bourne and Coworkers named this oncogene as gsp (for stimulatory G protein).

Site directed mutagenesis studies on Gas have shown that replacement of Gln 227 by Leu activates Gas by inhibiting its GTPase activity (Masters et al., 1989; Graziano and Gilman, 1989). Expression of Gas Q227L in Swiss 3T3 cells was found
to elevate cAMP levels in the cell and to augment DNA synthesis in serum depleted medium containing insulin (Landis et al. 1989).

In the light of the above observations with the mutationally activated Gαs in tumorigenesis, we decided to check if any such mutation existed in ZAH. We checked for mutation in Gαs in ZAH "C" at both positions, 201 and 227. As described in the result section (see fig. 29-30), we did not find any mutation in Gαs in ZAH. This observation rules out the possibility of mutations at positions 201 and 227 being causative determinants for ZAH C.

We have examined the possible changes in the expression and organization of the G protein subunit genes upon transformation. Gα3 and Gβ2 were found to be overexpressed in ZAH, a rat hepatoma. In the light of the known functions of these subunits, we have discussed possible ways by which their overexpression could play a role in ZAH cell proliferation. It would be of interest to examine which of the mechanism(s) (see sections 4.7.1-4.8.2) may actually be altered in ZAH, and thus, contribute to its tumorigenic property. Experiments designed to study the steady state levels of the second messengers may provide important clues for further investigation.

We have observed high steady state levels of G protein subunits in ZAH. It would be of great interest to examine the levels of these subunits in other chemically induced tumors and also in other type of tumors. Such a study would be needed in order to see the generality of this phenomena.