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I am submitting herewith a dissertation written by Kausik Ray entitled "Molecular Analysis of the Structure, Organization and Expression of the *Drosophila* G protein γ-subunit Gene". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Ph.D, with a major in Life Sciences.

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MOLECULAR ANALYSIS OF THE STRUCTURE, ORGANIZATION AND EXPRESSION OF THE *DROSOPHILA* G PROTEIN γ-SUBUNIT GENE

A Dissertation
Presented for the
Doctor of Philosophy
Degree
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Kausik Ray
May, 1993

DEDICATION

This dissertation is dedicated to .....

my mother, Mrs. Mina Ray, for her love, support & encouragement

and

the memory of my father, (late) Mr. Samir K. Ray, who is always in my heart as a source of inspiration

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ABSTRACT

The present study was undertaken to identify and characterize a gene which is expressed in the nervous system of *Drosophila*. Since adult head of the fly is enriched with neural tissues, a genomic clone 536, containing a gene predominantly expressed in the head of *Drosophila melanogaster* was characterized. DNA sequencing showed that a gene present in this clone encodes a protein similar to mammalian G protein γ subunits. G proteins are heterotrimeric, GTP-binding proteins which mediate transduction of extracellular stimuli to intracellular effector molecules. The putative *Drosophila* γ- subunit (D-Gγ1) protein shares 46, 43, and 28% identity and 59, 52, and 60% similarity with the γ2, γ3 and γt subunits of the bovine G proteins, respectively. The *D-Gγ1* gene produces 2.6-, 1.3-, and 1.1-kb mRNAs which show differential expression pattern but encodes the same D-Gγ1 protein. *In situ* hybridization and RNA blot analyses showed that (1) the 2.6-kb RNA is expressed only in the central nervous system; (2) the 1.1-kb RNA is expressed in all cells but the highest level is found in the central nervous system; and (3) the 1.3-kb RNA is expressed only in the ovary. In embryos, the 1.1-kb D-Gγ1 RNA is the predominant mRNA species. Structure and organization study of the *D-Gγ1* gene demonstrated that two exons and one intron are present in the *D-Gγ1* gene and

the transcription start site of all three D-Gγ1 mRNAs is the same. The putative promoter region of *D-Gγ1* gene possesses sequences similar to the GC-box but no TATA-box was found. The 3′-untranslated regions of the D-Gγ1 mRNAs vary due to differential utilization of polyadenylation signals. Also, the 5′-untranslated region of the ovary-specific 1.3-kb RNA has an extra 188 nucleotide sequence which originates by alternative splicing. The present study has indicated that *D-Gγ1* gene is expressed predominantly in neural tissues and may play important role in neuronal transduction processes. The size differences in the 5′- and 3′-untranslated regions of the D-Gγ1 mRNAs may regulate translatability and differential expression of the *D-Gγ1* gene.

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CHAPTER 1

GENERAL INTRODUCTION

In multicellular organisms, communication between cells is required for growth, differentiation and diverse cellular function. Individual cells need to be able to sense both the general status of the whole organism in relation to the external environment, and the functional status of other cells which create the internal environment. Appropriate intercellular communication systems are therefore important for different cellular responses. Neuronal communication in the brain, where chemical and electrical synapses represent an additional step of intercellular communication pathways, provides the most complex system of intercellular communication or cell signaling. There are also various other cell signaling mechanisms which are non-neuronal in nature. Recent advances in molecular neurobiology indicate that at the cellular level the neuronal and non-neuronal cell signaling processes converge and utilize various common signaling molecules. Thus, these two types of cell signaling mechanisms together involve a large number of proteins which maintain the structure and function of the communication network. Although a large number of genes encoding many of these proteins have been identified by

biochemical and genetic means, a complete understanding of the cell communication process requires other approaches, such as reverse genetics. The present investigation was undertaken to study the molecular genetic aspects of cell signaling in *Drosophila*. The gene encoding a γ-subunit of *Drosophila* G protein was isolated and characterized during the course of this investigation. In view of the nature of this study, the emphasis in the review of the literature below is on the cellular and molecular aspects of cell signaling processes, with special emphasis on the G proteins.

1. SIGNALING VIA NEURONAL CIRCUITS

The perception of a sensory signal by the brain can be divided into two processes, signal recognition by sensory cells and neural processing. While the initial molecular and biochemical events of transmembrane signal recognition by the photoreceptor, olfactory, and taste receptor cells are beginning to be understood (Kinnamon 1988; Reed 1992; DeVries & Baylor, 1993), many intriguing questions remain unanswered: How does the brain know what has been transduced? How do the central circuits and systems in the brain process the incoming information? How is this information translated into subsequent motor responses?

In the brain, millions of neurons with chemical and electrical synapses contribute to brain function,

establishing the most unique and complex system of intercellular communication. This complex system seems to function by two basic processes: axonal transmission and the ability of cells to generate and receive chemical messages.

Axonal transmission depends on a change in the resting membrane potential brought about by charge transfers carried by ionic fluxes through gated pores or channel proteins (Armstrong, 1992). Inside a neuron, resting membrane potential is maintained by Na+/K+ pumps driven by ATP, which selectively allows Na+ ions to exit and K+ ions to enter the cell across the neuronal membrane. For every three outgoing Na+ ions, two K+ ions enter the cell. As a result, the neuron becomes more negatively charged inside relative to the outside of the cell. Changes in ionic fluxes are brought about by graded potentials generated at the post-synaptic terminal by selective opening of Na+ channels, either through direct interaction between these channels and neurotransmitters or by their interaction with second messenger systems. When voltage-gated Na+ channels along the axonal axis respond to an adjacent drop in voltage and open to exceed a defined threshold potential, the depolarization potential (axon potential) spreads along the axonal axis. As the axon potential translocates along the axon, depolarization is reversed by self-closure of the Na+ channels and opening of voltage-gated K+ channels, making the membrane permeable to K+ and thus repolarizing the

axonal membrane. This rapid depolarization and repolarization of axonal membranes with the influx and efflux of sodium and potassium ions, repeated over and over again, fires an action potential propagating along the full extent of a nerve cell. When the nerve impulse or action potential reaches a synapse, the pre-synaptic neuron releases neurotransmitters, such as serotonin, catecholamines, etc. These neurotransmitters then interact with their respective receptors on the surface of post-synaptic neurons or other target cell types in order to promote the electrical signal to the next axon (Jessel & Kandel, 1993). These processes are diagrammatically represented in Fig. 1.

Nerve cells have the ability to generate and receive chemical as well as electrical signals, i.e., neurons are both electrically and metabolically coupled. Other than electrical signaling, the basic metabolism and survival of an individual nerve cell depends on its response to a variety of chemical signals, including different growth factors and hormones. Transmembrane signaling of these chemicals are often mediated by receptor-mediated signaling pathways in nerve and sensory cells. At least three different major classes of receptor have been implicated in neuronal signaling processes: (1) ligand-gated ion channels (e.g. $GABA_A$, NMDA-type receptors etc.), (2) growth factor-type neuropeptide receptors (e.g. nerve growth factor,

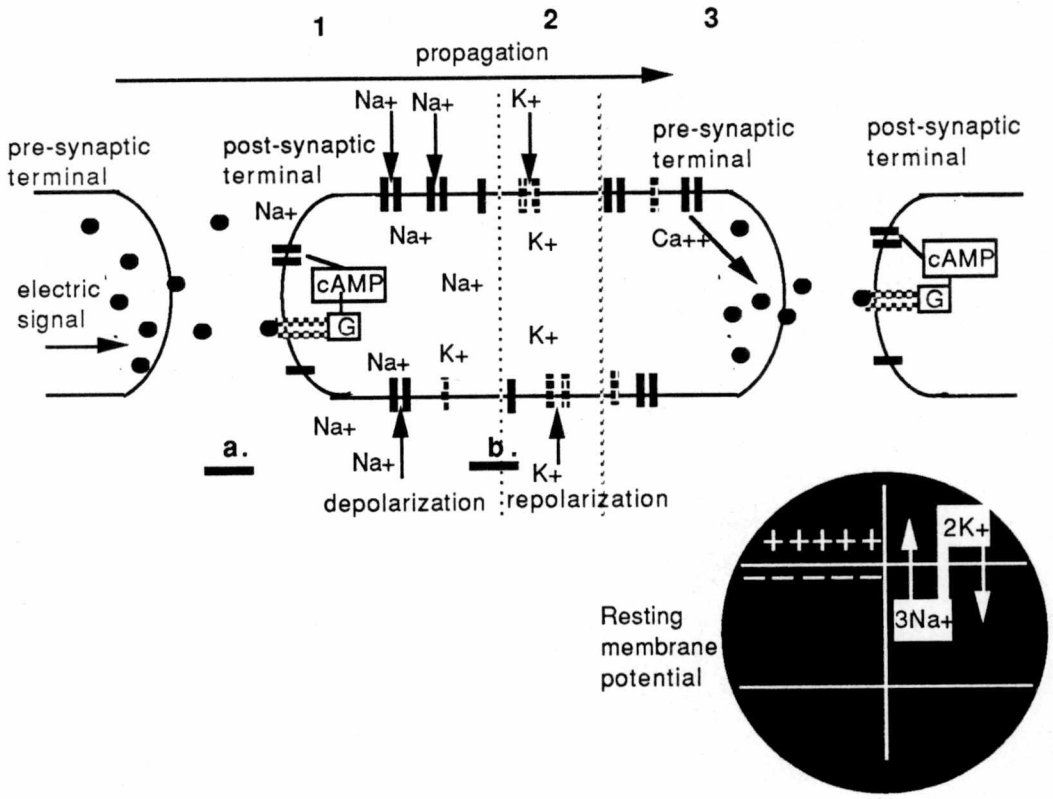


Fig. 1. Schematic representation of electric and synaptic transmission along the nerve cells. In this example, Na^+, K^+, and Ca^{2+} ions are shown in the transfer of information. (1) **a.** Binding of neurotransmitters (black dots) on the post-synaptic membrane opens Na^+ channels and influx of Na^+ ions initiates depolarization of the plasma membrane. **b.** Repolarization of the membrane takes place when Na^+ channels close and voltage-gated potassium channels open, making the membrane permeable to K^+ ions. Rapid depolarization and repolarization of axonal membrane with the influx and efflux of Na^+ and K^+ ions, repeated over and over again, fires an action potential, which is propagated. (2) At the pre-synaptic terminus, depolarization of plasma membrane opens voltage-gated Ca^{2+} channels, leads in increase of free Ca^{2+} in the cytoplasm which promotes secretion of neurotransmitters (shown as black dots). (3) In synapse, secreted neurotransmitters interact with their receptors which are often G-protein coupled. G protein subunits can then regulate opening of ligand-gated Na+ channels by interacting directly or indirectly with cAMP or with cAMP activated protein kinases. A part of neuron highlighted inside the black circle shows the resting membrane potential of a neuron which is restored by Na+/K+ pumps, described in the text.

insulin receptors etc.) and (3) G-protein-coupled receptors (e.g. acetylcholine, α and β-adrenergic receptors) (reviewed in: Birnbaumer et al., 1990; Bixby, 1992; Catterall, 1992). The superfamily of growth factor receptors responds to neuron-specific growth or survival trophic factors (Bixby, 1992). Ligand-gated ion channels mediate rapid excitatory and inhibitory synaptic transmission in the nervous system. Binding of ligand to these receptors can regulate directly the rapid opening and closing of Na+ and K+ ion channels, thereby altering the membrane potential of the post-synaptic cell (Catterall, 1992). Alternatively, the G-protein-coupled receptors are used by several hormones and neurotransmitters in order to exert their effect on nerve cells. G protein subunits or G protein-induced cAMP or cAMP-activated protein kinases induce the opening of different ion channels (for review, Birnbaumer et al., 1990).

Receptor-mediated transmembrane signaling is also involved in perceiving sensory signals by the various sensory cells, such as photoreceptor, olfactory and taste sensory neurons (Kinnamon, 1988; Reed, 1992; DeVries & Baylor, 1993). Recent biochemical and molecular studies indicate that sensory signals (light, odor, taste modalities) in vertebrates use predominantly G-protein-mediated signaling pathways (Lagnado & Baylor, 1992; McLaughlin et al., 1992; Reed, 1992). For example, in the

retina, the photoreceptor cells convert light energy into membrane hyperpolarization via a G-protein-mediated signal transduction process. Transducin, a photoreceptor cell-specific G protein (Gt), couples photolyzed rhodopsin with the cyclic GMP phosphodiesterase to initiate this signal transduction pathway. Transducin activates a cGMP phosphodiesterase, which in turn hydrolyses cGMP to GMP. Since light-activated ion channels are opened by cGMP, this transient reduction in cGMP level causes closure of cGMP-gated channels and subsequently hyperpolarize a photoreceptor cell. This hyperpolarization is known to decrease neurotransmitter release at the photoreceptor synapse, which represents the final output of neuronal transmission (reviewed in Lagnado & Baylor, 1992; DeVries & Baylor, 1993). Similarly, the biochemical events involved in signal transduction in olfactory neurons have recently been tested (for review: Reed, 1992). When olfactory receptor cells bearing cilia are exposed to odorant, the odor molecule binds the receptor. The activated receptor then interacts with the olfactory cell-specific G protein (G_{olf}) which in turn activates adenyl cyclase. As a result the cAMP level rises, which then directly opens the cationic channels (Reed, 1992). This causes membrane depolarization and initiation of neuronal action potential which is then transmitted to the brain. Recently, a taste cell-specific G protein, gustducin, has been cloned; it is believed to be

a component of G-protein-mediated signal transduction pathways used to perceive different tastes (McLaughlin et al., 1992). However, the specific signaling mechanism for gustducin has not yet been elucidated.

2. SIGNALING VIA NON-NEURONAL CIRCUITS

While neuronal circuits are used to transmit nerve impulse and sensory signals, non-neuronal circuits of communication bring other types of biological compounds to the target cells, such as, hormones and growth factors. Hormones are produced by various endocrine organs, such as pituitary, adrenal, thyroid glands etc., and instead of neurons, the circulatory system is used for transmission of hormones to their target organs. Growth factors usually act locally and influence neighboring cells. While growth factors promote cell division and cell proliferation, hormones induce growth as well as various physiological changes in the target organs or cells.

It is now known that target cells perceive the hormonal and growth factor signals via specific receptors which fall into two definable categories: growth factor receptors and G-protein-coupled receptors. The general scheme of the molecular mechanism of signal transduction via these two categories of receptor is shown in Fig. 2.

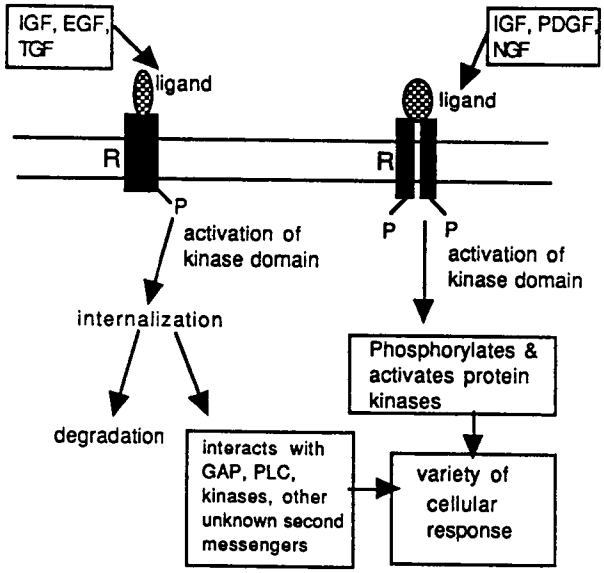
A growth factor receptor has a kinase domain in the intracellular carboxyl-terminal region. This domain

Fig. 2. Schematic representation of growth factor receptor-mediated and G-protein-mediated signal transduction.

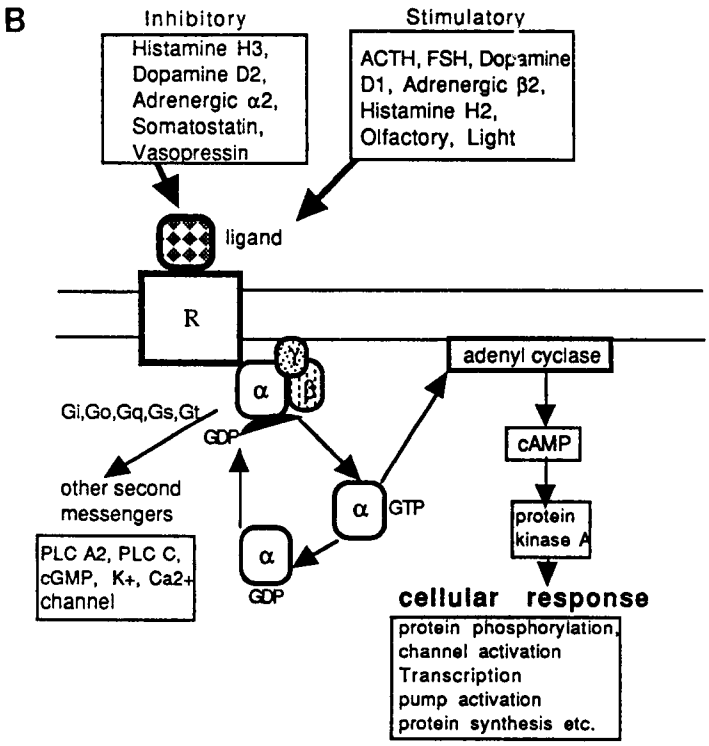
A. Schematic representation of growth factor receptor-mediated signal transduction. Two types of generalized receptor actions are shown here. In one case, ligand-bound receptor is internalized into the cytoplasm by endocytosis. Phosphorylation of tyrosine by ATP activates the receptor kinase domain which can directly interact with several cytoplasmic protein kinases, GAP, PLC etc., and in turn, activates these second messengers. In the other case, ligand interaction can dimerize receptor subunits thereby activating its RTK domain. The activated RTK interacts directly with other second messengers (such as protein kinases) without being internalized into the cytoplasm. Activated second messengers then generate several cellular activities. Schematic representation of G protein-mediated signal transduction.

B. Schematic representation of G-protein-mediated signal transduction. Various receptors are linked to various second messengers (effectors) via G proteins. Receptor-ligand interaction allows GTP to replace GDP on the α subunit of a G protein. The activated α-GTP dissociates from $\beta\gamma$ dimer and interacts directly with effectors (adenyl cyclase, shown as one example). The intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP, and α-GDP recombines with $\beta\gamma$, ending the activation cycle. Activated second messenger, on the other hand, can generate several cellular responses directly, or via other components of the cascade.

A


IGF, EGF, TGF
ligand
IGF, PDGF, NGF
ligand
R
R
P
P
P
activation of kinase domain
activation of kinase domain
internalization
Phosphorylates & activates protein kinases
degradation
interacts with GAP, PLC, kinases, other unknown second messengers
variety of cellular response

B


Inhibitory
Histamine H3, Dopamine D2, Adrenergic α2, Somatostatin, Vasopressin
Stimulatory
ACTH, FSH, Dopamine D1, Adrenergic β2, Histamine H2, Olfactory, Light
ligand
R
adenyl cyclase
α
β
Gi,Go,Gq,Gs,Gt
GDP
cAMP
other second messengers
α
GTP
protein kinase A
PLC A2, PLC C, cGMP, K+, Ca2+ channel
α
GDP
cellular response
protein phosphorylation, channel activation Transcription pump activation protein synthesis etc.

phosphorylates a tyrosine residue following ligand-receptor interaction (for review: Yarden & Ullrich, 1988). A large family of non-receptor type protein-kinases, e.g., serine, threonine, and tyrosine kinases, have also been shown to play important roles in cell signaling pathways (reviewed in Hanks et al., 1988). The receptor tyrosine kinase activity (RTK) of growth factor receptors results in activation of several second messengers, including non-receptor protein kinases, to modulate different biochemical and biological events within the cell (Yarden & Ulrich, 1988). Transcriptional activation, DNA synthesis and cell division are the three major responses of RTK signaling (for review: Pawson & Berstein, 1990).

The second category of receptors does not possess receptor tyrosine kinase activity and are coupled to the G proteins. These receptors include rhodopsin, β-adrenergic, α2-adrenergic, muscarinic cholinergic receptors etc. These receptors bind to a variety of unrelated hormones (adrenaline, acetylcholine, glucagon and ACTH), and neurotransmitters. Some of them even interact with light and odorant molecules (Gilman, 1987; Birnbaumer et al., 1990; Simon et al., 1991). The polypeptide hormones, such as adrenaline, ACTH, epinephrin etc., bind their respective receptors and regulate the production of cyclic AMP which is considered to be the most important intracellular second messenger in the hormonal signaling pathways. The cAMP

influences various cellular activities, such as metabolism, growth and differentiation (Birnbaumer et al., 1990). It is interesting to find that not only hormone signaling pathways but also different neuronal and sensory receptors such as acetylcholine receptor, rhodopsin, odorant receptor and taste cell receptor utilize the G-protein-coupled signal transduction pathway to activate various effectors, such as adenyl cyclase, phosphodiesterase, ion channels.

In summary, neuronal circuits are basically involved in the propagation of sensory stimuli from the receiving sensory organs, such as eye, ear, tongue, etc. to the brain and from the brain to many other non-neuronal target cells. Non-neuronal circuitry, on the other hand, utilizes the circulatory system and extracellular fluid to carry hormones and growth factors to specific target organs or cells. Although hormonal and neuronal circuitry subserve different functions, many of the molecules that are used to carry information between cells are common in the nervous and endocrine systems. Transmembrane signaling of hormones and neurotransmitters via receptors are often coupled to G-protein-mediated signal transduction pathways. The salient features of G proteins are briefly described in the next section.

3. G PROTEINS AND THE MECHANISM OF THEIR ACTION

G proteins are a family of guanine-nucleotide-binding

proteins that function as intermediaries in transmembrane signaling pathways. They are composed of α (39-52 kD), β (35-36 kD), and γ (8-10 kD) subunits (Gilman, 1987; Neer & Clapham, 1988; Birnbaumer et al., 1990). The α subunits contain a GTP-binding site, an intrinsic GTPase activity, and regions that interact specifically with receptor and effector molecules (for review: Gilman, 1987; Simon et al., 1991). When a ligand binds its cell-surface receptor, the activated receptor interacts with the α subunit of the G protein. As a result, the α subunit exchanges bound GDP for GTP, dissociates from the $\beta\gamma$ complex, and interacts with various intracellular effectors, such as adenyl cyclase, phospholipase A2, ion channels, and phosphodiesterases. Following this interaction, the intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP, and inactivates the α subunit. The GDP-α subunit then reassociates with the $\beta\gamma$ complex to form the heterotrimeric configuration. These events are represented diagrammatically in Fig. 2 B.

Besides its role in efficient membrane attachment of the G protein, the $\beta\gamma$ dimer is thought to activate the α subunit (reviewed in Birnbaumer, 1992). Recently it has been shown that the $\beta\gamma$ complex can also modulate effector functions, including adenylylcyclase type II and IV (Tang & Gilman, 1991; Federman et al., 1992) phospholipase A2 (Kim et al., 1989) and plasma membrane Ca+ pump (Lotersztajn et al., 1992). However, direct interaction of the $\beta\gamma$ complex

with the effectors has not been demonstrated yet, and the mechanism of its action is still not clear.

4. DIVERSITY OF G PROTEINS

Historically, G proteins have been named on the basis of the function or suggested function of their α subunits. Rodwell and his colleagues (1971), first demonstrated a GTP requirement for hormonal stimulation in liver and hypothesized that the site of action of GTP might be a protein distinct from the hormone receptor and the effector, adenyl cyclase. After this GTP binding protein was purified from liver, it was named Gs, adenylyl cyclase stimulatory G protein (Sternweis et al., 1981). Later, it was observed that certain hormones inhibit adenyl cyclase and act via another GTP binding protein. This second G protein was purified and named as Gi or adenylyl cyclase inhibitory G protein (Bokoch et al., 1983). Subsequently, a different G protein was purified from brain and named as Go for G "other" protein (Sternweis & Robishaw, 1984). The Go is not related to the adenyl cyclase system, suggesting that G proteins may regulate effectors other than adenyl cyclase. Further support of this view came from the purification of fourth G protein from retina which is required for photo activation of cGMP phosphodiesterase (Fung et al., 1981). Subsequently, molecular cloning has provided information on the primary structure of several other forms of α subunits.

The diversity amongst the α subunits is enormous and to date at least twenty different forms of α-subunits have been identified. Many of these α subunits show high degree of amino acid sequence similarity with Gs, Gi or Go proteins, however, the specific functions of most of these α subunits are not known precisely (for review: Simon et al., 1991; Birnbaumer, 1992).

In mammals, at least twenty different α subunits, four different β-subunits and six different γ-subunits have been identified so far. It has been speculated that different combinatorial associations of the three subunits will generate diverse types of G proteins which may differ functionally (Simon et al, 1991). Different forms of α and β subunits showed a high level of amino acid sequence identities which ranges from 80-90%, while different forms of γ subunits are widely divergent and share only 27-75% identity at the amino acid level. This greater diversity in γ subunits has been speculated to provide for the uniqueness of G protein function (Birnbaumer, 1992; Cali et al., 1992).

Signals required for developmental processes, such as cell migration, differentiation, and spatial organization, are believed to be mediated by signal transduction pathways involving G proteins. Sensory signals (light, odor, taste) also use G-protein-mediated signaling pathways (Birnbaumer, 1992). Different lines of evidence support the notion that G-protein-coupled signal transduction also plays a key role

in the development of the nervous system in mammals (Lankford et al., 1987; McCobb et al., 1988; Strittmatter et al., 1990). Since several forms of α and β subunits are expressed in the central nervous system, in other tissues, and during developmental stages of *Drosophila*, G protein-mediated signal transduction pathways seem to be involved in a variety of cellular and developmental processes.

5. CELL SIGNALING IN *DROSOPHILA* NEURONAL DEVELOPMENT

The adult central nervous system of *Drosophila* arises from neuronal precursors (neuroblasts) residing in a specialized ectodermal territory of early embryos called the neurogenic region (for review: Campos-Ortega, 1990; Simpson, 1990). Within this single-layered epithelium, a dispersed subset of about 25% of the cells become neuroblasts, while most other cells left at the surface become epidermis. Genetic evidence has suggested that the cellular signals for the segregation process of neuroblasts involves a lateral inhibition mechanism, in which the developing neuroblasts induce surrounding cells to become epidermis rather than entering a default neuroblast pathway (for review: Simpson, 1990). Inductive processes similar to those described in vertebrates have been ruled out in case of *Drosophila* neuronal development (Leptin, 1991).

Genetic and molecular evidence suggests that neurogenic genes, such as *Notch* (*N*), *Delta* (*Dl*) *Enhancer of split*

complex [E(spl)-C], *neuralized (neu)*, *big brain (bib)* etc., are expressed in developing neuroblasts and that their products determine the developmental fate of the neuroblasts (reviewed in Woods & Bryant, 1992). In embryos lacking function of any of these neurogenic genes, more cells are recruited as neuroblasts at the expense of epidermal cells (for review: Campos-Ortega 1990). Although the exact mechanism of action of the neurogenic gene products is not known, a few neurogenic genes have been shown to encode membrane-bound proteins. For example, the *Notch* gene encodes a protein with a few domains similar to epidermal growth factor receptor (Wharton et al., 1985). *Delta* encodes a transmembrane protein with multiple EGF receptor domains in the extracellular part (Vassin et al., 1987). The *E(spl)* gene encodes a product has showed amino acid sequence similarity with the β-subunit of G protein (Hartley et al., 1988). Another neurogenic gene, *shaggy*, encodes a potential kinase (Bourouis et al., 1990). These proteins are candidates for involvement in the signal transduction processes during neurogenesis. It has been proposed that the *Notch* gene product acts as a receptor for a lateral inhibition mechanism. Upon activation by its ligand, *Notch* transduces the inhibitory signal. Once the signal is received by the ectodermal cell, the cell will follow the default pathway to become a neuroblast (for review: Xu et al., 1990). The transduction pathway of the inhibitory

signal is unknown. However, other neurogenic genes encoding membrane-bound products may also function within this signal transduction process of neuroblast segregation.

Besides an important role in neurogenesis, the *Notch* gene is also involved in other developmental functions. For example, *Notch* function is necessary for photoreceptor neurons as well as for a number of non-neuronal cells (Cagan & Ready, 1989). Both *Notch* and *Delta* are involved in the development of the ovarian follicle cells of adult females (Ruohola et al., 1991). These observations suggest that genes required for neuronal development may also function in other cell signaling pathways.

The compound eye of *Drosophila* is also ideal for studying how the developmental fate of a cell is determined by its neighboring cells. The compound eye is composed of approximately 800 repeating units called ommatidia. Each ommatidium contains eight photoreceptor cells and twelve accessory cells. In the *sevenless* (*sev*) null mutation, the ommatidia lack the seventh photoreceptor cell (R7) (for review: Banerjee & Zipursky, 1990). Developmental analysis has shown that in *sev* mutants the precursor of the R7 cell develops as a nonneuronal lens-secreting cone cell instead of a photoreceptor cell (Tomlinson & Ready, 1987). Molecular analysis demonstrated that sev protein has extracellular transmembrane and cytoplasmic domains. Interestingly, the cytoplasmic domain has tyrosine kinase

activity (Simon et al., 1989). It has been proposed that the sev protein acts as a receptor to trigger R7 photoreceptor cell differentiation. Molecular and genetic studies also revealed that the ligand for the sev protein is located on the membrane of the adjacent R8 cell. This ligand is also a transmembrane protein and is the product of *boss* gene. The boss protein in the R8 cell triggers sev receptor activity (Reinke & Zipursky, 1988; Kramer et al., 1991). This cell signaling process is essential for the presumptive R7 cell to become a photoreceptor cell.

6. OBJECTIVES OF THE PROPOSED RESEARCH

Thus a large number of genetic loci in *Drosophila* have been identified as playing important roles in the normal function or development of the nervous system (reviewed in Hall, 1985). Studies directed toward identification of these genes may contribute toward better understanding of cell signaling processes in the nervous system. Therefore, the objective of this investigation was to identify neurobiologically important genes in *Drosophila* by a reverse genetic approach.

Since the head of *Drosophila* is rich in neural tissues, studies of genes expressed exclusively or predominantly in the head might improve the possibility of identifying a gene whose product functions in neural tissue. Therefore, twenty genomic clones isolated by Levy et al., (1982) were

examined. These twenty clones contain genes of *Drosophila melanogaster* which are predominantly expressed in the head, rather than in the body of the fly (Levy et al., 1982). Since the major portion of the head is occupied by eye and brain, it was expected that genes present in these clones may code for eye- and/or brain-specific proteins. Indeed, genes in four of these twenty clones, showing expression in the head of the fly but virtually no expression during embryogenesis (Levy & Manning, 1982), were found to encode eye-specific proteins (Montell et al., 1985; Zipursky et al., 1985; Fryxell & Meyerowitz, 1987; Montell & Rubin, 1988; Krishnan & Ganguly, 1990; Yamada et al., 1990; Krishnan et al., 1991). It was assumed that the genes present in the second class of clones, those that showed peak expression during embryogenesis as well as in the adult fly head, might have neural cell-specific function, since genes with similar functions are known to be highly active during embryogenesis (Campos-Ortega, 1990). Indeed, clone 549, belonging to the second class of clones, was found to encode a product similar to the mammalian 14-3-3, an activator protein of tyrosine and tryptophan hydroxylases, the two key enzymes regulating neurotransmitter biosynthesis (Swanson & Ganguly, 1992). In view of this, clone 536, belonging to the second class of clones was chosen for the present investigation with the following specific objectives:

(1) Identification and analysis of the gene(s) present in clone 536, which is predominantly expressed in the nervous system of *Drosophila melanogaster*. DNA sequencing analysis was planned to determine the nature of the encoded product of these genes.

(2) Determination of the expression pattern of the gene(s) isolated above in different tissues during development. This objective was undertaken to determine whether the isolated gene is expressed predominantly or specifically in the neural tissues of adult fly and different developmental stages.

(3) Examination of the structure and organization of the isolated gene and prediction of the probable mechanisms that may regulate the expression of the gene isolated in objective 1.

CHAPTER 2

DETERMINATION OF THE POLYPEPTIDE ENCODED BY THE HEAD-SPECIFIC TRANSCRIPTION UNIT WITHIN CLONE 536 0F *DROSOPHILA MELANOGASTER*

1. INTRODUCTION

The main objective of the present investigation was to study the structure and organization of a gene which is predominantly or exclusively expressed in the nervous system of *Drosophila melanogaster*. For this purpose a head-specific clone, designated 536, was chosen. The rationale for choosing this clone has been detailed in Chapter 1.

Clone 536 was originally isolated by Levy et al. (1982) from a genomic library of *D. melanogaster* made in λ-Charon 4 phage (Maniatis et al., 1978). It was reported that the average size of inserts in the clones in this library is approximately 15-kb (Maniatis et al., 1978). Therefore, genomic clone 536 was expected to contain approximately 15-kb of insert DNA. Although it is quite possible that a 15-kb genomic DNA fragment of *Drosophila* may contain only one gene, the results of several molecular analyses make this assumption doubtful. First, the size of the *D. melanogaster* haploid genome is 1.3×10^8 bp (Fristrom and Yund, 1973) and

70% of this DNA belongs to single copy class (Manning et al., 1975). Second, RNA complexity analysis has shown that about 16,000-17,000 genes are expressed throughout *D. melanogaster* development (Zimmerman et al., 1980; Levy and Manning, 1981). Interestingly, 70% of these genes are expressed in the tissues of adult head (Levy and Manning, 1981). By using these data on the size of the genome and the number of genes expressed in the fly, it can be estimated that a 15-kb genomic DNA of *D. melanogaster* may contain more than two genes. In fact there are many examples in the literature which support this notion (Lifton et al., 1978; Ait-Ahmed et al., 1987; Kuhn et al., 1991). Therefore, clone 536 may also contain more than one gene which might be expressed in the head of the fly. In view of this possibility, initially two basic objectives were planned: the first was to determine the number and location of transcription unit(s) of clone 536 which were expressed exclusively or predominantly in the fly head; and second objective was to determine the organization of the transcription unit expressed in the head and its protein product. The results described in this chapter demonstrate that a transcription unit present in clone 536 expressed predominantly in the head encodes a protein showing significant sequence similarity with several forms of γ-subunits of the mammalian G proteins. A major portion of the results in this chapter has been published (Ray and

Ganguly, 1992).

2. MATERIAL AND METHODS

The details of the methods used in each chapter are given in Appendix I.

2.1. *Drosophila* strains and cultures

The wild type *D. melanogaster* strain (Canton S) was obtained from Dr. J.C. Lucchesi, Emory University, Atlanta. Flies were raised on cornmeal-agar-molasses medium at 23^0 C and 60% humidity with a 12 hour dark/light cycle.

2.2. Nucleic acid isolation, radiolabeling and blot hybridization

Fly genomic DNA and RNA were isolated according to Levy and Manning (1982) and Jowett (1986), respectively. Plasmid DNA was isolated according to standard procedure (Maniatis et al., 1982), and bacteriophage lambda DNA was isolated as described by Helms et al. (1982). DNA fragments to be used as probes were labeled with radioactive deoxynucleotides by nick translation (Maniatis et al., 1982), or by random priming (Cobianchi and Wilson, 1987). For Southern-blot hybridization, restriction fragments were blotted onto HyBond nylon membrane (Amersham Corp.), baked, and hybridized with ^{32}P-labeled probes with specific activities ranging from 2x 10^7 to 1x 10^8 cpm/μg.

2.3. Isolation of cDNA clones complementary to the head-specific transcription unit in genomic clone 536

A *D. melanogaster* (Oregon R^+ strain) head cDNA library made against head poly(A)$^+$ RNA in λ-SWAJ2 vector, was a gift from Seymour Benzer, CALTECH. In this library, the cDNA fragments were directionally cloned as *Eco*RI-*Xba*I inserts, with *Eco*RI at the 5′- and *Xba*I at the 3′-end of the mRNAs (Palazzolo & Meyerowitz, 1987). This head cDNA library was screened by *in situ* plaque hybridization (Benton and Davis, 1977) using the head-specifically transcribed genomic DNA fragment of clone 536 as a probe. The clones hybridizing with the probe were purified and the cDNA inserts present in these clones were isolated by digesting with *Eco*RI and *Xba*I. Two of the longest cDNA inserts (536-B2 and 536-D2) were subcloned into the pBluescript (pBSK+) plasmid vector.

2.4. Determination of direction of transcription

To determine the direction of transcription relative to the restriction map, first, the genomic DNA fragment found to contain the transcription unit subcloned into the pBSK+ plasmid vector. Single-stranded ^{32}P-labeled RNA, complementary to the sense and antisense strands of the DNA were individually generated by using the T3 and T7 promoters present in the vector using the respective RNA polymerases. The labeled RNA (riboprobe) was then hybridized to Northern blots containing head and body poly(A)$^+$ RNA.

2.5. *In situ* hybridization to polytene chromosomes

In situ hybridization to polytene chromosomes was performed according to Levy et al. (1982). DNA fragments to be hybridized were labeled with ^{3}H-CTP by nick-translation. The site of hybridization on the polytene chromosomes was determined by autoradiography followed by Geimsa staining.

2.6. Deletion, DNA sequencing and sequence analysis

The cDNA and genomic DNA fragments to be sequenced were subcloned in pBSK+ plasmid and overlapping deletions of these DNA fragments were generated by using *Exo*III/*S*1 nuclease deletion system (Promega). The deleted DNA fragments were then self-ligated, used to transform bacteria and sequenced by the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase version-II kit (U.S. Biochemical Corp.). Wherever needed, specific oligonucleotide primers were used for sequencing. Nucleotide and amino acid sequence analyses were performed by using the tFASTA, GAP, MAP and PILEUP programs available with the University of Wisconsin Genetics Computer Group (UWGCG) software package.

3. RESULTS

3.1. Clone 536 has only one head-specific transcription unit

By hybridizing immobilized DNA of clone 536 with ^{32}P-labeled

head and body poly(A)$^+$ RNA of adult flies, Levy et al. (1982) showed that the RNA transcribed by the gene(s) present in this clone are at least 10-fold more abundant in the head than in the body. This experiment, however, did not reveal the number and location of different genes that are expressed exclusively or predominantly in the head or their levels of expression in the head and body of the fly. As a first step toward resolving these issues, a detailed restriction map of clone 536-1 i.e. clone 536 originally isolated by Levy et al. (1982), was made (Fig. 3). The clone was then digested with *Eco*RI, Southern blotted onto Nylon membrane and hybridized with ^{32}P-labeled single stranded cDNA probes made against head poly(A)$^+$ RNA of adult flies. Hybridization of the cDNA probe was seen only with the 1.5- kb and 2.0-kb *Eco*RI fragments, located next to each other in the genome (Fig. 3, fragments A and B, respectively). These results indicated that the 1.5- and 2.0-kb *Eco*RI DNA fragments contain the transcribed region(s) of a gene(s), which is expressed abundantly in the fly head.

The above experiment, however, did not resolve whether the transcription units present in these *Eco*RI fragments (Fig. 3, fragment A and B) are expressed more abundantly in the head than in the body or exclusively in the head of the fly. Another question which could not be resolved with this experimental strategy was the number of transcription units present in these two *Eco*RI fragments. It is possible that

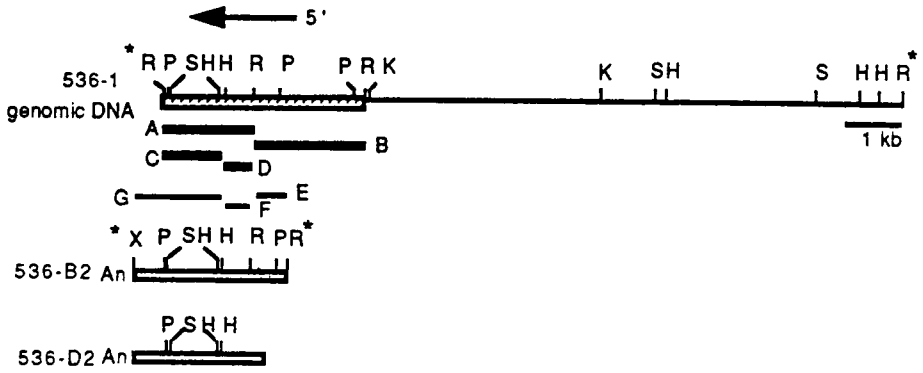


Fig. 3. Restriction maps of 536 genomic and 2.6- kb RNA-specific cDNA clones. Genomic clone 536-1 was isolated by Levy et al. (1982). The *hatched bar* in the genomic clone indicates the transcribed regions, the boundaries of which have not yet been determined. The *open bars* represent different cDNA clones isolated by using the transcribed DNA fragments as probes. The direction of transcription was shown by an *arrow*. Fragments A-D are from clone 536-1, and fragments E-F are from 536-B2 cDNA clone. The E-F fragments correspond to the respective restriction fragments shown in the map of 536-1. Restriction sites with *asterisks* are the cloning sites in λ-Charon 4 vector. R, *Eco*RI; H, *Hind*III; S, *Sst*I; P, *Pst*I; K, *Kpn*I.

these two DNA fragments contain more than one transcription unit, one of which is expressed only in the adult head. To find answers to these questions, the 1.5- and 2.0-kb *Eco*RI fragments were individually cloned into the pBSK+ plasmid, so that these fragments and their sub-fragments could be isolated in pure form and used as probes for the experimental strategy described in the following section.

3.1.1. Further characterization of the 1.5- and 2.0-kb EcoRI fragments

To resolve the questions raised in the previous section, the 2.0-kb *Eco*RI fragment (Fragment B) and sub-fragments of the 1.5-kb *Eco*RI fragment (Fig. 3, fragments C, D) were isolated. Each of these fragments was then individually used as probes on Northern blots of head and body poly(A)$^+$ RNA (Fig. 4). In order to detect the hybridization signals with the body RNA, five times more body RNA than head RNA was used. While fragment C hybridized only with a 2.6-kb RNA (Fig. 4, panel a), both fragments B and D hybridized with RNAs of three different lengths: 2.6-, 1.3- and 1.1-kb (Fig. 4, panels b, c). The results also showed that the 2.6-kb RNA is head-specific, the 1.3-kb RNA is body-specific, and the 1.1-kb RNA is expressed both in the head and body, but its level is much greater in the head of the fly.

It is not clear from the above results whether all

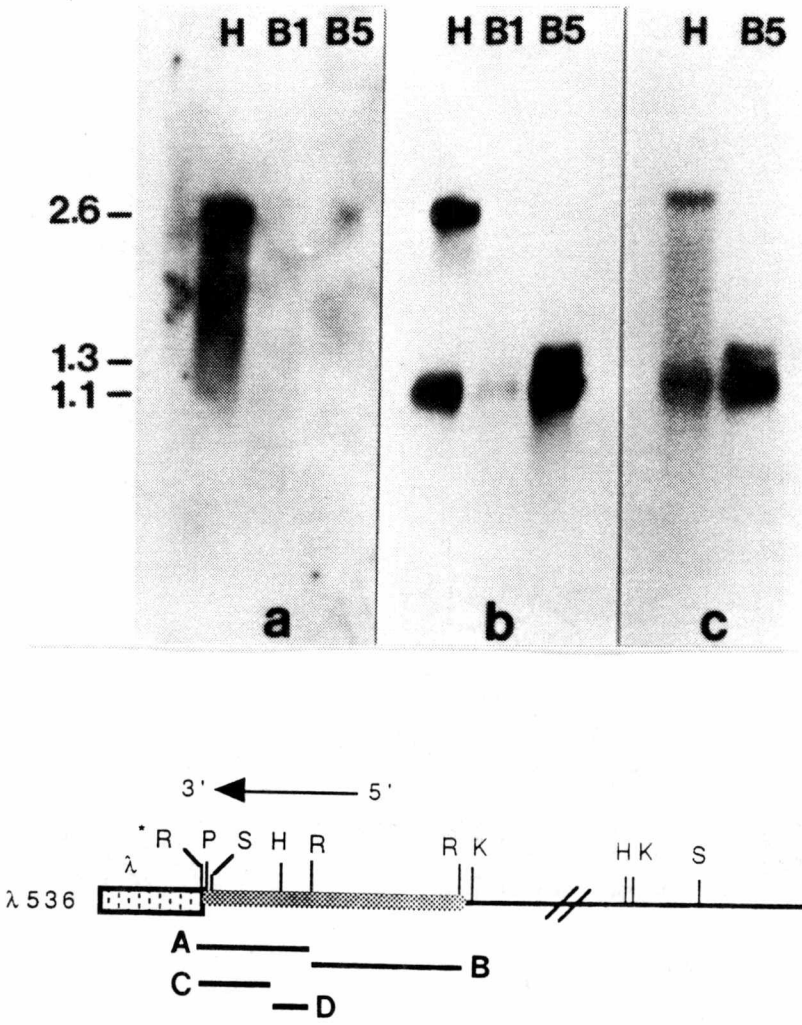


Fig. 4. Hybridization of head and body poly(A)$^+$ RNA with different regions of the transcribed genomic DNA fragments of clone 536-1 shown in the map below. All three DNA fragments used as probes were labeled with ^{32}P by the nick-translation procedure. The blot shown in *panel b* was reused after removal of the probe from *panel a*. DNA fragments used as probes were: *panel a*, fragment C; *panel b*, fragment D; *panel c*, fragment B. Lanes H, 1 μg of head RNA; Lanes B1, 1 μg of body RNA; lanes B5, 5 μg of body RNA.

three RNA species originate from a 3.5-kb genomic DNA (1.5-kb plus 2.0-kb *Eco*RI DNA fragments) within clone 536 (Fig. 3). It is possible that the 3.5-kb genomic DNA contains two transcription units fully but the other transcription unit partly. If this were the case, then why does the 0.5- kb *Eco*RI fragment (Fig. 3, fragment D) hybridize with all three RNA species? Perhaps these transcription units overlap within this DNA fragment. Alternatively it is possible that two RNAs are transcribed from one DNA strand and the third one is made from the other, as it is found in the case of nested genes in *Drosophila* (Henikoff & Eghtedarzadeh, 1987). To test these alternative hypotheses, ^{32}P-labeled RNA (riboprobes) were synthesized from opposite strands of the 0.5-kb *Eco*RI (fragment D, Fig. 3) fragment. Northern blots containing head and body Poly(A)$^+$ RNA were then individually hybridized with these single-stranded riboprobes. The result (Fig. 5) shows that the riboprobe made from only one DNA strand hybridizes with all three RNAs. Thus, these RNAs are transcribed from the same DNA strand of the 0.5-kb *Eco*RI fragment and it is not a case of nested genes. These results also determined the direction of transcription in relation to the restriction map and it is shown in Fig. 3.

The above results lead to the conclusion that all three RNAs are products of a single gene. In order to have a clear idea about how three RNAs are produced from a single

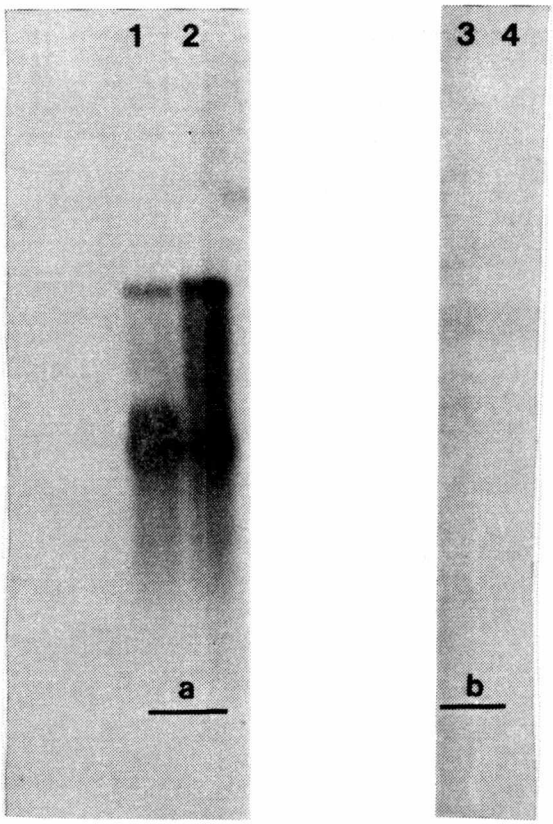


Fig. 5. Determination of the direction of transcription of three mRNA species. The 0.5-kb EcoRI-HindIII DNA (fragment D, Fig. 3) was first cloned in pBSK+ plasmid, and strand-specific riboprobes were synthesized using the T3 and T7 promoters present in the vector. These riboprobes were individually hybridized with Northern blots shown in panel a and b. *panel* a, blot hybridized with the riboprobe generated from T3 promoter; *panel b*, blot hybridized with the riboprobe generated from T7 promoter. *Lane* 1 and 3, 5 μg of body Poly(A)$^+$ RNA; *lane* 2 and 4, 1 μg of head Poly(A)$^+$ RNA. On the basis of this experiment the direction of transcription of 2.6-, 1.1- and 1.3- kb RNA species was determined and indicated by an *arrow* in Fig. 3. This experiment also showed that all three RNAs are transcribed from the same strand of DNA.

gene, it became necessary to isolate the cDNA clones for each of these three RNAs and use them to analyze the genomic organization of this transcription unit. As a first step, cDNA clones specific for the longest mRNA (2.6-kb) were isolated because this RNA is expressed only in the head. Therefore, characterization of this cDNA may reveal whether the polypeptide encoded by this RNA has any similarity with proteins known to be involved in neural function in other organisms.

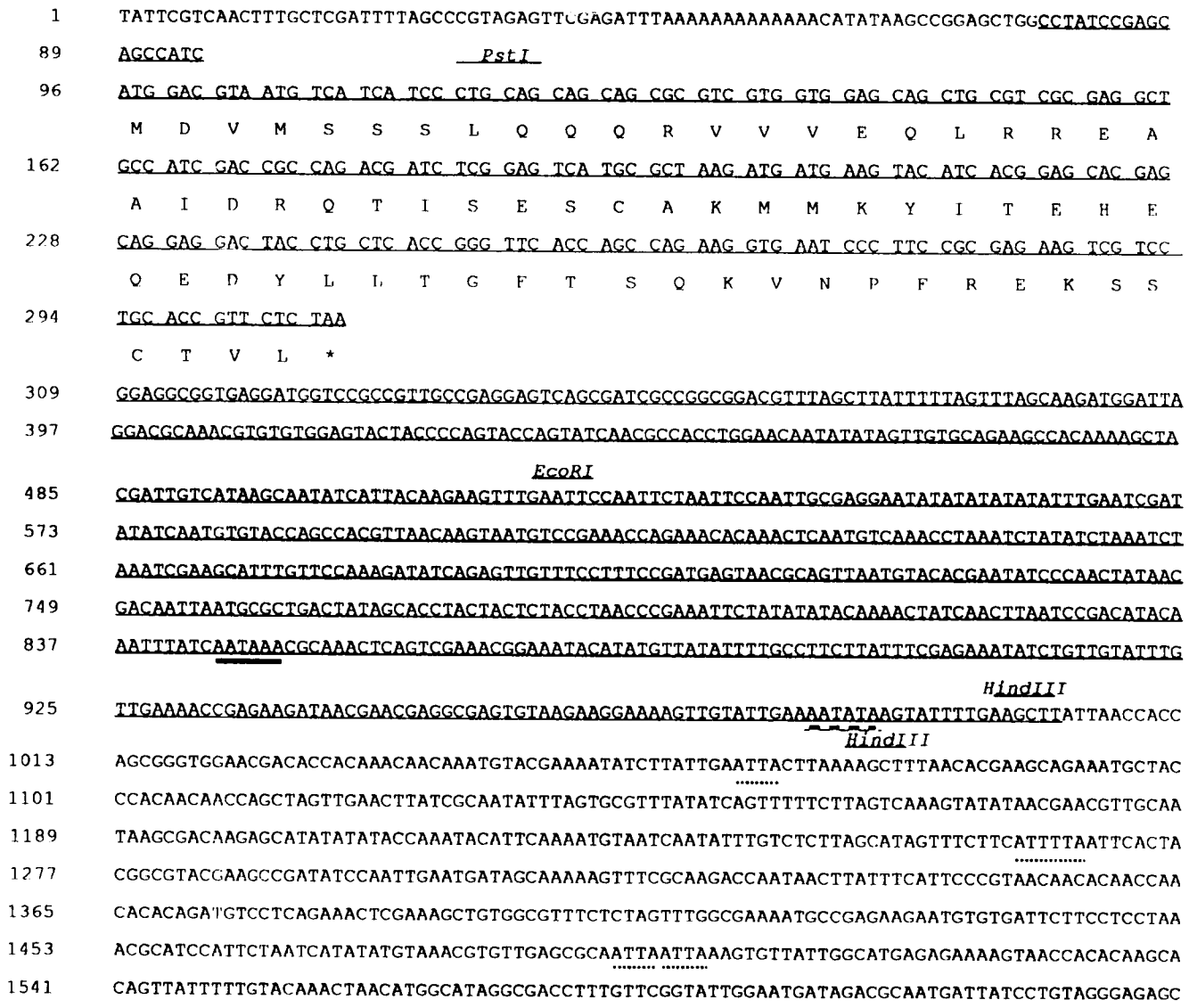
3.2. Nucleotide sequence analysis of the cDNA for the head-specific 2.6-kb mRNA

The cDNA clones specific for the 2.6-kb mRNA were isolated by screening a *Drosophila* head cDNA library with fragment C (fig. 3) which has been shown to hybridize only with the 2.6-kb RNA (see Fig. 4, panel a). Screening of about 75,000 plaques yielded several positive clones. The extent of homology of these cDNAs to the genomic DNA was determined by restriction mapping (Fig. 3) and Southern blot hybridization analysis. The restriction pattern of one of the longest cDNA clones, 536-B2, was found to be identical to the restriction pattern of the transcribed regions within genomic clone 536 (Fig. 3). This suggests that the transcribed region does not contain introns larger than 100 base pair, which could have been detected by agarose gel electrophoresis. Also, the sub-fragments of 536-B2 cDNA,

i.e. fragments G, F, and E (Fig. 3), showed identical patterns of hybridization with respect to the sizes and levels of the head and body RNA, to those observed with the genomic DNA fragments C, D, and B of 536 clone (Fig. 3), respectively (data not shown).

Nucleotide sequence analysis of the 536-B2 cDNA was carried out using the method of Sanger et al. (1977). The results (Fig. 6) showed that this cDNA is 2535 base pairs long and is almost a full length copy of the 2.6-kb RNA. Conceptual translation of the nucleotide sequence in three reading frames was done using the MAP program of the GCG software package. The results showed that in the first reading frame, the longest ORF contains only 46 codons which do not conform to typical *Drosophila* codon usage, making it unlikely that this ORF is used for translation. In the second reading frame, the longest ORF extends to 50 codons with poor context for the *Drosophila* translational initiation sequence (Cavener, 1987). In this same reading frame a 65 codon ORF is found starting at 2 nt without an ATG start codon, which may be present in the missing 5′-upstream sequence. Sequencing of the genomic DNA corresponding to the 5′-upstream region of the cDNA showed that the 65 codon ORF is preceded by a stop codon just 7 codons upstream from the 5′-end of the cDNA. Also no ATG start codon in this open reading frame was found in the upstream genomic sequence.

Fig. 6. Nucleotide sequence of the 2.6-kb mRNA-specific 536-B2 cDNA. The conceptual open reading frame starts at nt 96. Amino acids are denoted by single-letter codes. The AATAAA polyadenylation signals are underscored with *solid lines*. A potential polyadenylation signal (AATATA) is underscored with *broken lines*. A few of the A(T)nA motifs in the 3′-untranslated region are underscored with *dots*. Underlined nucleotides denote that these sequences were also determined in the corresponding genomic DNA (see chapter 4, section 3.2 for details).


1
TATTCGTCAACTTTGCTCGATTTTAGCCCGTAGAGTTCGAGATTTAAAAAAAAAAAAAACATATAAGCCGGAGCTGGCCTATCCGAGC
89
AGCCATC
PstI
96
ATG GAC GTA ATG TCA TCA TCC CTG CAG CAG CAG CGC GTC GTG GTG GAG CAG CTG CGT CGC GAG GCT
M D V M S S S L Q Q Q R V V V E Q L R R E A
162
GCC ATC GAC CGC CAG ACG ATC TCG GAG TCA TGC GCT AAG ATG ATG AAG TAC ATC ACG GAG CAC GAG
A I D R Q T I S E S C A K M M K Y I T E H E
228
CAG GAG GAC TAC CTG CTC ACC GGG TTC ACC AGC CAG AAG GTG AAT CCC TTC CGC GAG AAG TCG TCC
Q E D Y L L T G F T S Q K V N P F R E K S S
294
TGC ACC GTT CTC TAA
C T V L *
309
GGAGGCGGTGAGGATGGTCCGCCGTTGCCGAGGAGTCAGCGATCGCCGGCGGACGTTTAGCTTATTTTTAGTTTAGCAAGATGGATTA
397
GGACGCAAACGTGTGTGGAGTACTACCCCAGTACCAGTATCAACGCCACCTGGAACAATATATAGTTGTGCAGAAGCCACAAAAGCTA
EcoRI
485
CGATTGTCATAAGCAATATCATTACAAGAAGTTTGAATTCCAATTCTAATTCCAATTGCGAGGAATATATATATATATTTGAATCGAT
573
ATATCAATGTGTACCAGCCACGTTAACAAGTAATGTCCGAAACCAGAAACACAAACTCAATGTCAAACCTAAATCTATATCTAAATCT
661
AAATCGAAGCATTTGTTCCAAAGATATCAGAGTTGTTTCCTTTCCGATGAGTAACGCAGTTAATGTACACGAATATCCCAACTATAAC
749
GACAATTAATGCGCTGACTATAGCACCTACTACTCTACCTAACCCGAAATTCTATATATACAAAACTATCAACTTAATCCGACATACA
837
AATTTATCAATAAACGCAAACTCAGTCGAAACGGAAATACATATGTTATATTTTGCCTTCTTATTTCGAGAAATATCTGTTGTATTTG
HindIII
925
TTGAAAACCGAGAAGATAACGAACGAGGCGAGTGTAAGAAGGAAAAGTTGTATTGAAAATATAAGTATTTTGAAGCTTATTAACCACC
HindIII
1013
AGCGGGTGGAACGACACCACAAACAACAAATGTACGAAAATATCTTATTGAATTACTTAAAAGCTTTAACACGAAGCAGAAATGCTAC
1101
CCACAACAACCAGCTAGTTGAACTTATCGCAATATTTAGTGCGTTTATATCAGTTTTTCTTAGTCAAAGTATATAACGAACGTTGCAA
1189
TAAGCGACAAGAGCATATATATACCAAATACATTCAAAATGTAATCAATATTTGTCTCTTAGCATAGTTTCTTCATTTTAATTCACTA
1277
CGGCGTACGAAGCCGATATCCAATTGAATGATAGCAAAAAGTTTCGCAAGACCAATAACTTATTTCATTCCCGTAACAACACAACCAA
1365
CACACAGATGTCCTCAGAAACTCGAAAGCTGTGGCGTTTCTCTAGTTTGGCGAAAATGCCGAGAAGAATGTGTGATTCTTCCTCCTAA
1453
ACGCATCCATTCTAATCATATATGTAAACGTGTTGAGCGCAATTAATTAAAGTGTTATTGGCATGAGAGAAAAGTAACCACACAAGCA
1541
CAGTTATTTTTGTACAAACTAACATGGCATAGGCGACCTTTGTTCGGTATTGGAATGATAGACGCAATGATTATCCTGTAGGGAGAGC


1629 ACGCTATGATATCAGCATACACCCACCAAGTCGTACATACACACATATTGCATCCCGAACTTACGATTCTCGTCCCGCTGAGACGAAC
1717 TGTCCAAGTCGCCAGCTCAGTGTGGATGGGAATGAGGAAGGTATAGTTTATTTGATCGGTTCTTCCAAAATTGCATTTAACAAGAGTT
1805 TTTGAATTGGCGGTGATTTTAGTTCTTAACGTAGCAAAAATTCAGACAACGTTTACTTAGTACCAAGAATCACACGTTTTTGGAGACG
SstI
1893 GACATTTTCTATTCGGAAACCTATATAAAAGGTTTTGAGCTCCAGTTTGCTGTCGTGAGCAAACAGCAAAATGTGGTCCAAAGCCGCC
PstI
1981 ATTGCGAAAGGCTTTCATGGGAGCCGCCCTATCAATCGAAACTGCAGTCGGTTACGGGAGTCCTCCAGTTTCGGCTTAAACATTCCGC
2069 ATTCAACAGTTTTCGATCCAACGGACACTTTGCATCCAAACTTTAGTTATCCTGAATGTTATTAGCCCGAGGACAGATTGAACCACCC
2157 AATGGAGCACGGATGGTTTGGTTTCGTCCTCAGATGTGTCATTTTCTATAACTTATCCATAGAGGGTAAATCGCATTTGGAAAGGAAG
2245 GGCATTAGATGCACACGTACTCAAAATCAATCATGGTCATCGAATACACTTTAACACCTTTCACTTCAAATGAAAAGCGACAACGAGA
2333 TCGTATTTTATTCGAAAATTAATACAACTCTTTTGCGATGCAAACGGGGACGAGTCCTCACAAATATTATTGAAATGTATCTGCGAAT
2421 AAGCATTTGAAATATTGTACTCAACAAAATGAATAATCGTTTGCTTGAATTTCAAAAATAAAAAGCGATAACTTACTTAGATTCGTTA
2509 TCTAGCGGTGGCACCGGGTTCGGTGCGA20

When the sequence was translated using the third reading frame, two long ORFs were found. One of these with 85 codons is in poor context with *Drosophila* translational initiation sequence (Cavener, 1987) and does not conform to typical *Drosophila* codon usage (Ashburner, 1990) (Table 1). However, the second ORF in the 3rd reading frame, which is 70 codons long and starts with an ATG at 96 nt (Fig. 6), seems more likely to encode a protein in *Drosophila*. For three reasons it is believed that this ORF is meaningful. First, the ATG start codon of this ORF is preceded by a tetranucleotide sequence, 5'-CATC-3', which shows a close match with the consensus for *Drosophila* (C/A)AA(A/C) and vertebrate (CANC) translation initiation (Cavener, 1987). Second, the sequence upstream of the first ATG has stop codons in all three open reading frames. Third, the codon usage within the predicted open reading frame shows a bias to the *Drosophila* codon usage frequency (Table 1). Lastly, when the conceptually translated polypeptide was compared with other polypeptides available in the protein data bases (EMBL, GenBank, etc), this ORF indeed became biologically meaningful. The data base search revealed that this polypeptide is 46, 43, and 28% identical (and 59, 52, and 60% similar) to the γ2, γ3 and γt subunits of bovine G proteins, respectively (Fig. 7).

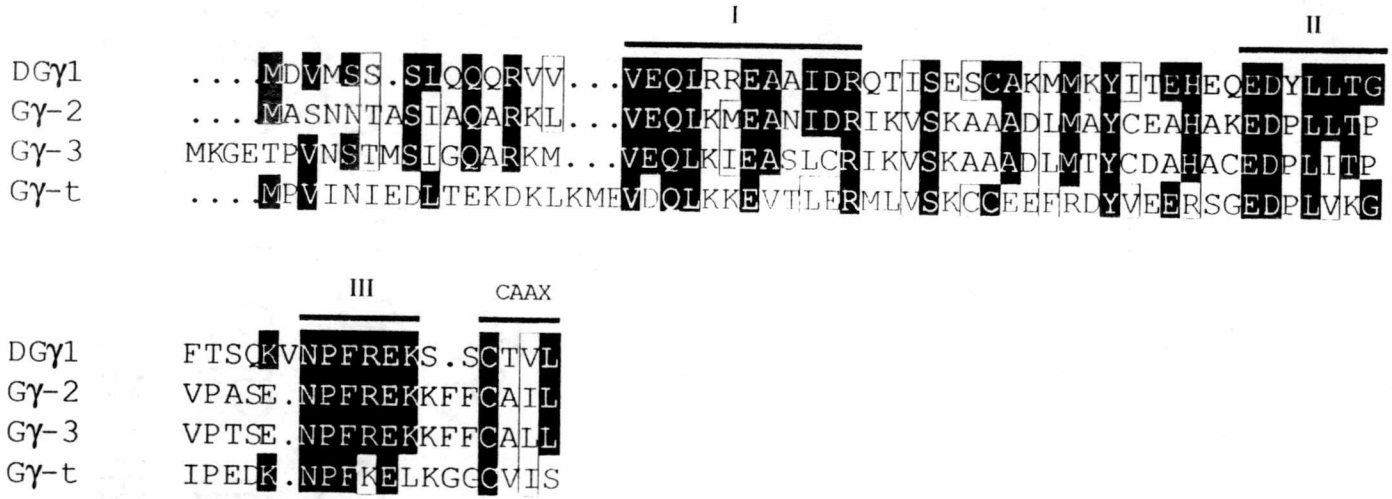
The above findings led to a more detailed comparison between the predicted *Drosophila* protein and bovine G

Table 1. Comparison of codon usage bias

amino acid	codon	pref	70 aa	80 aa
Glu	GAG	74%	7	1
Glu	GAA	26%	0	7
Val	GTG	48%	3	0
Val	GTA	7%	1	1
Val	GTT	17%	1	2
Val	GTC	27%	1	1
Lys	AAG	78%	4	3
Lys	AAA	22%	0	2
Ile	ATA	12%	0	6
Ile	ATT	31%	0	1
Ile	ATC	51%	3	3
Thr	ACG	24%	2	3
Thr	ACA	15%	0	2
Thr	ACT	15%	0	5
Thr	ACC	47%	3	0
Tyr	TAT	23%	0	3
Tyr	TAC	77%	2	3
Arg	CGG	13%	0	0
Arg	CGA	14%	0	4
Arg	CGT	19%	1	0
Arg	CGC	35%	4	0
Leu	CTG	47%	3	1
Leu	CTA	7%	0	1
Leu	CTT	8%	0	1
Leu	CTC	15%	2	0

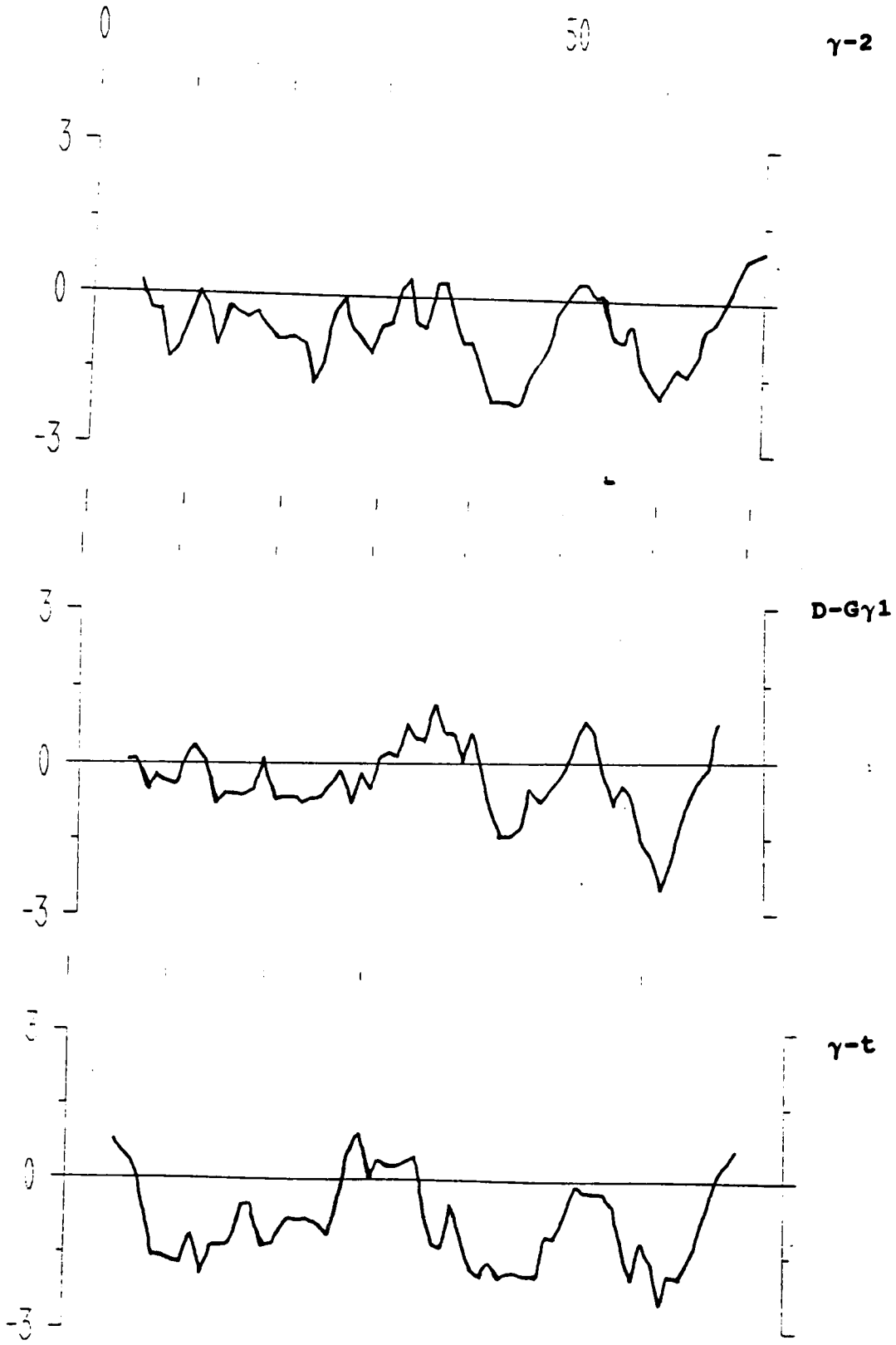
Codon Usage Bias between 70 aa ORF and 85 aa ORF in 3rd reading frame are shown in this table. Drosophila codon usage frequency (Ashburner, 1990) of eight different amino acids are shown here. Number of times each of these codons are used by 70aa and 85aa ORFs are shown in column 4 and 5 respectively. The data shows that the 70aa ORF shows more codon bias towards Drosophila codon usage frequency.

Fig. 7. Comparison of the deduced amino acid sequence of the D-Gγ1 protein with the published amino acid sequence of bovine γ-subunits of G proteins: γ2 (Robishaw et al, 1989; Gautam et al, 1990), γ3 (Gautam et al, 1990), and retinal γt (Hurley et al, 1984). The *dark* and *open boxes* represent identical and similar amino acids between *D-Gγ1* and any one bovine γ-subunit. Regions *I*, *II*, and *III* showing greater similarities are *overlined*. A *CAAX* box present in the c-terminal end of all γ subunits is the potential site of post-translational modification by isoprenylation. The PILEUP program of the UWGCG software package was used for sequence alignment.


I
II
DGγ1 ...MDVMSS.SLQQQRVV...VEQLRREAAIDRQTISESCAKMMKYITEHEQEDYLLTG
Gγ-2 ...MASNNTASIAQARKL...VEQLKMEANIDRIKVSKAAADIMAYCEAHAKEDPLLTP
Gγ-3 MKGETPVNSTMSIGQARKM...VEQLKIEASLCRIKVSKAAADLMTYCDAHACEDPLITP
Gγ-t ...MPVINIEDLTEKDKLKMEVDQLKKEVTLERMLVSKCCEEFRDYVEERSGEDPLVKG
III
CAAX
DGγ1 FTSQKVNPFREKS.SCTVL
Gγ-2 VPASE.NPFREKKFFCAIL
Gγ-3 VPTSE.NPFREKKFFCALL
Gγ-t IPEDK.NPFKELKGGCVIS

protein γ-subunits, i.e., γ2, γ3, and γt (Fig. 7). The deduced molecular mass (8 kDa) of the *Drosophila* protein is very similar to that of the bovine γ-proteins which ranges between 7-10 kDa (Gilman, 1987). Like the mammalian γ-subunit proteins, the *Drosophila* protein is also an acidic protein with a calculated pI of 4.6. Hydropathy analysis showed that the *Drosophila* and the bovine gamma proteins share similarly placed hydrophobic and hydrophilic domains (Fig. 8). Three regions of the *Drosophila* protein (Fig. 7), one in the amino-terminal and the other two in the carboxyl-terminal halves, show greater than 75% identity with the bovine brain γ2 and γ3 proteins. These three regions, I, II and III, are highly conserved in all known Gγ proteins. However, less similarity between the *Drosophila* protein and bovine retinal γt proteins are found in these regions. Finally, as in all three bovine γ-subunits (Yatsunami, 1985; Robishaw et al., 1989; Gautam et al., 1990), the *Drosophila* protein also has a CAAX motif (where C is cysteine, A represents aliphatic amino acids and X is any amino acid) at the carboxyl-terminal end (Fig. 7). Based on these similarities it has been concluded that the 70 amino acid polypeptide encoded by the second ORF of the third reading frame of 536-B2 cDNA is a putative *Drosophila* homologue of bovine G protein γ-subunits. Thus, the locus corresponding to this cDNA clone has been designated *D-Gγ1*.

Fig. 8. Hydropathy analysis of the D-Gγ1 and bovine γ2 and γt subunits of G proteins. The comparison shows the presence of similarly spaced hydrophilic and hydrophobic domains in all three γ-subunits. Positive values on the ordinate indicate hydrophobicity and negative values indicate hydrophilicity (Kyte & Doolittle, 1982). All curves were generated by the PEPPLOT program of UWGCG.


0
50
γ-2
3
0
-3
D-Gγ1
3
0
-3
γ-t
3
0
-3

3.3. The *Drosophila* genome has only one *D-Gγ1* gene which produces three different mRNAs

Previous data demonstrated that a 2.0- kb *Eco*RI transcribed DNA fragment of the *D-Gγ1* gene hybridizes with three mRNAs (section 3.1; Fig. 4, panel c). To determine whether all three mRNAs originate from the *D-Gγ1* gene or if sequences similar to the *D-Gγ1* gene are present elsewhere in the genome, Southern blots of the genomic DNA of adult flies digested with various enzymes were probed with the 2.0- kb *Eco*RI genomic DNA under low and high stringency conditions (fragment B; Fig. 3). Under both these conditions major hybridization was observed with 2.0- kb *Eco*RI, 8.0- kb *Hin*dIII, and 2.0- and 1.4- kb *Pst*I fragments (Fig. 9). According to the restriction map at the bottom of Fig. 9, these DNA fragments are expected to hybridize because they include the probe DNA. Besides the major hybridization signals, faint hybridization of an 8.0- kb *Eco*RI fragment was also noticed (Fig. 9, lane 1), suggesting the presence of another locus with similar sequence in the genome. Although low signal intensity may reflect the possibility of weak homology with another locus in the genome, absence of similar bands with faint signals in other lanes urgues against this idea. It is possible that in a small population of flies, the *Eco*RI site marked with a *black dot* (Fig. 9) is missing. Such *Eco*RI polymorphism will then generate a 5.0- and a 8.0- kb *Eco*RI fragment, showing

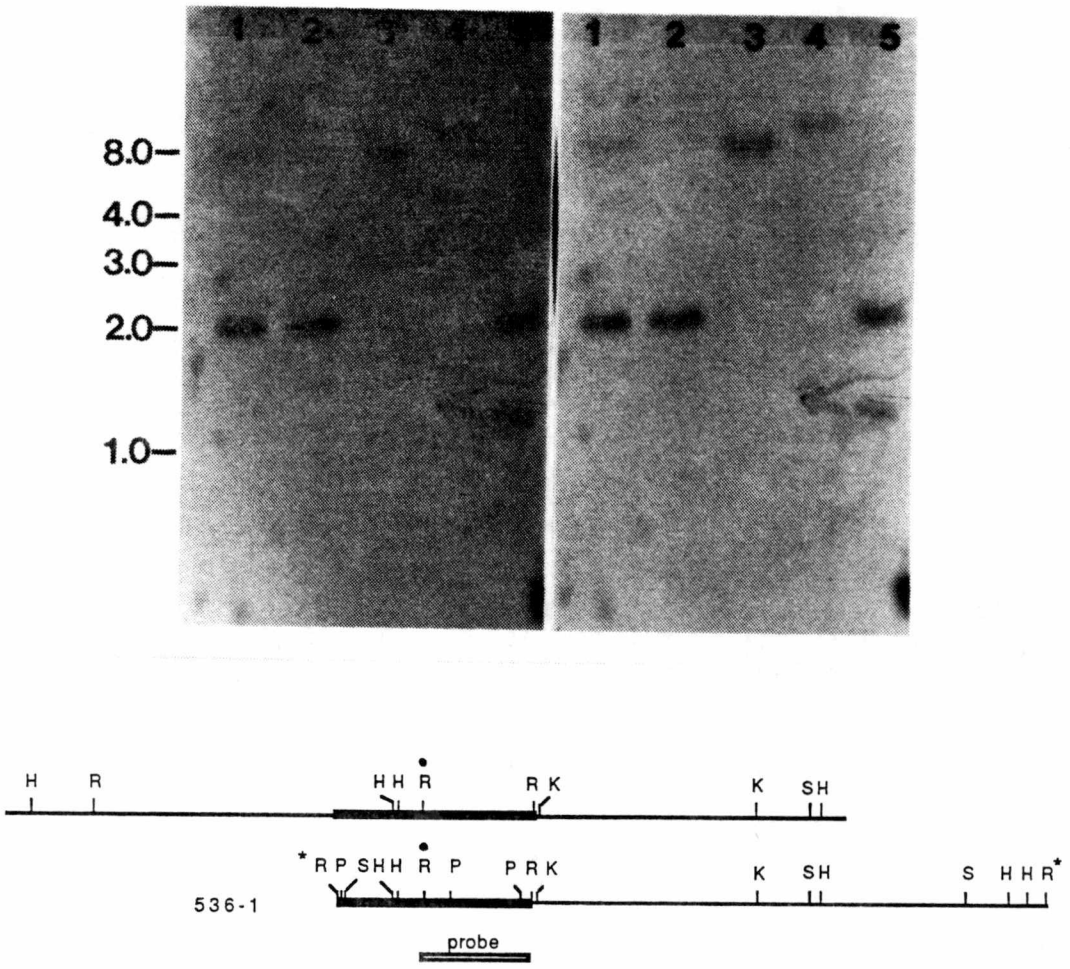


Fig. 9. Southern-blot analysis of the genomic DNA digested with various restriction enzymes. Genomic DNA isolated from adult flies were digested with various restriction enzymes, blot transferred and hybridized with ^{32}P-labeled genomic DNA fragment B shown in the map above. Left *panel*, the blot was hybridized and washed under low stringent condition as described under "Materials and Methods"; right *panel*, rehybridization of the blot shown in the left *panel* under high stringent condition. Both blots were exposed for 48 h. Lane 1, *Eco*RI; lane 2, *Eco*RI-*Hind*III; lane 3, *Hind*III; lane 4, *Bam*HI; lane 5, *Pst*I. All lanes contained approximately 4 μg of DNA except lane 3 and 4, which had 2 μg of DNA. Abbreviations for the restriction enzymes are same as in Fig. 3.

stronger and weaker hybridization signals, respectively.

In order to examine this issue further, the 2.0- kb *Eco*RI fragment was hybridized to the polytene chromosomes. As reported earlier (Levy et al., 1982), the probe hybridized to a single site on the second chromosome (Fig. 10). Taken together, these results strongly suggest that there is only one copy of *D-Gγ1* gene in the haploid genome of *Drosophila*.

4. DISCUSSION

The results presented in this chapter indicate that the *D-Gγ1* gene located within clone 536 encodes a *Drosophila* homologue of mammalian G protein γ-subunit. Amino acid sequence comparison revealed significant similarities between the D-Gγ1 and different mammalian γ-subunits of G proteins. Although a wide range of heterogeneity exists among all known mammalian γ-subunits (Yatsunami et al., 1985; Gautam et al., 1989; Robishaw et al., 1989; Gautam et al., 1990), the overall degree of similarity in specific conserved domains between *Drosophila* D-Gγ1 and mammalian γ-subunits expressed in brain is significant. Three regions of the D-Gγ1 protein of *Drosophila* show greater than 75% identity with the corresponding regions of the bovine γ2 and γ3 proteins, suggesting that these regions are functionally important. The D-Gγ1 protein shows greater amino acid sequence similarity with the bovine Gγ subunits (γ2, and γ3)



Fig. 10. ***In situ*** **hybridization to the polytene chromosomes of** ***D. melanogaster.*** ^{3}H-labeled 2.0-kb *Eco*RI fragment (fragment B, Fig. 3) containing the coding region of *D-Gγ1* gene was hybridized with the polytene chromosomes. Hybridization grains were observed over the 44E region of the second chromosome, as indicated by an *arrow*.

than with the γ-subunit of transducin (γt). Interestingly, γ2 and γ3 are expressed predominantly in the bovine brain while γt shows eye-specific expression. Since there is only one γ-subunit gene present in *Drosophila*, it is expected to be expressed in both the brain and eye. The detailed analysis of the expression of the *D-Gγ1* gene described in the later chapter has resolved this issue.

The carboxyl terminus of the D-Gγ1 matches the CAAX motif, which is known to be required for prenylation of all Gγ proteins by a farnesyl or a longer and more hydrophobic geranylgeranyl isoprenyl group (Clarke et al., 1988). Prenylation is a post-translational modification of proteins and it is found in mammalian G protein γ-subunits, the yeast homologue STE 18 of Gγ, ras and other ras-related GTP-binding proteins (Lai et al., 1990; Finegold et al., 1991; Sanford et al., 1991; Kinsella et al., 1991). This modification has been shown to be necessary for membrane anchorage and function of the ras protein and the G-protein βγ complex (Powers et al., 1986; Sanford et al., 1991). It has also been shown that if the carboxyl terminal amino acid of the CAAX motif is leucine, a gernylgerynl group but not a farnesyl group will be attached to the proteins (Lai et al., 1990; Stanford et al., 1991; Finegold et al., 1991). Since the last amino acid of the CAAX motif of the D-Gγ1 protein is leucine, it is likely to be post-translationally modified by geranylgeranyl group. Attachment of this

hydrophobic group may help membrane anchorage of the D-Gγ1 protein.

The results presented in this chapter established that a single *D-Gγ1* gene produces 2.6- , 1.1- and 1.3-kb long RNAs. Transcription of multiple mRNA from a single gene is not a new phenomenon and a single gene transcribing multiple transcripts in stage- and tissue-specific manner has been reported for many organisms including *Drosophila* (Theurkauf et al., 1986; Nagoshi et al., 1988; Amrein et al., 1990; Sharp, 1987). Occasionally, multiple transcripts of a single gene may differ in coding sequence and produce isoforms of a single protein (Schejter et al., 1986; de Sousa et al., 1989; Amrein et al., 1990). In the other cases, all transcripts have the same coding region but they vary in their 5′- or 3′-UTRs (Benyajati et al., 1983; Schejter et al., 1986; Mattox et al., 1990; Smith et al., 1989 for review). In all cases, however, multiple transcripts are generated by utilization of alternative promoters, alternative splice junctions or alternative polyadenylation signals (for review: Smith et al. 1989; Maniatis, 1991). In the case of the *D-Gγ1* gene, the mechanism by which multiple transcripts are generated is not known. However, characterization of the cDNA clones for all three mRNAs and the genomic organization of the *D-Gγ1* gene may shed some light in this regard.

The 2.1- kb long 3′-UTR of the 2.6-kb RNA is of

particular interest. In eukaryotic mRNAs, the length of the 3′-UTR sequence generally varies between 50-1000 nt. However, very long (>1000 nt) 3′-UTRs have been identified in mammalian and *Drosophila* genes (review: Kozak, 1988; Saez et al., 1990; Grens & Scheffler, 1990; Code & Olmsted, 1992). There are also examples of multiple mRNAs produced from a single gene which differ only in the length of their 3′-UTR (reviewed in Kozak, 1988). While it is not clear whether the translational efficiency of mRNAs is influenced by the length of their 3′-UTRs, the 3′-UTR has been shown to have a role in the stability of mRNAs and therefore may indirectly influence the level of encoded polypeptides (reviewed in Kozak 1983). The 3′-UTR of the 2.6-kb RNA was examined for structural features relating to RNA stability and degradation. Certain structural features, such as sequences with the potential to form stem-loop structures and the A-T rich sequences in the 3′-UTR are believed to play a role in the decay of mRNAs (review: Brawerman, 1989). It has been hypothesized that $A(T)_nA$) sequences and stem-loop structures in the 3′-UTR are primary targets of nucleases (Brawerman, 1989). Alternatively, interaction of protein factors with A-U-rich sequences and with the stem-loop structures may reduce accessibility of the nucleases and thereby prevent mRNA degradation (Brawerman, 1989; Cleveland & Yen, 1989). Consistent with this hypothesis, it has been found that the 3′-UTR of the 2.6-kb D-Gγ1 RNA is

65% A-T rich and has several stretches of $A(T)_nA$ sequence (Fig. 4) which are known to cause RNA degradation (Shaw & Kamen, 1986). This mRNA also has several potential stem loop structures in the 3'-UTR region (Fig. 11). Similar "bulged" nucleotides (unpaired bases within a duplex stem) found in many biologically significant RNAs are thought to be controlled by factors that limit accessibility of stem-loops to nuclease attack (Ross et al., 1986; Casey et al., 1988; Mullner et al., 1989). Therefore, the 2.6-kb D-Gγ1 mRNA may also be susceptible to degradation by exo- or endo-nuclease activity when not protected by RNA-protein interaction. If this is the case, it can be assumed that the protein factor(s) that protects the 3'-UTR of the 2.6-kb RNA must be present only in the head, but not in the body. Lack of this hypothetical protein factor(s) in the body may cause degradation of the 2.6-kb RNA in the body. A similar stem-loop structure-mediated cell-specific stability has been speculated for mouse histone mRNA in dividing and nondividing cells (Ross et al., 1986; Levine et al., 1987).

The sequence data showed that the 3'-UTR of the 2.6-kb RNA contains two polyadenylation signals at position 845 nt and 2477 nt, and a putative polyadenylation signal at position 986 (Fig. 6). The polyadenylation signal sequences at 845 nt and 2477 nt match the consensus AATAAA sequence, while AATATA at position 986 nt is a variant form of the consensus sequence. The latter sequence is, however,

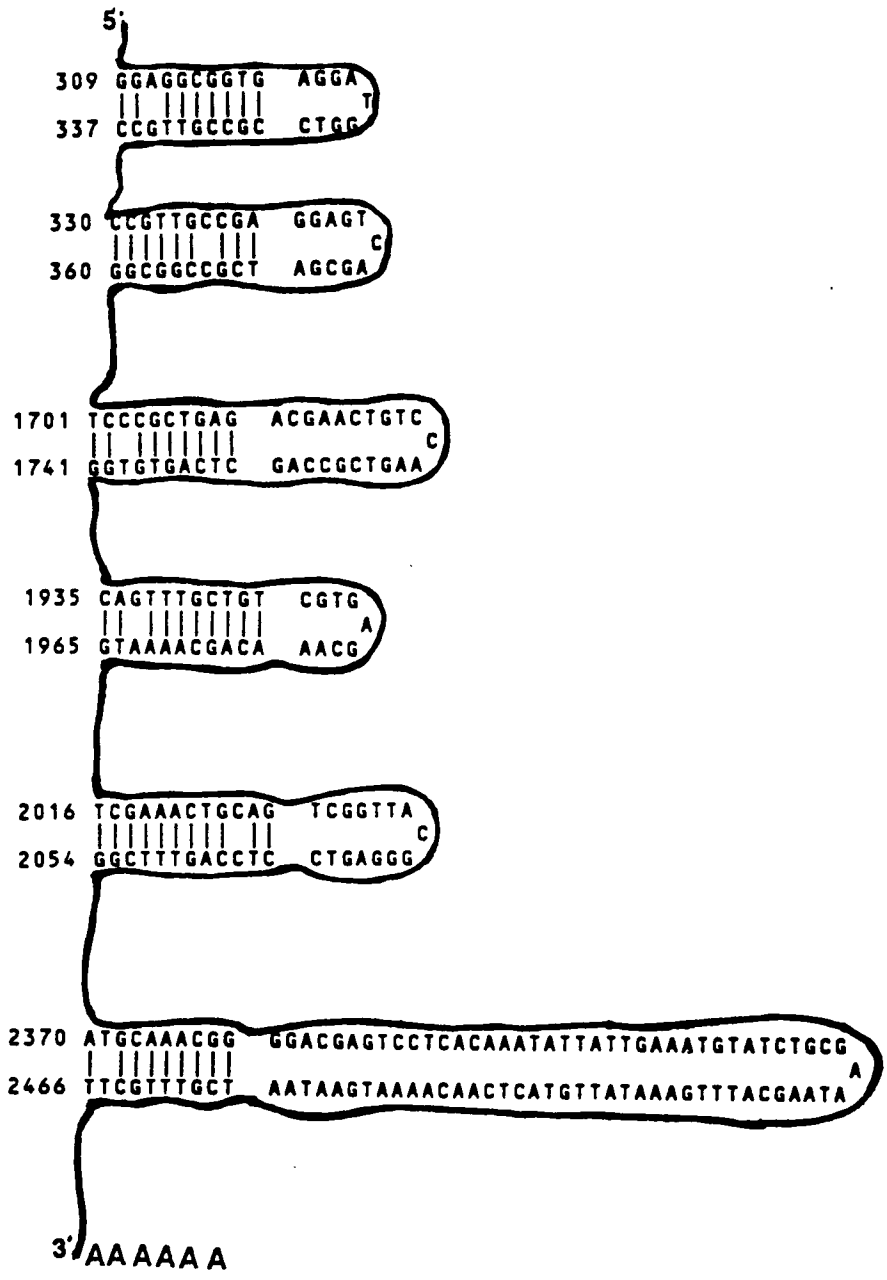


Fig. 11. Potential stem-loop structures in the 3′-UTR of the 2.6- kb D-Gγ1 mRNA. The 3′-UTR of the 2.6-Kb RNA was subjected to stem-loop analysis using stem-loop program of UWGCG. Some of the stem-loop structures shown, may be susceptible to cleavage by nucleases otherwise protected by protein factors.

believed to be used for polyadenylation of the *Xenopus* maternal mRNA D7 (Smith et al., 1988). It appears that the polyadenylation signal at position 2477 nt is used by the 2.6-kb RNA since it is the closest one to the poly(A)$^+$ tail. The other putative polyadenylation signals are probably utilized by the 1.1- and 1.3-kb mRNAs. If both these RNAs use the same polyadenylation signal at position 845 nt or 986 nt, the lengths of their 5'-UTRs have to be different to account for their size difference. Sequencing of the 1.1- and 1.3-kb RNA-specific cDNAs and comparison of the corresponding genomic DNA sequences will resolve these questions and reveal how three mRNAs are originated from one *D-Gγ1* gene. Results of these analyses are presented in chapter 4.

CHAPTER 3

TEMPORAL AND TISSUE-SPECIFIC EXPRESSION OF THE *D-Gγ1* GENE

1. INTRODUCTION

In *Drosophila*, G-protein-mediated signal transduction has been implicated in phototransduction and neuronal processes (Quan et al., 1989; Thambi et al., 1989; Guillen et al., 1990; Ranganathan et al., 1991; Wolfgang et al., 1991). Involvement of G-protein-coupled pathways has also been speculated in cell signaling in non-neuronal tissues and in a variety of developmental processes in *Drosophila,* such as the establishment of embryonic position, development of the nervous system and organogenesis (Parks & Wieschaus, 1991; Wolfgang, et al., 1991). These speculations are, however, mostly based on the expression studies of various G protein subunits (Dαs, Dαi, Dαo, D-Gβ1, D-Gβt) or their transcripts in various tissues during developmental stages (Hartley, et al., 1988; Provost et al., 1988; Yarfitz et al., 1988; de Sousa et al., 1989; Quan et al., 1989; Thambi et al., 1989; Wolfgang et al., 1991; Yarfitz et al., 1991). Since the results in the previous chapter showed that the *D-Gγ1* gene codes for a putative *Drosophila* G protein γ-subunit, one obvious question is whether the expression pattern of the

D-Gγ1 gene is also similar to the other G protein-subunit genes. Therefore, in this chapter reports studies whereby the patterns of expression of the *D-Gγ1* gene in various tissues and developmental stages are examined. The results showed that the three D-Gγ1 RNAs are differentially expressed in different tissues and developmental stages and these expression patterns seem to be under stringent control. The significance of these findings in relation to G-protein function, and gene regulation via RNA processing will be discussed.

2. MATERIALS AND METHODS

2.1 *Drosophila* strains and cultures

Wild type *Drosophila melanogaster* (Oregon R+ strain) was obtained from Dr. John C. Lucchesi, Emory University, Atlanta. Eye missing (*eym*) flies were obtained from Y. Inoue, Mishima, Japan. Flies were raised on cornmeal-agar-molasses medium at 23^0C and 60% humidity with a 12 hour dark/light cycle.

2.2. Nucleic acid isolation, radiolabeling and blot hybridization

Staged embryos (0-3 h, 3-6 h, 6-9 h, 9-12 h, 12-15 h, 15-18 h, 18-24 h), larvae and pupae, were frozen and pulverized in liquid nitrogen. Total RNA was isolated from these samples according to Jowett (1986). DNA probes were prepared either by

nick translation, using a commercially available kit (Bethesda Research Laboratories, MD) or by the random hexamer-primed labeling method of Feinberg and Vogelstein (1984). These procedures are detailed in Appendix I.

2.3. Tissue localization of D-Gγ1 mRNAs

D-Gγ1 RNAs in various tissues were localized by *in situ* hybridization technique described by Ashburner (1989). Eight-micron sections of frozen tissues were fixed, dehydrated and subsequently hybridized in a solution containing 50% formamide, 0.6 M NaCl, 1x Dendardt solution, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2 mg tRNA/ml, 10% dextran sulfate, 10 mM DTT, and approximately 5x 10^5 cpm of labeled DNA probe per slide. Hybridization was carried out overnight at 40^0C. After hybridization the slides were washed at 37^0C in 50% formamide, 0.6 M NaCl, Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM DTT, with several changes of the wash solution, and air dried. After immersion in Kodak NTB2 emulsion, the slides were exposed at 4^0C for 2-10 days, developed, and stained with Giemsa. The slides were examined both under bright- and dark-field of a Zeiss microscope.

3. RESULTS

3.1 Blot hybridization analysis of the *D-Gγ1* gene expression in adult tissues and different developmental stages.

3.1.1. Adult head

Northern blot analysis (chapter 2, section 3.1, Fig. 4) indicated that the 2.6-kb and 1.1-kb mRNAs of the *D-Gγ1* gene are expressed predominantly in the fly head. To determine whether expression of any of these two RNAs is eye-specific, RNA isolated from the heads of eye-missing (*eym*) mutant flies was probed with fragment D (see Fig. 3) which hybridizes with the 2.6-kb and 1.1-kb RNAs. Since the *eym* flies lack the compound eyes (Inoue, 1980; Ray and Ganguly, unpublished observation), mRNAs not expressed in *eym* flies are expected to be eye-specific transcripts. The results (Fig. 12) showed that both the 2.6- and 1.1-kb mRNAs are present in the *eym* flies, indicating that these transcripts are not eye-specific; they are either expressed in the brain and the eye, or in the brain only. *in situ* hybridization to head tissue sections can resolve this issue (see section 3.2.1).

3.1.2. Thorax

Northern blots of the total RNA extracted from male and female thorax (Fig. 13) were probed with the common probe (fragment D, see fig. 3). The results showed that both the 2.6- and

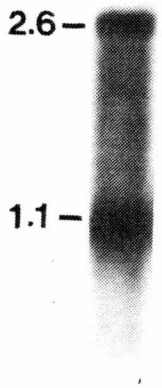


Fig. 12. Northern blot analysis of the head poly(A)$^+$ RNA of *eym* (eye missing) strain. One microgram of RNA was electrophoresed and hybridized with ^{32}P-labeled *D-Gγ1* common probe (fragment D, Fig. 3).

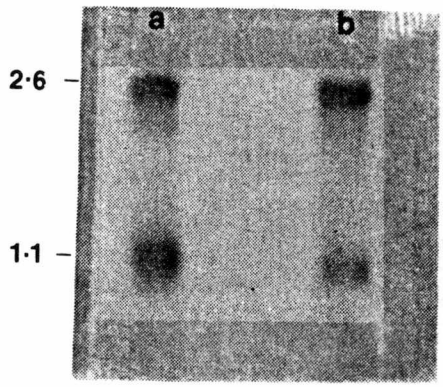


Fig. 13. Hybridization of male and female thorax RNA with the common D-Gγ1 probe. Northern blot containing total RNA extracted from male and female thoraces was probed with *D-Gγ1* common probe (fragment D, Fig. 3). *Lane a*, 10 μg of male thorax RNA; *lane b*, 10 μg of female thorax RNA. The blot was exposed for 8 days to detect the hybridization signals.

1.1-kb RNAs are present in the tissues of thorax at low levels. Since head RNAs were not used in the same blot, the levels of the 2.6- and 1.1-kb RNAs in the thorax can not be compared directly with their levels in the head. However, visual comparisons of hybridization signals generated by equivalent amounts of head and thorax RNAs on separate Northern blots indicated that the levels of 1.1- and 2.6-kb RNAs are higher in the heads. It is possible that a smaller number of tissues in the thorax express these two RNAs and/or the levels of expression are much lower in the tissues of the thorax than in the head. This possibility is examined later by *in situ* hybridization to thorax sections (see section 3.2.2).

3.1.3. Abdomen

It is important to mention here that the earlier Northern blot analysis of body (thorax + abdomen) was done with RNA isolated from mixed flies (males + females) and the results showed that both 1.1- and 1.3-kb RNAs are expressed in the bodies. In the present investigation, the patterns of expression of these two mRNAs were examined in the abdomens of male and female flies separately. The results presented in Fig. 14 demonstrate that while the 1.1-kb RNA is present in the abdomen of both sexes, the 1.3-kb RNA is expressed only in the female abdomen. When RNA extracted from abdomen without ovary was examined, no hybridization signal from the 1.3-kb RNA was observed (Fig.

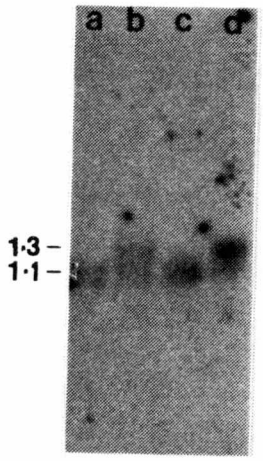


Fig. 14. Ovary-specific expression of the 1.3-kb D-Gγ1 mRNA. Northern blot containing 10 μg total RNA in each lane was hybridized with the *D-Gγ1* common probe (fragment D, Fig. 3). *Lane a*, male abdomen; *lane b*, female abdomen; *lane c*, female abdomen without ovary; *lane d*, ovary.

14, lane c). However, a high level of 1.3-kb RNA was found to be present in the RNA isolated from ovary (Fig. 14, lane d). Therefore, 1.3-kb RNA expression is ovary-specific.

3.1.4. Developmental stages

The expression pattern of three D-Gγ1 mRNAs in different stages of development were monitored by Northern blot analysis using the common probe (fragment D; see Fig. 3). The results shown in Fig. 15 demonstrate that the 1.1-kb RNA is the most predominant species during embryogenesis. This RNA is first detectable after 3 h of development and its level peaks between 9-12 hour of embryogenesis. In other stages of development, such larvae and pupae, the 1.1-kb RNA is the only detectable RNA species but its level seems to be much lower level than in the embryos. Since it is first detectable after 3 hour stage, it seems that the 1.1-kb RNA is zygotically expressed.

The 1.3-kb RNA is found as early as in the 0-3 hour stage (preblastoderm) of embryogenesis (Fig. 15). Since very few genes in *Drosophila* are transcribed in the first 3 hours of embryogenesis (Edgar & Schubiger, 1986), it is possible that the 1.3-kb RNA is maternally transcribed and deposited into the egg. This speculation is consistent with the ovary-specific expression of 1.3 kb mRNA (previous section; Fig. 14).

The 2.6-kb RNA is not detectable during early embryonic

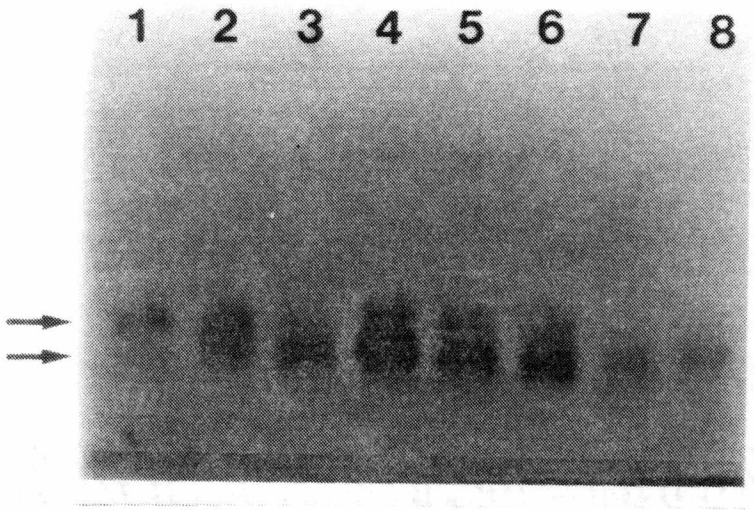


Fig. 15. Developmental expression of the D-Gγ1 gene. Approximately 10 μg of total RNA isolated from different stages were fractionated on formaldehyde-agarose gel, transferred to nylon membrane and hybridized with ^{32}P-labeled D-Gγ1 common probe (fragment D, Fig. 3). The *arrows* indicate the 1.3- and 1.1-kb RNAs. The 2.6-kb RNA was only seen faintly in lane 5, 6, 7 and 8, only after 10 days exposure (data is not shown). *Lane 1*, 0-3 h embryo; *lane 2*, 3-6 h embryos; *lane 3*, 6-9 h embryos; *lane 4*, 9-12 h embryos; *lane 5*, 12-15 h embryos; *lane 6*, 15-18 h embryos; *lane 7*, third instar larvae; *lane 8*, unstaged pupae.

development. Although it seems that this RNA is not expressed in late embryos, larvae and pupae (Fig. 15), a low level of the 2.6-kb RNA was detected when these blots were exposed for a longer time (not shown). However, when total RNA isolated from brain ganglia was examined, the 2.6-kb RNA was found to be present in this tissue (Fig. 16). Thus as in adults, the 2.6-kb RNA is expressed only in the larval brain ganglia, whereas the 1.1-kb RNA is expressed in brain as well as in other larval tissues.

3.2. Tissue localization of D-Gγ1 transcripts

3.2.1. Adult head

The Northern blot experiment described earlier showed that the 2.6-kb mRNA of the *D-Gγ1* gene is expressed only in the head and the thorax of the fly (Chapter 1, Fig. 4, panel a; Fig. 13). In this study, the site of expression of this RNA in the head was determined by *in situ* hybridization using a 2.6-kb RNA-specific probe (fragment C, Fig. 3) to head sections. Heavy hybridization was observed over the cortical regions of the brain and the optic lobe (Fig. 17, A and B), which contain the cell bodies of neurons. No significant hybridization signals were observed over the neuropil region of the brain, which contains the axonal processes of neurons. Low level of hybridization were also found over the lamina and retina of the compound eye (Fig. 17, A and B). When similar head

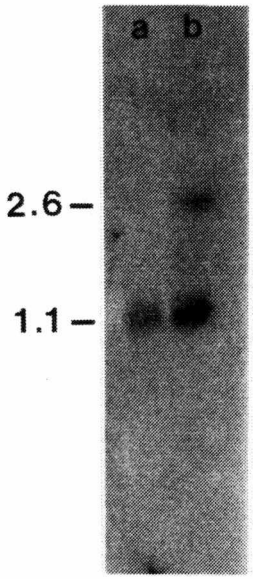


Fig. 16. Northern blot hybridization showing larval brain ganglia-specific expression of the 2.6-kb D-Gγ1 mRNA. Total RNA isolated from larval carcasses without brain ganglia (*lane a*) and hand-dissected brain ganglia of third instar larvae (*lane b*) were fractionated in a formaldehyde-agarose gel, blotted and hybridized with *D-Gγ1* common probe (fragment C, Fig. 3). *Lane a*, 10 μg of larval carcasses RNA; *lane b*, 10 μg of brain ganglia RNA.

Fig. 17. ***In situ*** **hybridization of the** *D-Gγ1* **probe to the RNA in head sections.** Cryostat sections of adult *Drosophila* head were probed with ^{35}S- and ^{3}H-labeled probes of *D-Gγ1* DNA fragments. *Panels A* and *C*, bright field; *B* and *D*, dark field. The 2.6-kb RNA-specific probe (^{35}S-labeled fragment C, Fig. 3) was used for the section shown in *panel A*. For the section in *panel C*, ^{3}H-labeled fragment D (see Fig. 3) was used as the common probe. *r*, retina; *l*, lamina; *o*, lobula; *n*, neuropile of the brain. The arrows indicate cortical regions of brain and lobula.

B
D
A
n
r
o
C
n
r
o

sections were probed with the common probe, heavy hybridization were observed over the same sites as seen with the 2.6-kb RNA-specific probe (Fig. 17, C and D). No additional sites of hybridization were observed with the common probe, indicating that the expression patterns of 2.6- and 1.1-kb mRNAs are very similar. Although whether the 1.1-kb mRNA expression pattern is a subset of the 2.6-kb RNA expression pattern could not be resolved by *in situ* hybridization technique, these results indicate that both 2.6- and 1.1-kb mRNAs of the *D-Gγ1* gene are expressed predominantly in the central nervous system of adult *Drosophila melanogaster*.

3.2.2. Thorax

Previous Northern blot analysis showed that both 2.6-kb and 1.1 kb mRNAs are expressed in the thorax of adult fly (Fig. 13). Specific tissue localization of these mRNAs in the thorax was established by the technique of *in situ* hybridization. As before, the 2.6-kb RNA-specific probe (fragment C) and the common probe (fragment D) were used to monitor the tissue-distribution pattern of these two mRNAs. Fig. 18 shows that both 2.6- and 1.1-kb mRNAs are expressed in the cortical regions of thoracic and abdominal ganglia, which represent the neuronal cell bodies in the ganglia. No significant hybridization signal was detectable in the thoracic muscles with either probe. Interestingly, the Goα

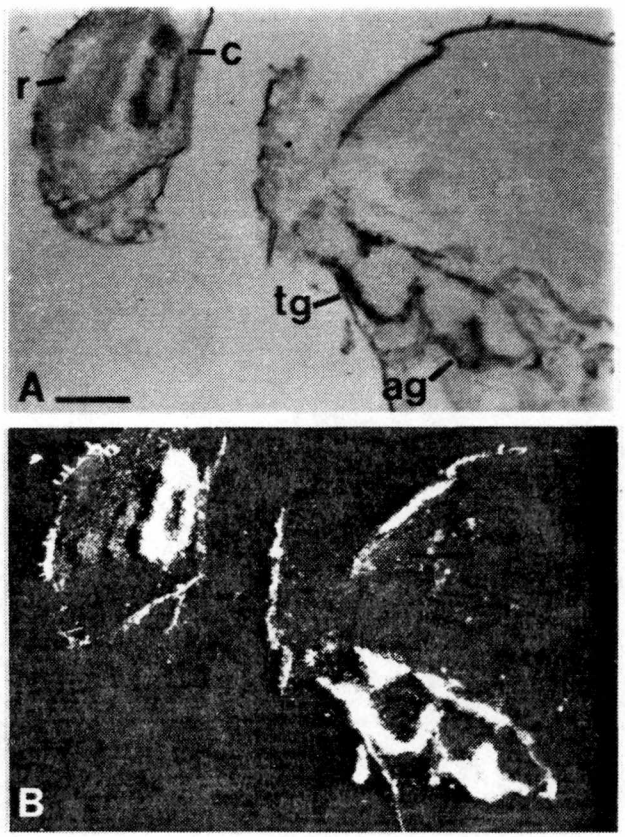


Fig. 18. Localization of D-Gγ1 transcripts in *D. melanogaster* adult thorax and head. ^{35}S-labeled D-Gγ1 common probe (fragment D, Fig. 3) was hybridized with the RNA in the tissue section. *panel A*, bright field; *panel B*, dark field; *c*, brain cortex; *r*, retina; *tg*, thoracic ganglia; *ag*, abdominal ganglia.

subunit mRNA of *Drosophila* is also expressed in the cortical regions of the brain, optic lobe, thoracic and abdominal ganglia (de Sousa et al., 1989; Yoon et al., 1989; Shortridge et al., 1991). This raises the possibility that the products of these two genes are coupled in the same neuronal signaling pathway.

3.2.3. Ovary

Ovary-specific expression of the 1.3-kb RNA was shown earlier by Northern blot analysis (Fig. 14). To localize the 1.3-kb RNA more precisely in the ovary, *in situ* hybridization to ovary sections was done using the common probe (fragment D). Since the 1.3-kb RNA is the only D-Gγ1 transcript found in the ovary (see Fig. 14, lane d), hybridization signals generated by the common probe should represent the localization pattern of this RNA in the ovary. The results (Fig. 19) showed that the 1.3-kb RNA is present in high levels in the nurse cells of developing egg chambers of the ovary as well as in developing oocytes. In both these cell types, hybridization signals are found to be uniformly distributed in the cytoplasm. Interestingly, this expression pattern matches the ovary-specific expression pattern of *Drosophila* Goα and *concertina* genes which encode two different Gα- subunits (de Sousa et al., 1989; Wolfgang et al., 1991; Parks & Wieschaus, 1991).

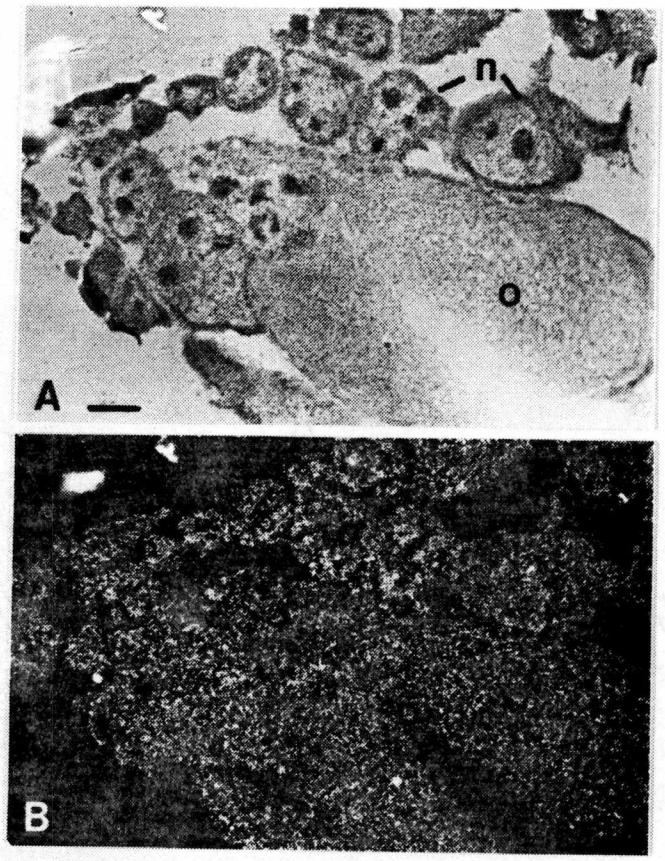


Fig. 19. Localization of the 1.3-kb D-Gγ1 RNA in the ovary. Cryostat sections of isolated ovaries were probed with ^{3}H-labeled D-Gγ1 common probe (fragment D; Fig. 3). *o*, mature oocyte; *n*, nurse cells.

3.3.4. Embryos

Initially, the expression patterns during embryogenesis of D-Gγ1 mRNAs was followed by Northern blot analysis (Fig. 15). Here the spatial distributions of D-Gγ1 transcripts were examined in the embryos by *in situ* hybridization. The 2.6-kb RNA-specific probe and the common probe were used in these experiments. As a negative control experiment some slides were treated with RNase before hybridization (data not shown).

Sections of three different embryonic stages were examined : 0-5 , 7-12 and 18-21 h embryos, which represent early cellular division stages, mid germ band extension stages, and the organogenesis stages, respectively (Campos-Ortega, 1985). With the common probe the D-Gγ1 transcripts were found to be uniformly distributed throughout the cellular blastoderm stages (Fig. 20, a and b). It is to be noted that the intense glow over the yolk material under dark field (Fig. 20, b) was not due to specific hybridization; this was probably due to some technical problem or due to yolk. When observed under bright field, no silver grains were found over the yolk material but they were readily detectable over the cellular region (Fig. 20, a). Since both the 1.3-kb maternal RNA and the zygotically expressed 1.1-kb RNA are found in 0-5 h embryos (see Fig. 15), the hybridization signals shown in Fig. 20, a and b are probably due to both these RNAs. Whether these RNAs are differentially distributed in the embryos at this developmental stage could not be resolved with the common

Fig. 20. Localization of D-Gγ1 transcripts in early embryos. Sagital section of a blastoderm embryo (3 h) hybridized with ^{35}S-labeled *D-Gγ1* common probe (fragment D, Fig. 3). The anterior end is to the right. Bright field (*a*) and dark field (*b*) micrographs are shown. The glowing yolk area in the dark field micrograph is artifactual, no hybridization signals were observed under bright field. *y*, yolk; *c*, cell layers.

b
a
c
v

probe. In contrast, no hybridization with the 2.6-kb RNA-specific probe was observed (data not shown) because the 2.6-kb RNA is not expressed during early embryogenesis. This is consistent with the Northern blot analysis of embryonic RNAs.

During the late germband extension stage (7-12 h), major structures, such as brain, ganglia and gut begin to form. At this stage, uniform hybridization of the common probe was seen over most of these structures of the developing embryos (Fig. 21, panels, A, C, E). Since Northern blot analysis has shown that only the 1.1-kb RNA is expressed during this stage, the hybridization seen in Fig. 21 must be due to the presence of 1.1-kb transcript only. No hybridization was observed with the 2.6-kb RNA-specific probe, indicating that this RNA is not expressed at this stage of embryogenesis.

In the late stages of embryogenesis (18-21 hour), the hybridization signal with the common probe was found to be primarily concentrated over the ventral nerve cord and brain proper (Fig. 22, A, B). However, low level hybridization was also detected over other cell types of the embryos. Although the developmental Northern analysis (Fig. 15) detected a very low level of 2.6-kb RNA after a long autoradiographic exposure (data not shown), *in situ* hybridization studies showed a strong and localized hybridization of the 2.6-kb RNA-specific probe over the ventral nerve cord and brain (Fig. 22, C, D). Since tissues other than the brain and nerve cord do not express the 2.6-kb RNA, the relative concentration of this RNA

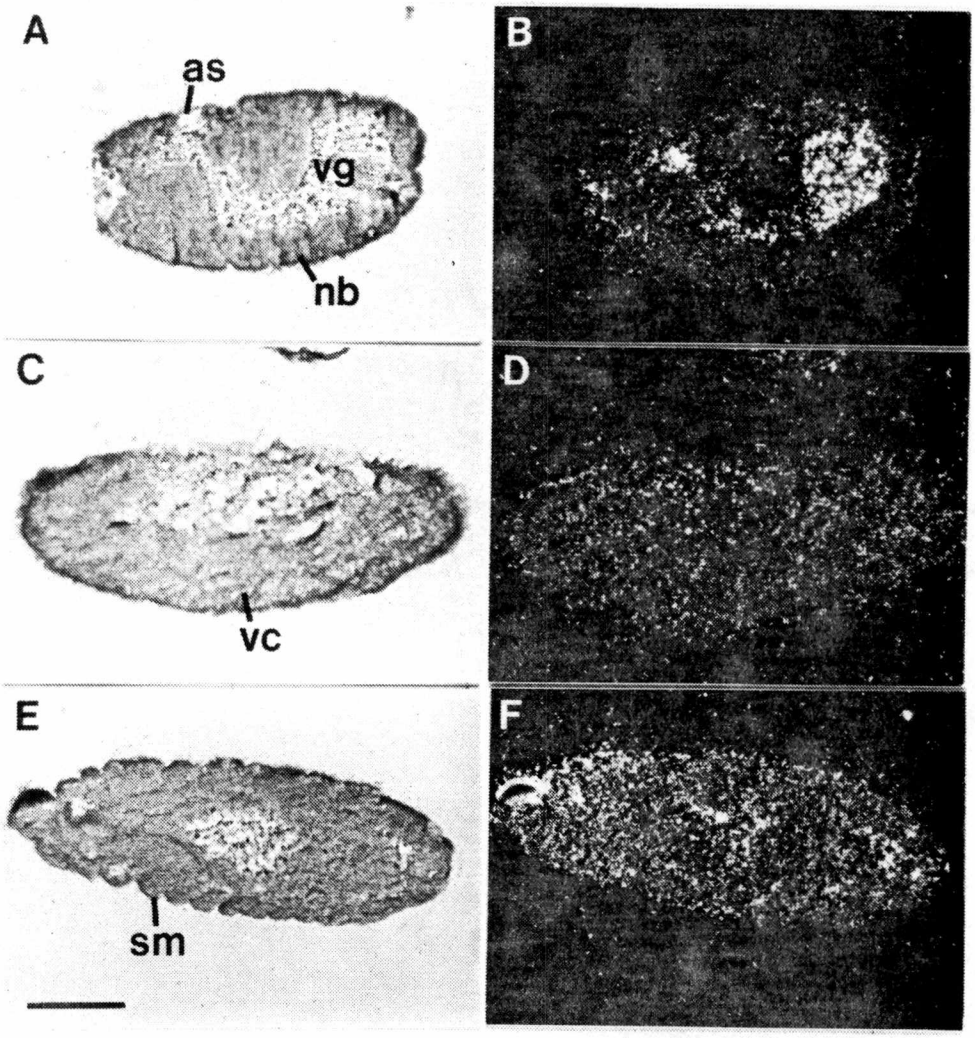
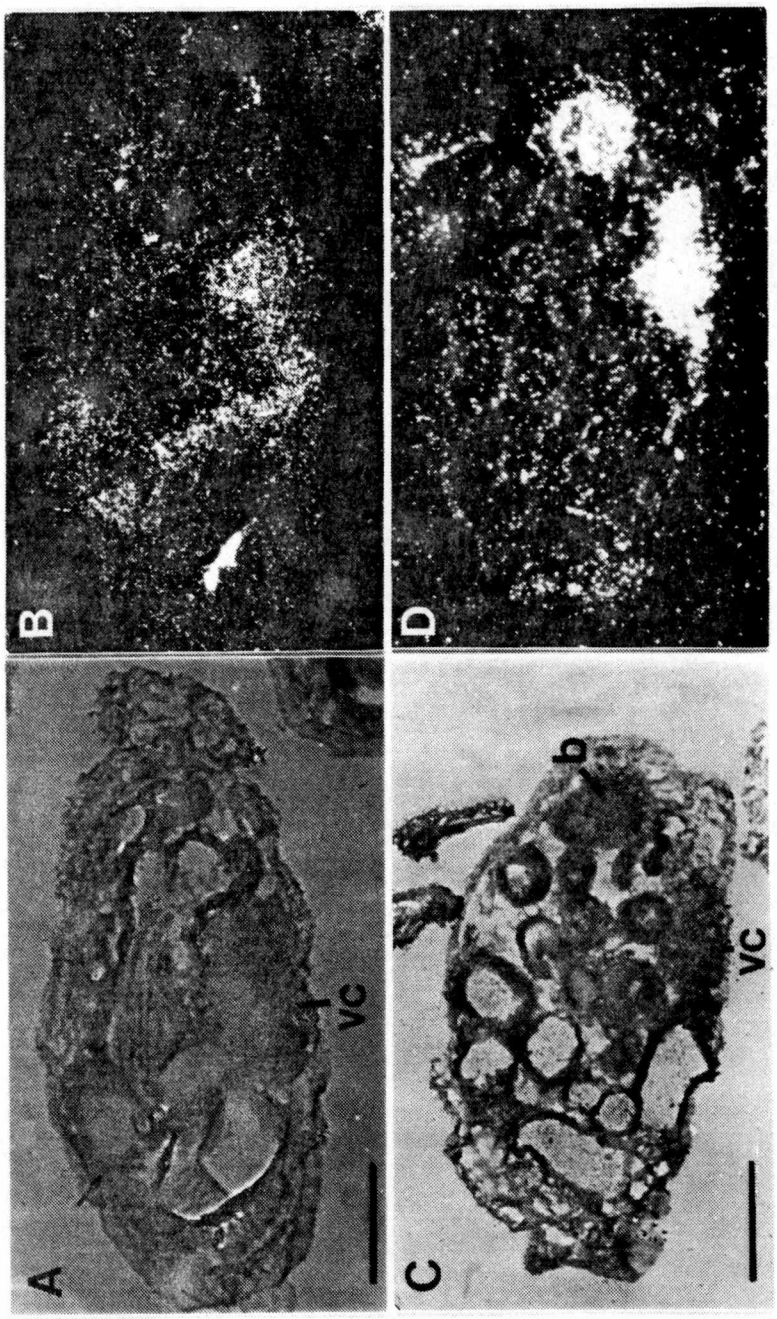


Fig. 21. Localization of D-Gγ1 transcripts in mid-stage embryos. ^{3}H-labeled common probe (fragment D, Fig. 3) was hybridized with the RNA in the embryo sections and autoradiographed. Panels A, C, E, bright field; B, D, F, dark field; A, 7 h embryo; C, 9.5 h embryo; E, 12 h embryo; *as*, amnioserosa; *nb*, neuroblasts; *vg*, vitelophages (yolk); *vc*, ventral nerve cord; *sm*, somites.

Fig. 22. Localization of D-Gγ1 transcripts in late-stage embryos. Cryostat sections of embryos were probed with ^{3}H-labeled D-Gγ1 common probe (fragment D, Fig. 3) in panel A, and with ^{35}S-labeled 2.6-kb RNA-specific D-Gγ1 probe (fragment C, Fig. 3) in panel C. Panels A, C, bright field; B, D, dark field; *vc*, ventral nerve cord.


A
VC
B
C
b
VC
D

in the total RNA extracted from this stage is expected to be low. This may explain why such a faint hybridization signal was observed from this RNA in the developmental Northern blot experiment. The results also suggest that the hybridization signals observed over other cell types with the common probe must be due to the expression of 1.1-kb RNA. The high level of expression of the 1.1-kb and 2.6-kb RNAs in the nervous system proper suggests that the D-Gγ1 protein plays an important role in the differentiation and/or the function of the nervous system.

3.2.5. Larvae

The Northern blot analysis using a common probe showed that in third instar larvae the 2.6-kb RNA expression is restricted to the brain ganglia (see Fig. 16). This experiment also indicated that the 1.1-kb RNA is expressed in brain ganglia as well as in other tissues of the larvae (Fig. 16). Consistent with this expression pattern, *in situ* hybridization of the 2.6-kb RNA-specific probe to larval tissue sections showed that this RNA is expressed only in the cortical region of the developing larval brain ganglia (Fig. 23). When the common probe was used, hybridization over the brain ganglia and other larval tissues, such as imaginal discs, primordial gut tissues, etc., was observed (Fig. 24). These results suggest that the 1.1-kb RNA is expressed in the nervous system and other tissues of the larva.

Fig. 23. Localization of the 2.6-kb D-Gγ1 transcript in larvae. Cryostat section of a larva was probed with ^{35}S-labeled 2.6-kb RNA-specific D-Gγ1 probe (fragment C, Fig. 3) Panels A, bright field; B, dark field; *bg*, brain ganglia.

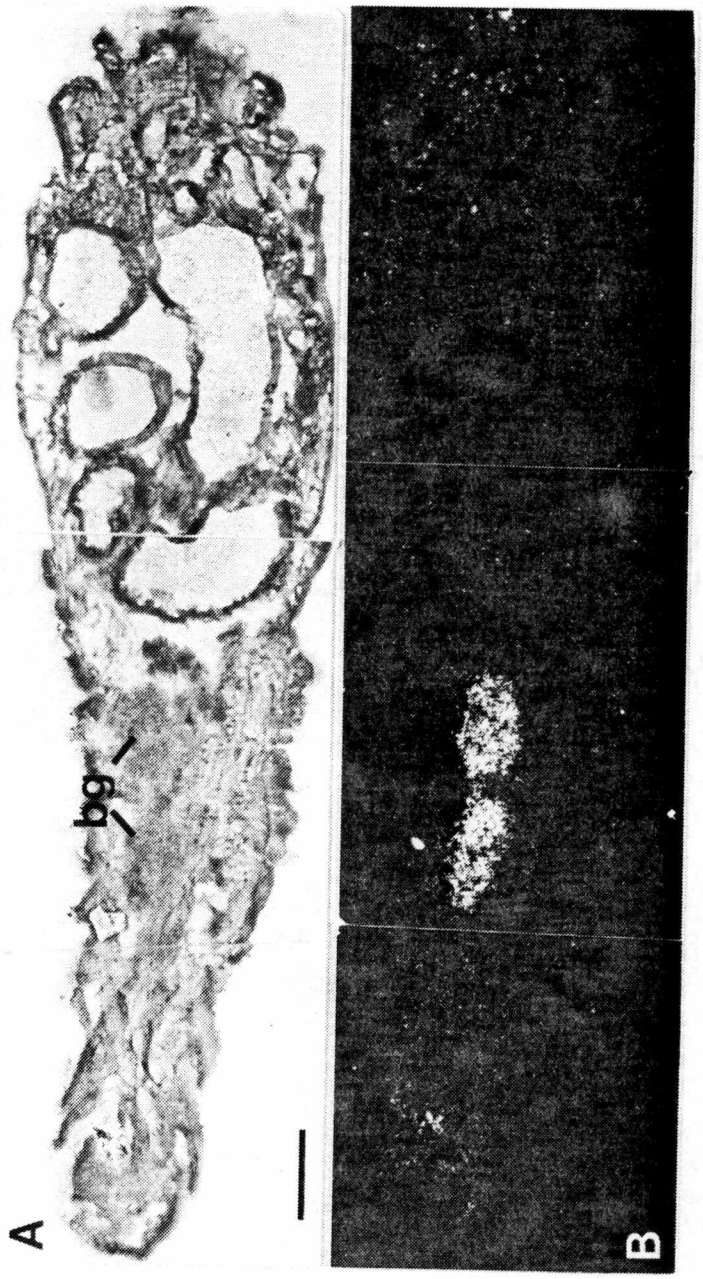

A
bg
B

Fig 24. Localization of other D-Gγ1 transcripts in larvae. Cryostat section of a larva was probed with ^{3}H-labeled common probe (fragment D, Fig. 3). Panel A, bright field; B, dark field; *bg*, brain ganglia.

A
bg
B

4. DISCUSSION

The current study confirms that the *D-Gγ1* gene is expressed predominantly in the central nervous system of the adult flies, indicating its possible role in neuronal signal transduction pathways. Abundant expression of D-Gγ1 transcripts in the developing CNS of larvae also suggests that D-Gγ1 protein is coupled to the regulatory cascade driving the differentiation process of the nervous system. It is also possible that D-Gγ1 subunit needs to be coupled with various other G-protein-mediated signaling pathways during various developmental stages and for these reasons the D-Gγ1 gene is expressed in a variety of tissues of embryo, larva, and adult.

In metazoan organisms, the developmental role of G-protein-coupled signal transduction pathways has not been clearly elucidated. Studies in simpler organisms, such as yeast and *Dictyostelium*, indicate that G-protein-coupled pathways may play an important role in mediating signals for the activation of developmental programs. For example, in yeasts, developmental processes initiated by exposure to pheromones are mediated by G-protein-coupled signal transduction events (Dietzel & Kursan, 1987). In *Dictyostelium*, receptors responsible for initiating cellular aggregation and differentiation have been shown to be coupled to developmentally regulated G proteins (Hadwiger, 1991). In both these organisms, G-protein-coupled transduction is predominantly used to convey signals for developmental

consequences. Since a wide variety of signals (peptides, small molecules, light, hormones, neurotransmitters, odorants etc.) are used in *Drosophila*, different forms of signaling pathways may be used in this organism and some of the pathways may be G-protein-mediated. This diversity may explain the complex pattern of expression of the *D-Gγ1* gene during development.

Roles of G proteins in early development and oogenesis have been indicated in *Drosophila* and *Xenopus* (Kroll et al., 1991; Wolfgang et al., 1992). For example, in *Drosophila*, Gαi, Gαo and a maternal effect gene *concertina*, encoding an α-subunit of a G protein, are expressed during oogenesis (Parks & Wieschaus, 1991). Interestingly, mutation of the *concertina* gene causes female sterility and embryonic lethality in early gastrulation stage (Parks & Wieschaus, 1991). In *Xenopus*, it has been shown that the α subunit of Go protein induces oocyte maturation (Kroll et al., 1991). Although the pattern of expression of D-Gγ1 protein is not known, the presence of a 1.3-kb D-Gγ1 transcript in oocytes and early embryonic stages implies that G-protein-mediated signaling processes may be required for early development and oogenesis in *Drosophila*.

The 1.1-kb mRNA is expressed zygotically but not maternally. Interestingly, expression of this RNA begins at the time (6 h) when the level of the 1.3-kb RNA starts declining. After 6 h, the 1.1-kb RNA becomes the predominant

D-Gγ1 mRNA species in the embryonic stages. The level of this RNA is also highest throughout development and in adults. Besides its high level of expression in the cortical regions of the brain and thoracic ganglia, the 1.1-kb RNA is also expressed in every cell type in embryos, larvae and adults. This suggests that the 1.1-kb RNA-encoded protein plays an important role in a variety of signaling pathways in neuronal and other cell types. Analysis of the 1.1-kb RNA-specific cDNA will reveal whether this RNA encodes the same polypeptide encoded by the 2.6-kb RNA or a variant of it. These studies have been included in the next chapter.

The pattern of expression of the 2.6-kb mRNA is different from the other two RNAs. It is expressed only in the nervous system of late embryos, larvae and adults, suggesting that the D-Gγ1 protein encoded by this RNA is involved only in neuronal transduction and differentiation processes. A possible role of cell signaling molecules in neuronal differentiation has been suggested in different organisms. For example, nerve growth factors have been shown to play an important role in the induction of nerve cell differentiation (Alema et al., 1985). In *Drosophila* and in other systems, in *vivo* and in *vitro* observations support the view that G proteins play a crucial role in axonal outgrowth and establishing neuronal connections within the developing nervous system (Wolfgang et al., 1992; Haydon et al., 1987; McCobb et al., 1988; Lankford et al., 1987). For example, in *Drosophila*, expression of Gsα

and Goα proteins were detected in the neuropil of the CNS as it begins to form during mid embryogenesis (Wolfgang, 1992). In PC-12 neuronal cultured cells and brain of neonatal rats, Gαo has been detected in growth cones and shown to interact with a growth cone-specific protein (Strittmatter et al., 1990). In other invertebrates, serotonin, which has been shown to modulate axonal outgrowth and neuronal connections, is coupled with G-protein-mediated pathways (Haydon et al., 1987; McCobb et al., 1988). The high level of expression of the *D-Gγ1* gene in the CNS of embryos, larvae and adults may thus be a reflection of the participation of G protein-mediated signaling pathways in neural function and differentiation.

The results of *in situ* hybridization in this investigation have shown that at least one of the three D-Gγ1 RNAs is present in *Drosophila* cells at all stages of development. Although this makes the expression of this gene ubiquitous, the level of expression of these RNAs varies in different tissues. The highest level of expression of the D-Gγ1 gene is found in the neural tissues, and this matches well with that of Gαi, Gαs , Gαo, Gβ-subunits of *Drosophila* G proteins (Table 2). This similarity is quite expected because α, β, and γ subunits together are needed to form G protein and G protein is known to be involved in signal transduction. It is, however, interesting to note that the levels of expression of α, β, and γ subunit genes of *Drosophila* is not constant

Table 2. Expression of mRNAs for Gα, Gβ and D-Gγ1 subunits during *Drosophila* development:

Stage	D-Gγ1	Gαo	Gαs	Gαi	Gβ
Adult	CNS, cortex	CNS, cortex	*	*	CNS, cortex
Oogenesis					
	oocytes, nurse cells	*	oocytes, nurse cells	*	oocytes, nurse cells
Embryos					
early	*	-	uniform	*	uniform
mid	*	cortex, CNS	uniform	*	uniform
late	*	cortex, CNS	-	*	uniform, CNS-cortex
Larva	*	*	-	*	uniform, CNS-cortex

* indicates mRNA is detected; tissue distribution unknown

\- indicates absence or lack of knowledge about RNA or tissue distribution

during embryogenesis, indicating that the G protein may play important roles in certain stages of development. Moreover, it is intriguing to find that D-Gγ1, Gαo (de Sousa et al, 1989), and phospholipase C (Shortridge et al., 1991) transcripts co-localize in the thoracic ganglia, abdominal ganglia, brain, ovarian nurse cells, oocyte and embryonic stages, indicating that G-protein-coupled signals may also act via phospholipase C (second messenger) in these tissues.

The present investigation has demonstrated that the D-Gγ1 transcripts show differential tissue-specific and developmental expression patterns. The significance of having three transcripts from one *D-Gγ1* gene and the relationship among these transcripts are not clear at this point. It is possible that the three RNAs of different sizes are products of alternative splicing and also that these RNAs differ from one another in their coding regions. Such differences can produce protein isoforms with different functional specificities. However, the length difference may also be a result of differential RNA processing in different tissues and developmental stages without involving any change in the coding region. To determine the actual mechanisms used for generating these D-Gγ1 mRNAs, cDNA clones specific for all three mRNAs need to be characterized and a detailed genomic organization study of *D-Gγ1* gene has to be done. The next chapter discusses the results of these investigations.

CHAPTER 4

ORGANIZATION OF THE *D-Gγ1* GENE OF *DROSOPHILA MELANOGASTER*

1. INTRODUCTION

Different subtypes of γ-subunits encoded by different genes have been identified in mammals which are encoded by different genes (Yatsunami et al., 1985; Gautam et al., 1989; Robishaw et al., 1989; Fisher & Aronson, 1992; Cali et al., 1992). In contrast, this work has shown that only one γ-subunit gene (*D-Gγ1*) is presumably present in *Drosophila*; it produces three mRNAs of different lengths and with differential tissue-specific and temporal expression pattern. It is possible that these mRNAs encode different isoforms of γ-subunit which need to be differentially distributed in development. If this is the case, the *D-Gγ1* gene must possess alternative coding exons which are spliced differentially to produce three mRNAs coding for three protein isoforms. Therefore, it is of particular interest to determine the mechanism by which these three D-Gγ1 mRNAs are formed and whether they differ in their coding regions. Thus, cDNA clones specific for 1.1- and 1.3-kb RNAs of the *D-Gγ1* gene were isolated and characterized. In addition, a detailed analysis of the *D-Gγ1* gene organization was also

undertaken. The results show that transcription of all three D-Gγ1 mRNAs begins at the same site, and their size difference is a result of alternative splicing and differential usage of two alternative polyadenylation sites. The *D-Gγ1* gene organization study has shown that unlike in mammals, all three mRNAs encode the same D-Gγ1 protein. The significance of these findings will be discussed.

2. MATERIALS AND METHODS

2.1. Isolation of genomic and cDNA clones

The isolation of clone 536 has been described by Levy et al., (1982). An overlapping genomic clone with more 3′-downstream sequences was isolated from a genomic library of Canton S strain using a 0.4-kb *Pst*I-*Xba*I fragment located near the 3′-end of the 536-B2 cDNA as a probe. This library was made by cloning genomic DNA fragments at the *Bam*HI site of λEMBL3 phage (Krishnan et al., 1991).

To isolate the 1.1- and 1.3-kb mRNA-specific cDNA clones, the following cDNA libraries of *D.melanogaster* were screened: an oligo(dT)-primed head cDNA library made in the λSWAJ2 vector (Palazzolo & Meyerowitz, 1987), an oligo(dT)-primed 3-12 hour embryonic cDNA library made in λgt10 phage (from T.Kornberg), an oligo(dT)-primed 0-3 h embryonic cDNA library made in λgt10 (from T. Kornberg), and an oligo(dT) plus random primed ovary cDNA library made in λZap vector

(from T. Hazelrigg).

2.2. DNA sequencing

The cDNA and genomic DNA fragments were subcloned in pBSK+ plasmid and sequenced by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase version-II kit (U.S. Biochemical Corp). When needed, specific synthetic oligonucleotide primers were used for sequencing.

2.3. Determination of the transcription start site

The transcription start site was mapped by primer extension method as described by Sambrook et al., (1989). An oligonucleotide primer (20 mer) was 5'-end-labeled by using [γ^{32}P]-ATP and polynucleotide Kinase (Maniatis et al., 1982). Poly(A)$^+$ RNA (5-10 μg) was mixed with 5 pmol labeled primer in 30% formamide and incubated overnight at 20^0 C. The extension reaction was allowed to proceed in 2x reaction buffer with 1000u/ml AMV reverse transcriptase. The products were precipitated with ethanol and electrophoresed on sequencing gels.

An RNase protection assay as described by Zinn et al., (1983) was also used to map the transcription start site. Poly(A)$^+$ RNA (10-15 μg) precipitated with ethanol was redissolved in hybridization buffer. Antisense RNA probes were synthesized using 75 μCi of α^{32}P-UTP and 1 μg of DNA template. After synthesis of cRNA probe, the DNA template

was removed by DNase-I digestion. The probe was then ethanol precipitated and redissolved in hybridization buffer. For hybridization, $1x10^5$ cpm probe was added to the poly(A)$^+$ RNA solution and annealing was done overnight at 50^0C. The hybrids were digested with an RNase solution (RNase T1 + RNase A) and the resistant products were electrophoresed on sequencing gels for maximum resolution.

3. RESULTS

3.1. All three D-Gγ1 mRNAs encode the same protein and use alternative polyadenylation signals.

Since the 2.6-kb D-Gγ1 RNA is not expressed in 3-12 h embryos, and 1.1-kb RNA is the most prominent species at this stage of development, a 3-12 h embryo cDNA library was screened with the common probe to isolate the 1.1-kb RNA-specific cDNA clone. There was, however, a small chance that the clones hybridizing with the common probe might contain cDNAs for the 1.3-kb RNA which is present at a very low level in 3-6 h embryos. Therefore, before further characterization of the clones isolated from the 0-3 h embryonic library, a head cDNA library was also screened. The rationale and strategy of this screening are described below.

The previous sequence data identified a polyadenylation signal, located at 845 nt within the 2.6-kb

RNA (Chapter 2, Fig. 6). If this signal is used in production of the 1.1-kb RNA, in an oligo(dT)-primed cDNA library (e.g., the head cDNA library), the 1.1-kb RNA-specific cDNAs are expected to end 20-30 nt downstream from this polyadenylation signal at 845 nt. It is also expected that the cDNAs will hybridize with the common probe (fragment D, Fig. 3) but not with the 2.6-kb RNA-specific probe (fragment C, Fig. 3). Therefore, the head cDNA library was co-screened with these two probes. As expected, some clones hybridized only with the common probe and some with both probes. The latter clones, presumed to be 2.6-kb RNA-specific, were not characterized any further. The former clones were, however, isolated and characterized together with cDNA clones isolated from the 3-12 h embryo library.

Sequencing of an 817 nt long cDNA clone isolated from the 3-12 h cDNA library (536-C1, Fig. 25) revealed that this cDNA encodes the same polypeptide as the 2.6-kb RNA-specific cDNA (536-B2 cDNA, Fig. 25). Except for a single base substitution (Fig. 26, 312 nt) which is probably a result of polymorphism or allelic divergence, the cDNA is identical to the 817 nt of the 536-B2 cDNA (536-B2 sequence shown in Fig. 6). The 3'-end of the 536-C1 cDNA, however, terminates at 865 nt within the 3'-UTR of the 2.6-kb mRNA (Fig. 26). This was reconfirmed by sequencing another 1.1-kb RNA-specific cDNA clone which was isolated from the head cDNA library.

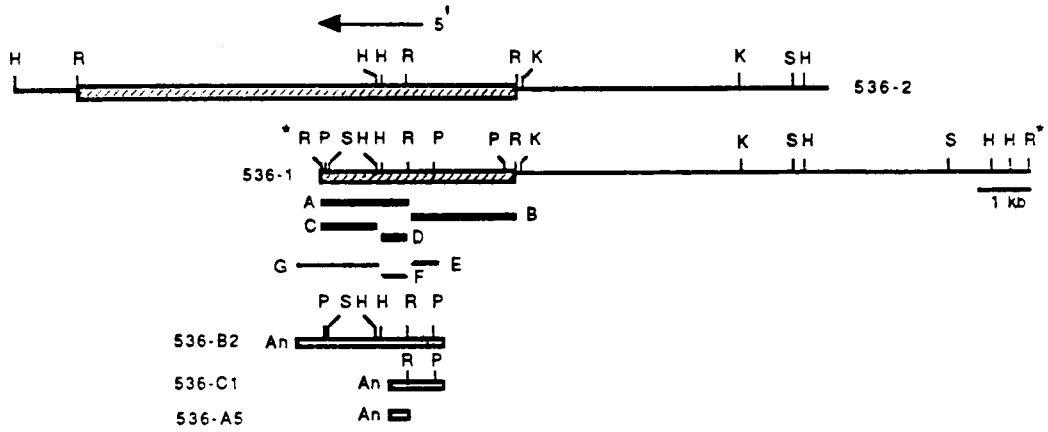
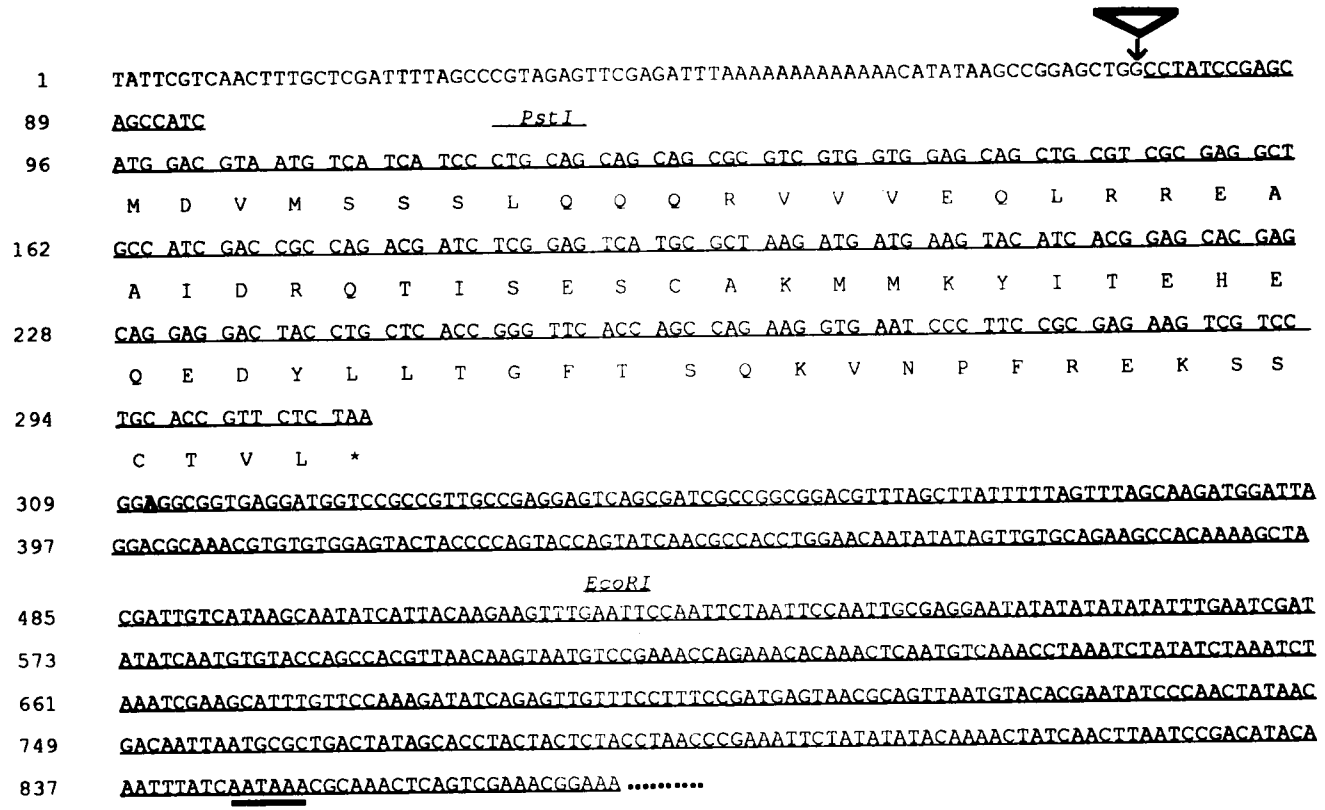


Fig. 25. Restriction maps of two D-Gγ1 genomic clones and different cDNA clones. The restriction map of clone 536-1 has been shown in Fig. 3. Using the 3′-end fragment of 536-B2 cDNA, a second overlapping genomic clone was isolated. The new genomic clone, designated as 536-2, was partially mapped for the restriction enzyme sites indicated above. The *hatched bar* in the genomic clones indicate the transcribed region, the boundary of which are not known at this point. The *open bars* represent different cDNA clones isolated by using the transcribed DNA fragments as probes (see text). The direction of transcription was shown by an *arrow*. Fragments A-D are from clone 536-1, and fragments E-F are from 536-B2 cDNA clone. Restriction sites with *astericks* are the cloning sites in λ-Charon 4 vector. R, *Eco*RI; H, *Hind*III; S, *Sst*I; P, *Pst*I; K, *Kpn*I.

Fig. 26. Nucleotide sequence of the 1.1- and 1.3-kb D-Gγ1 RNA-specific cDNA clones. The conceptual ORF is the same as that of the 2.6-kb D-Gγ1 RNA. A polyadenylation site AATAAA, at position 846 nt is used by both 1.1- and 1.3-kb RNAs. Underlined nucleotides denote that these sequences were also determined in the corresponding genomic DNA. The triangle in the 5′-UTR indicates the position of an extra 188 nt sequence which is present only in the 1.3-kb RNA-specific cDNAs.


1 TATTCGTCAACTTTGCTCGATTTTAGCCCGTAGAGTTCGAGATTTAAAAAAAAAAAAAACATATAAGCCGGAGCTGGCCTATCCGAGC
89 AGCCATC PstI
96 ATG GAC GTA ATG TCA TCA TCC CTG CAG CAG CAG CGC GTC GTG GTG GAG CAG CTG CGT CGC GAG GCT
M D V M S S S L Q Q Q R V V V E Q L R R E A
162 GCC ATC GAC CGC CAG ACG ATC TCG GAG TCA TGC GCT AAG ATG ATG AAG TAC ATC ACG GAG CAC GAG
A I D R Q T I S E S C A K M M K Y I T E H E
228 CAG GAG GAC TAC CTG CTC ACC GGG TTC ACC AGC CAG AAG GTG AAT CCC TTC CGC GAG AAG TCG TCC
Q E D Y L L T G F T S Q K V N P F R E K S S
294 TGC ACC GTT CTC TAA
C T V L *
309 GGAGGCGGTGAGGATGGTCCGCCGTTGCCGAGGAGTCAGCGATCGCCGGCGGACGTTTAGCTTATTTTTAGTTTAGCAAGATGGATTA
397 GGACGCAAACGTGTGTGGAGTACTACCCCAGTACCAGTATCAACGCCACCTGGAACAATATATAGTTGTGCAGAAGCCACAAAAGCTA
EcoRI
485 CGATTGTCATAAGCAATATCATTACAAGAAGTTTGAATTCCAATTCTAATTCCAATTGCGAGGAATATATATATATATTTGAATCGAT
573 ATATCAATGTGTACCAGCCACGTTAACAAGTAATGTCCGAAACCAGAAACACAAACTCAATGTCAAACCTAAATCTATATCTAAATCT
661 AAATCGAAGCATTTGTTCCAAAGATATCAGAGTTGTTTCCTTTCCGATGAGTAACGCAGTTAATGTACACGAATATCCCAACTATAAC
749 GACAATTAATGCGCTGACTATAGCACCTACTACTCTACCTAACCCGAAATTCTATATATACAAAACTATCAACTTAATCCGACATACA
837 AATTTATCAATAAACGCAAACTCAGTCGAAACGGAAA .....

This cDNA (536-A5, Fig. 25) was incomplete and only 352 nt long. Sequencing results showed that the 3′-end of this cDNA also ends at the same site where the 536-C1 cDNA ends. Further, the nucleotide sequence of the 536-A5 cDNA is identical to the last 352 nucleotides of the 536-C1 cDNA. Taken together these sequence data suggest that the 1.1-kb RNA is produced by the utilization of an alternative polyadenylation site, AATAAA, located at nt 845 and 20 nt away from the poly-A tail.

While the 2.6- and 1.1-kb RNAs are predominantly expressed in nervous tissues, the 1.3-kb RNA is expressed in 0-3 hour embryos and in the ovary. Also, Northern blot analysis (Fig. 15; chapter 3) showed that in 0-3 h embryos and in the ovary , 2.6- and 1.1-kb RNAs are not expressed. Four cDNA clones, two each from ovary and 0-3 h embryonic libraries, were isolated and analyzed by DNA sequencing. The results showed that the 3′- end of all these cDNAs ends at 865 nt within the 3′-UTR of the 2.6-kb RNA (Fig. 26). Therefore, the polyadenylation signal at 845 nt is presumably utilized by the 1.3-kb mRNA. This same polyadenylation signal is also utilized by the 1.1-kb RNA (Fig. 26).

The sequence data also revealed that the coding region of the 1.3-kb RNA is identical to those found in the other two D-Gγ1 RNAs. However, the 1.3-kb RNA-specific cDNA contains an extra 188 nt sequence in the 5′-UTR which is not

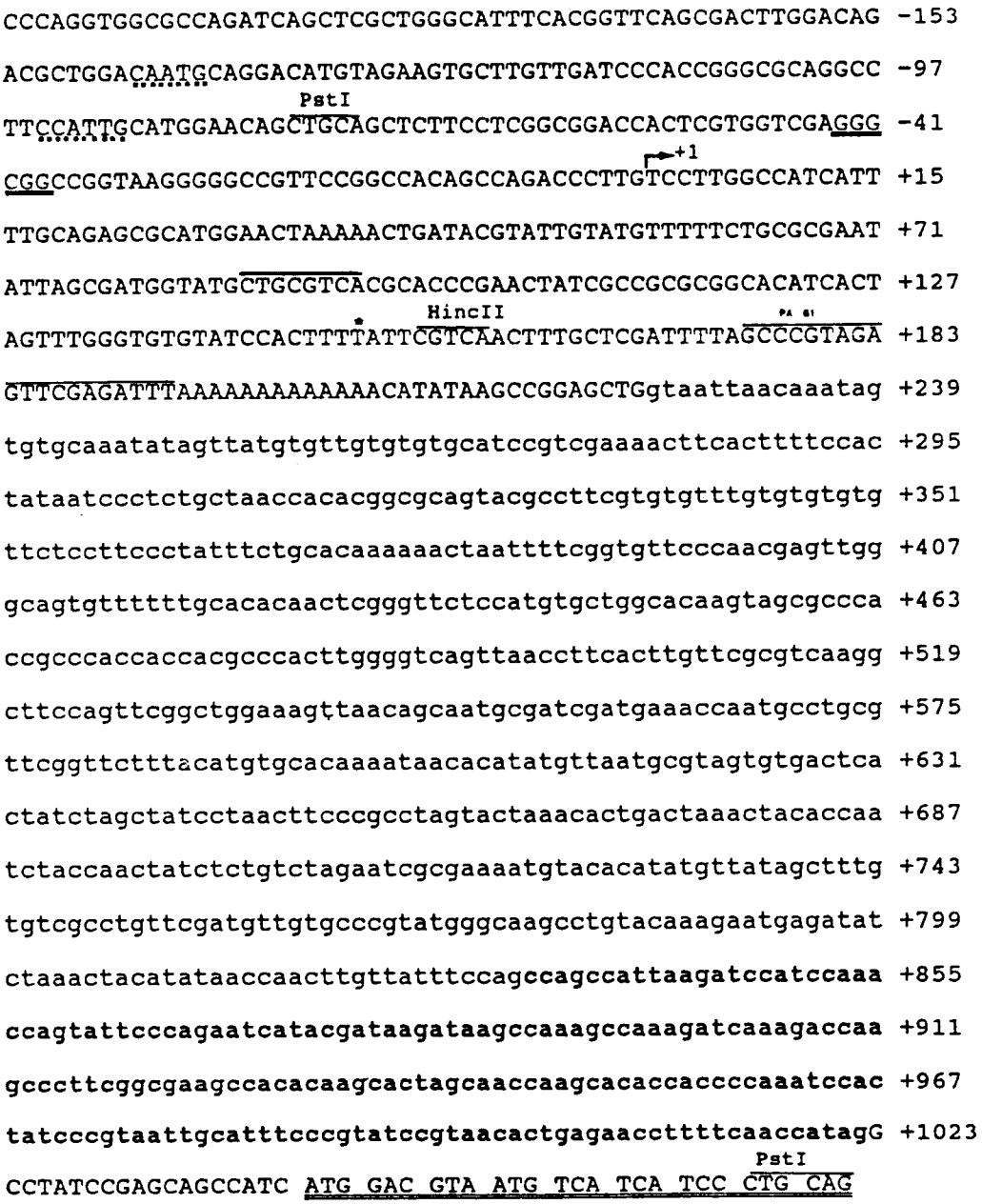
found in the 2.6- and 1.1-kb RNAs (Fig. 26). This suggests that the *D-Gγ1* gene has a 188 nt long 5′-untranslated exon which is retained in the 1.3-kb RNA but it is spliced out in the case of the 2.6- and 1.1-kb RNAs as an intron. This possibility was examined by sequencing of the corresponding genomic DNA and these results are described in the next section.

3.2. Alternative splicing in the 5′-UTR region of the *D-Gγ1* gene

To define the number and position of exons and introns more precisely within the *D-Gγ1* gene, a portion of the 2.6-kb *Eco*RI genomic DNA fragment containing the coding and 5′-upstream region of the *D-Gγ1* gene (Fig. 25) was sequenced. By comparing the nucleotide sequences of this genomic DNA and different cDNA clones, it was concluded that the coding region of the *D-Gγ1* gene does not have any intron. This confirms again that all three D-Gγ1 mRNAs code for the same D-Gγ1 protein.

The genomic sequencing data (Fig. 26 & 27) also revealed some interesting features of the *D-Gγ1* gene. First, the 5′-UTR of the *D-Gγ1* gene is interrupted by an intron. Second, the first exon representing part of the 5′-UTR, is present in all three RNAs. Third, while the same 3′ splice site of the intron is used for the 1.1- and 2.6-kb RNAs, in the case of the 1.3-kb RNA an alternative 3′

Fig. 27. Nucleotide sequence of the 1.4-kb *Pst*I genomic DNA encompassing first nine codons and the upstream region. The coding region of the *D-Gγ1* gene is shown by *double* underlines. The lower case *bold* letters comprise the 188 nt exon sequence of the 1.3-kb RNA, which is not present in the 2.6- and 1.1-kb D-Gγ1 RNAs. Intron sequences common for all three RNAs are shown in lower case letters. *PA 61* is the primer used for primer extension analysis. An *asterisk* on the top of nucleotide 150 indicates the 5′-extent of the longest cDNA clone. Potential GC and CAAT box sequences are underscored with *solid* and *broken* lines, respectively. A cyclic AMP-responsive element (CTGCGTCA) is overlined. Location of the *Pst*I genomic fragment in the genomic DNA is shown with *dashed* lines in the map above.


CCCAGGTGGCGCCAGATCAGCTCGCTGGGCATTTCACGGTTCAGCGACTTGGACAG -153
ACGCTGGACAATGCAGGACATGTAGAAGTGCTTGTTGATCCCACCGGGCGCAGGCC -97
PstI
TTCCATTGCATGGAACAGCTGCAGCTCTTCCTCGGCGGACCACTCGTGGTCGAGGG -41
+1
CGGCCGGTAAGGGGGCCGTTCCGGCCACAGCCAGACCCTTGTCCTTGGCCATCATT +15
TTGCAGAGCGCATGGAACTAAAAACTGATACGTATTGTATGTTTTTCTGCGCGAAT +71
ATTAGCGATGGTATGCTGCGTCACGCACCCGAACTATCGCCGCGCGGCACATCACT +127
HincII
AGTTTGGGTGTGTATCCACTTTTATTCGTCAACTTTGCTCGATTTTAGCCCGTAGA +183
GTTCGAGATTTAAAAAAAAAAAAAACATATAAGCCGGAGCTGgtaattaacaaatag +239
tgtgcaaatatagttatgtgttgtgtgtgcatccgtcgaaaacttcacttttccac +295
tataatccctctgctaaccacacggcgcagtacgccttcgtgtgtttgtgtgtgtg +351
ttctccttccctatttctgcacaaaaaactaattttcggtgttcccaacgagttgg +407
gcagtgtttttgcacacaactcgggttctccatgtgctggcacaagtagcgccca +463
ccgcccaccaccacgcccacttggggtcagttaaccttcacttgttcgcgtcaagg +519
cttccagttcggctggaaagttaacagcaatgcgatcgatgaaaccaatgcctgcg +575
ttcggttctttacatgtgcacaaaataacacatatgttaatgcgtagtgtgactca +631
ctatctagctatcctaacttcccgcctagtactaaacactgactaaactacaccaa +687
tctaccaactatctctgtctagaatcgcgaaaatgtacacatatgttatagctttg +743
tgtcgcctgttcgatgttgtgcccgtatgggcaagcctgtacaaagaatgagatat +799
ctaaactacatataaccaacttgttatttccagccagccattaagatccatccaaa +855
ccagtattcccagaatcatacgataagataagccaaagccaaagatcaaagaccaa +911
gcccttcggcgaagccacacaagcactagcaaccaagcacaccaccccaaatccac +967
tatcccgtaattgcatttcccgtatccgtaacactgagaacctttcaaccatagG +1023
PstI
CCTATCCGAGCAGCCATC ATG GAC GTA ATG TCA TCA TCC CTG CAG

splice site located 188 nt upstream from the other splice site is used (Fig. 27). Due to this, the length of the second exon is identical in 1.1- and 2.6-kb RNAs, but in the case of the 1.3-kb RNA, it is 188 bases longer at the 5′-end. Analysis of these data also showed that the splice sites used by all three mRNAs are in agreement with the GT and AG consensus splice junction sequences demarcating the 5′- and 3′- boundaries of introns (Padgett et al., 1986).

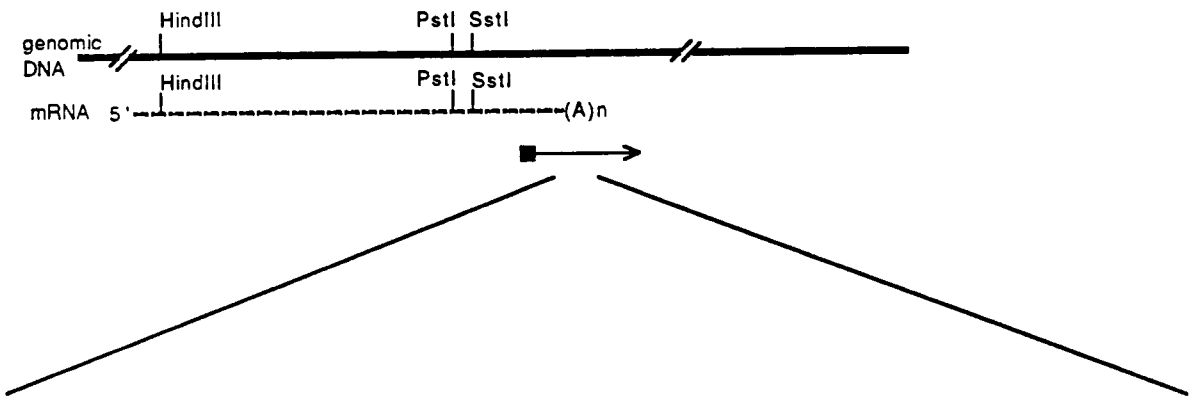
3.3. Mapping of the 3′-end of the *D-Gγ1* gene

Based on the results of restriction mapping of the genomic and cDNA clones, it was suspected that the original clone 536 (536-1; Fig. 25) does not contain the 3′- UTR of the *D-Gγ1* gene, and it must be present in downstream genomic DNA. Therefore, to locate the 3′-end of the *D-Gγ1* gene, genomic clones containing more downstream sequences were isolated by screening a genomic library with the 3′-end fragment (0.4-kb *Pst*I-*Xba*I, Fig. 25) of the 536-B2 cDNA as a probe. DNA of five of these genomic clones was digested with *Eco*RI, Southern blotted and hybridized with the 0.4-kb *Pst*I-*Xba*I probe. In three clones, the probe hybridized with a 5.5 kb-*Eco*RI fragment. One of these clones, named clone 536-2 was restriction mapped and found to overlap the original genomic clone 536-1 (Fig. 25). To determine whether the 3′-end of the *D-Gγ1* gene is located in this genomic clone, the 5.5-kb *Eco*RI fragment was subjected to DNA sequencing. For quick

analysis, a 15-mer oligonucleotide primer located near the 3′-end of the 2.6-kb RNA and directed downstream was used for sequencing (primer location shown in Fig 28). The results showed that sequences of the 2.6-kb mRNA downstream from the primer are present within the 5.5-kb *Eco*RI fragment of the genomic clone 536-2 (Fig. 28). Thus, the 3′-end of the *D-Gγ1* gene is present in the 5.5-kb *Eco*RI fragment but its exact location is not known. In this context it is to be noted that the restriction map of the 2.6-kb RNA-specific cDNA is very similar to that of the corresponding genomic DNA suggesting the absence of any large introns. If there is any intron present in this region, it must be smaller than 100 base pairs, which is difficult to resolve by agarose gel electrophoresis.

3.4. Identification of the transcriptional start site

To map the transcription start site (tsp), two techniques were used: a primer extension method and an RNase protection assay. A 20- mer oligonucleotide primer complementary to all three D-Gγ1 mRNA sequences between 176 nt to 195 nt (Fig. 27, PA 61), was used for primer extension assay. When head Poly(A)$^+$ RNA was used, the size of the major primer extension product was found to be 195 nt long (Fig. 29, a). The female body Poly(A)$^+$ RNA also yielded the same extension product (Fig. 29, b,c). The same results were obtained in three independent experiments with these two RNA samples.



TTTGCTTAAATTTCAAAAATAAAAAGCGATAACTTACTTAGATTCGTTATCTAGCGGTGGCACCGGGTT

CGGCTGAAAACCACGTTGTTTTATTTCAAAGGCCCCCGAAGATCACGCGTTTCAGTTCAGTTTTCGTAA

GTTAAGTTTATTAATACAAGAAACT

Fig. 28. Nucleotide sequence of the 3′-end of the *D-Gγ1* gene. The *solid* line shows the restriction map of a part of the 5.5-kb *Eco*RI genomic fragment isolated from genomic clone 536-2, and the *broken* lines below the genomic map shows the 3′-region of the 2.6-kb D-Gγ1 RNA-specific cDNA (see Fig. 25. for details). The black box with a arrow indicates the position of the primer and the position of the sequences shown below. The nucleotide sequence of the 3′-end of the *D-Gγ1* gene is highlighted, showing the polyadenylation signal utilized by the 2.6-kb D-Gγ1 mRNA (underscored with *solid* lines) and the arrows on the top of nucleotides indicate the potential poly(A) addition site.

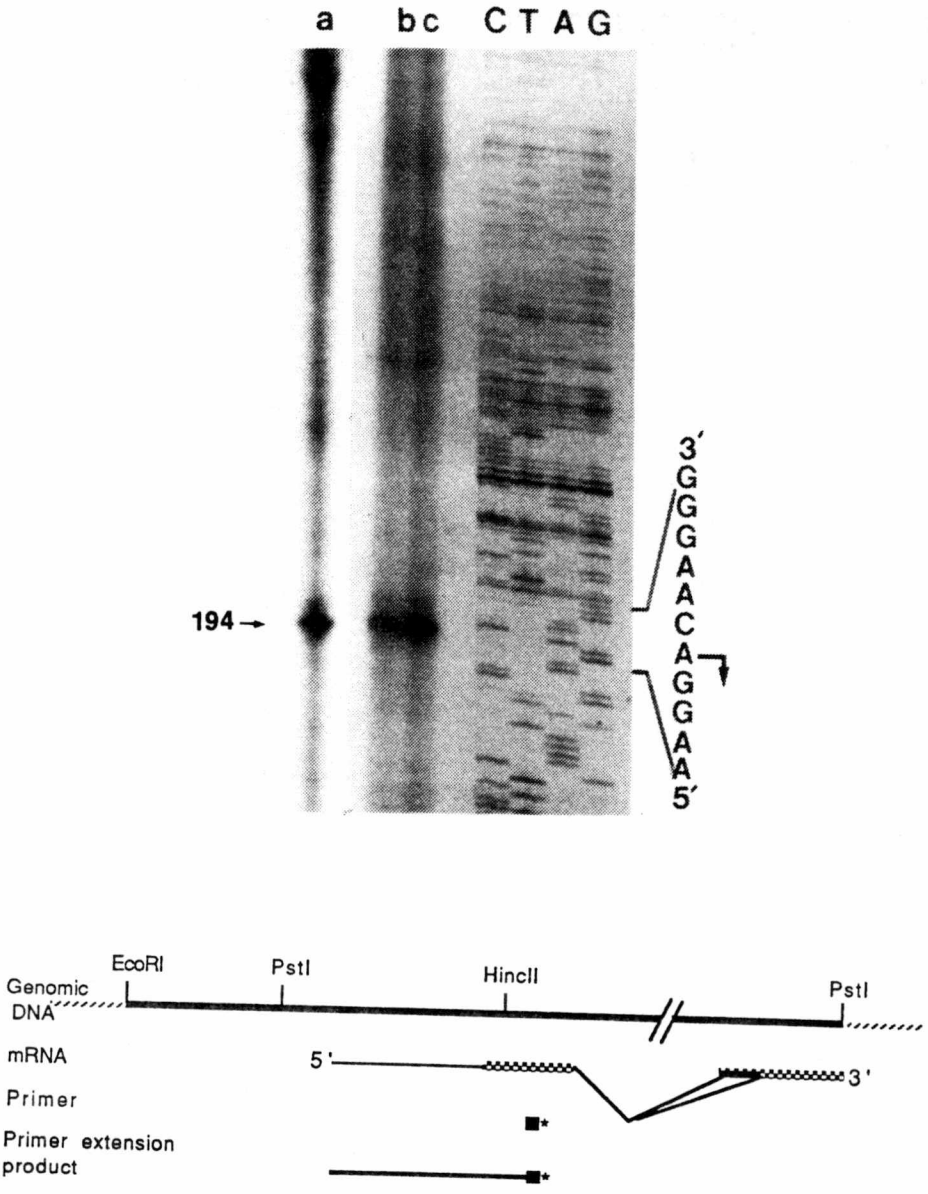


Fig. 29. Primer extension experiment to determine transcriptional start site. A ^{32}P-labeled 20-mer synthetic oligonucleotide (denoted as *PA 61*, Fig. 27) was used as a primer (shown here with a asterisk indicating 5′-end is labeled). Poly(A)$^+$ RNA isolated from head (lane a) and female body (lane b, c) were used as templates for primer extension. The synthesized cDNA was electrophoresed on an 8% denaturing polyacrylamide gel along with sequencing reaction products (C, T, A, G) initiated with the same primer. The number indicated by an arrow represents the putative transcriptional start site.

Since only one, but not three extended products was observed with these RNA samples which contain all three D-Gγ1 mRNAs, the transcription start site of all three RNAs appears to be located at the same site and at nt .

To further confirm the above observations, an RNase protection assay was used; the experimental strategy for this assay is shown in Fig. 30. A 240 base pair *PstI-HincII* genomic DNA fragment containing the first 10 nt of the 5′-UTR sequence of the *D-Gγ1* gene at the HincII end (Fig. 27) was used to synthesize a ^{32}P-labeled antisense RNA (riboprobe) *in vitro*. The poly(A)$^+$ RNA isolated from adult head and female body were individually annealed to the riboprobe and the hybrids were subsequently subjected to digestion by RNases A and T1. The RNase resistant product was then fractionated on a denaturing 8% polyacrylamide gel to determine the size of the protected riboprobe fragment. This experiment was repeated twice and in both cases a 159 nt fragment of the antisense RNA was protected by the head and body RNAs (Fig. 30). Therefore, the 5′-end of a D-Gγ1 mRNA is located 159 nt upstream from the *HincII* site. The same results were obtained with the primer extension assay. It is to be noted that the head and female body RNA used for protection together contained all three D-Gγ1 RNAs. However, only one RNase protected fragment was found, suggesting that transcription of all three mRNAs begins at the same site. These results and the results discussed in

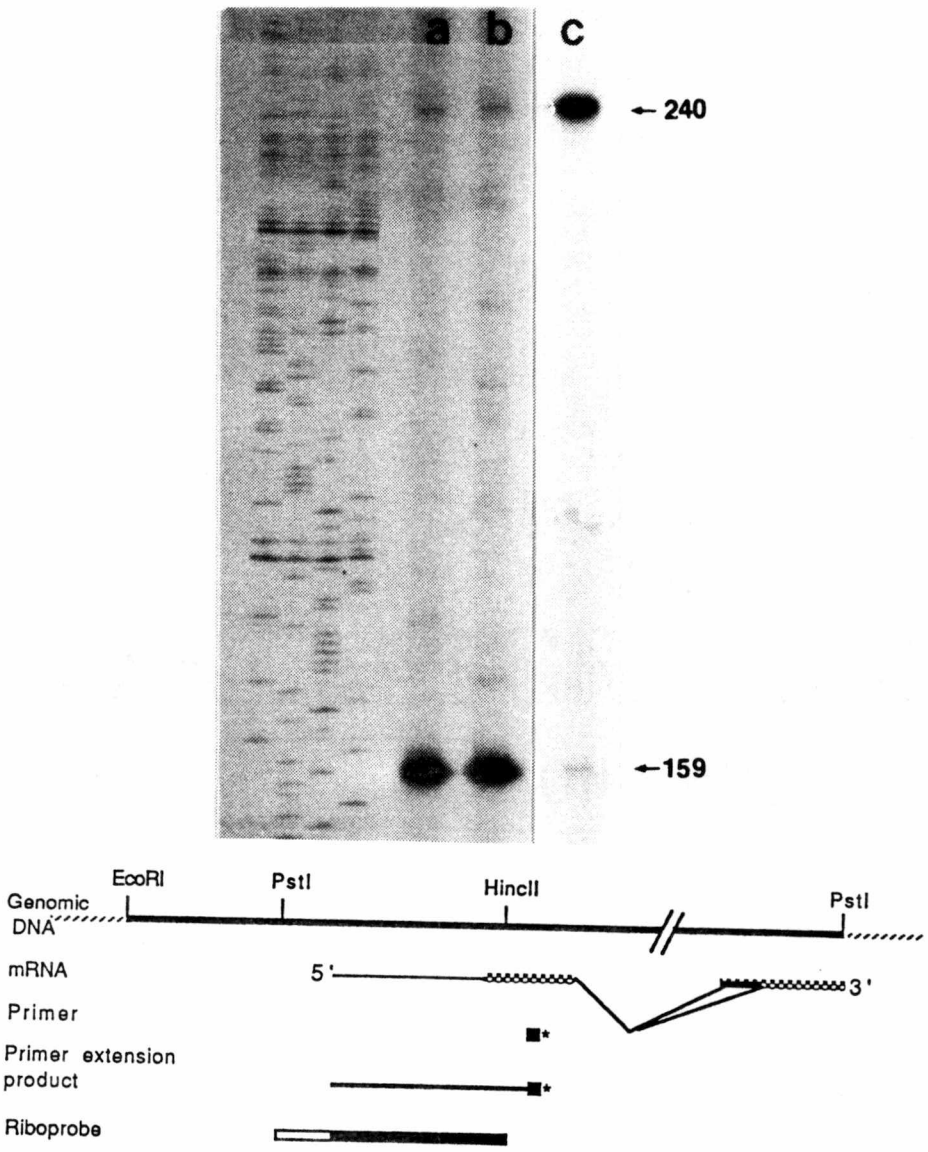


Fig. 30. RNase protection assay to confirm the transcriptional start site of the *D-Gγ1* gene. A 240 bp *Hinc*II-*Pst*I DNA template (see the map above) was used to generate ^{32}P-labeled anti-sense RNA (riboprobe). The riboprobe was annealed with the cellular RNA, digested with RNase A and RNase T1, and fractionated in a sequencing gel. Lane a, protected fragment after annealing with 5 μg of head total RNA; lane b, protected fragment after annealing with 5 μg female body total RNA; lane c, unprotected riboprobe as a control. The numbers indicated by arrows represent sizes in bp. The 240 nt long unprotected riboprobe is shown in the map, closed area in the riboprobe denotes the extent of protected fragment.

the previous sections now give a clearer picture of the organization of the *D-Gγ1* gene and it is shown in Fig. 31.

3.5. Analysis of the upstream DNA of the *D-Gγ1* gene

Since promoter sequences are usually present in the upstream DNA, upstream sequences of the transcription start site of the *D-Gγ1* gene were analyzed (see Fig. 27). Neither a typical TATA nor a CAAT-like motif was found in the upstream DNA sequence. However, a sequence (GCAAT) differing from the canonical CAAT box sequence, C-C-A-A-T, is present at -140 nt. Moreover, examination of the sequence in Fig reveals that in this upstream sequence, a typical GGCGGG (GC box) sequence is present 40 nt from the transcription start site. It has been reported that in many TATA-less genes, PolII promoters often contain a GGCGGG sequence or its reverse complement CCGCCC upstream (GC box), 30-50 nt upstream from the transcription start point (for review, Nussinov, 1990). In addition, the 5'-upstream region of the *D-Gγ1* gene has a high G+C content (63% G+C) which is another characteristic feature of the GC box containing promoters. Another interesting feature of this putative *D-Gγ1* promoter element is that it contains a cyclic AMP (cAMP) responsive element (CRE) within the leader sequence, indicating that transcription of the *D-Gγ1* gene may also be regulated by cAMP. The putative CRE sequence (CTGCGTCA) with CRE core

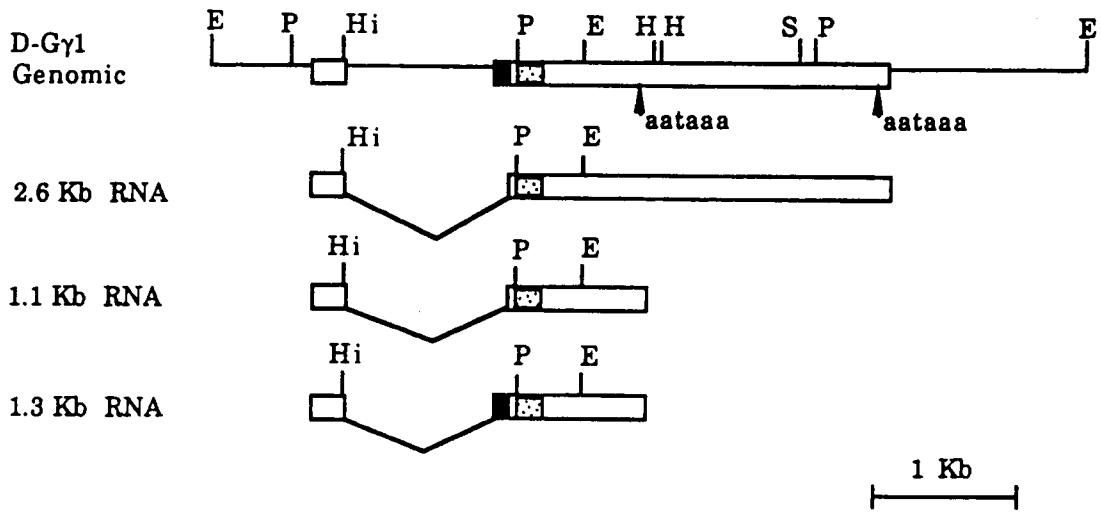


Fig. 31. A schematic representation of the structure and organization of the *D-Gγ1* gene. The open box represents the exons; dotted box represents the coding region; black box represents the extra 188 nt long exon region in the 5′-UTR of the 1.3-kb RNA, which is a part of the intron of 2.6- and 1.1-kb RNAs. The aataaa represents the location of alternative polyadenylation sites. E, *Eco*RI; P, *Pst*I; Hi, *Hinc*II; H, *Hind*III; S, *Sst*I.

motif, CGTCA, appears once in the +89 nt relative to the cap site. Although CRE elements are usually found in the upstream DNA, in the rat *prodynorphin (pro-DYN)* gene promoter, the CRE is located at 63 nt downstream to the cap site and has been proposed to impart cAMP responsiveness (McMurray et al., 1989).

4. DISCUSSION

Results presented in this section revealed certain features which may play crucial roles in the expression of the *D-Gγ1* gene. One is the difference in 3′-UTR length between 2.6-, 1.1- and 1.3-kb D-Gγ1 mRNAs. It is clear from the results that the differences in the length of the 3′-UTRs is a result of utilization of alternative polyadenylation signals. A similar mechanism of creating heterogeneity in the length of 3′-UTR has been observed in mRNAs of many other genes, such as chicken *pro α2(I) collagen*, mouse *MAP4*, human *myosin heavy chain* (Aho et al., 1983; Saez et a, 1990; Code & Olmsted, 1992). While the physiological significance of this size heterogeneity is not clear, the 3′-UTRs have been shown to be responsible for differential half lives of the mRNAs originating from a single gene, as seen in case of rat *IGF* gene (Lowe et al., 1989).

Another interesting feature of the *D-Gγ1* gene is the presence of a large untranslated leader sequence. In the case of 2.6- and 1.1-kb RNA, the 5′-UTR is 220 nt long

whereas, in case of 1.3-kb RNA the length is 420 nt (Fig. 27 & 31). 5′-UTRs longer than 200 nt are also found in other *Drosophila* genes, e.g., *yellow* (Chia et al., 1986), *Kruppel* (Rosenberg et al., 1986), *Notch* (Wharton et al., 1985), *Gld* (Whetten et al., 1988). Interestingly, a bovine G protein γ-2 subunit gene expressed only in the brain (J. Robishaw, personal communication) also has an mRNA with long 5′-UTR. Since the long 5′- UTRs of many of these genes contain multiple short upstream open reading frames (uORFs), the possibility exists that these 5′-UTRs regulate the translatability of the mRNA. The uORFs in the 5′-UTR have been documented to interfere with the "scanning" of ribosomal initiation complexes and thus inhibit initiation of translation (review: Kozak, 1989). The AUG triplets located in the 5′-UTR have been shown to significantly repress translation of several genes (Kozak, 1989). For example, 5′-UTR uORFs have been shown to repress translatability of the mammalian ODC mRNA (Manzella & Blackshear, 1990). When the 5′-UTR of the D-Gγ1 gene was examined, four uORFs were discovered, of which the longest uORF is 51 amino acids long (Fig. 32). It is thus possible that these uORFs are involved in regulating translatability of the D-Gγ1 mRNAs.

The 5′-UTR of the ovary-specific 1.3-kb D-Gγ1 mRNA contains an extra 188 nt sequence which is not found in the 2.6- and 1.1-kb RNAs. This extra 188 nt sequence is

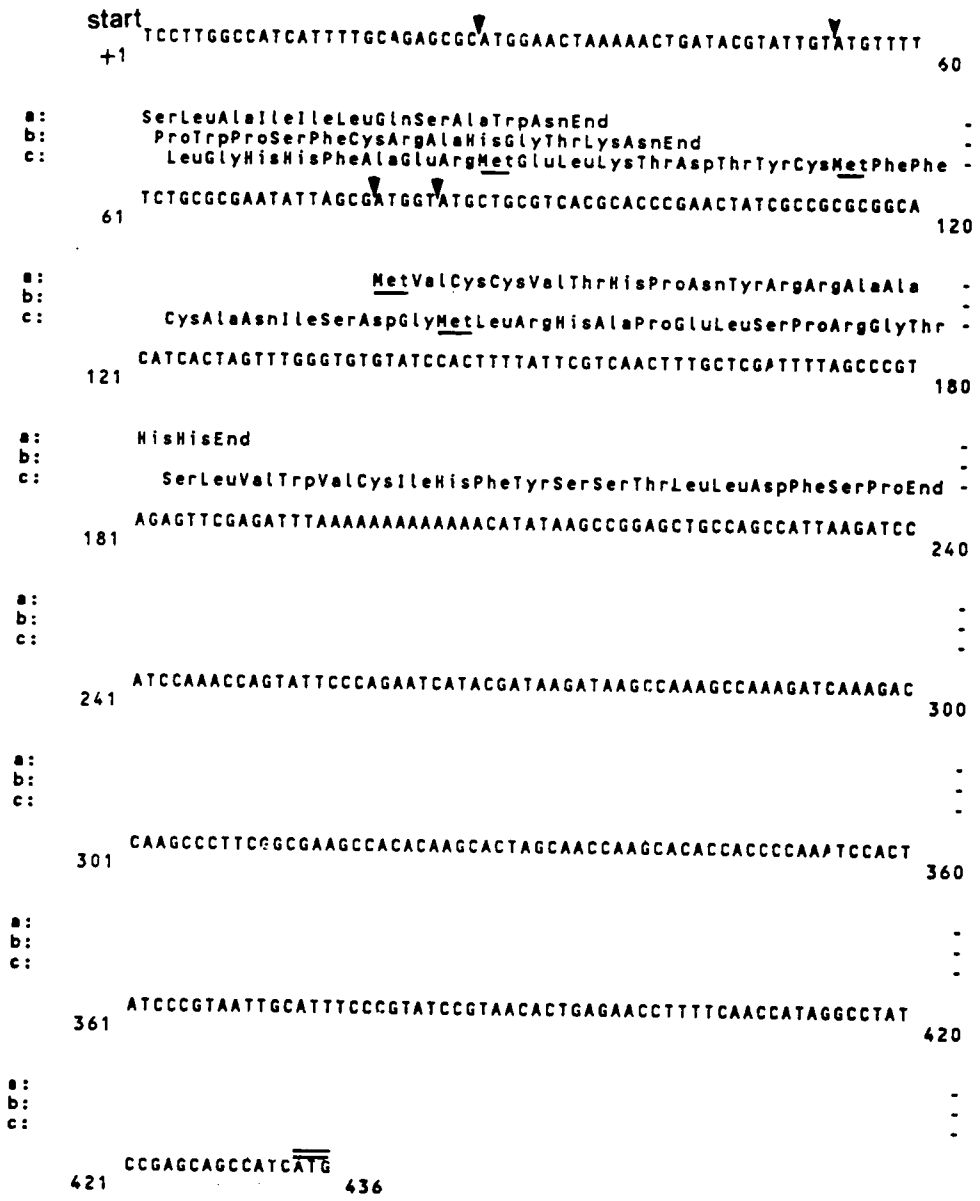


Fig. 32. 5′-UTR sequence of the *D-Gγ1* gene have several potential uORFs. The 5′-UTR of the *D-Gγ1* gene was examined for the presence of upstream open reading frames (uORFs) using the translate program of UWGCG. The upper nucleotide sequence is the 5′-UTR sequences present in D-Gγ1 mRNAs, which was translated in all three reading frames (shown as a, b, c). Four uORFs were identified, all of which start with a ATG start codon which are marked with *arrow* heads. The *D-Gγ1* translational start ATG codon marked with double lines is at position 433.

incorporated in the 1.3-kb RNA by differential selection of alternative 3′-splice site. This choice may be guided by an RNA binding protein which may be present only in the ovary. There are many examples of differential sex-specific pre-RNA splicing is in *Drosophila*. It has been shown that Sex lethal (Sxl), a *Drosophila* protein, regulates the choice between two alternative 3′- splice sites in transformer (tra) pre-mRNA (Sosnowski et al., 1989; Inoue et al., 1990). The Sxl protein binds specifically to one of the two sites and prevents it from being used. Similarly tra proteins appear to promote a female-specific 3′-splice site utilization in the doublesex *(dsx)* mRNA of *Drosophila* (Hedley & Maniatis, 1991). In the case of the *D-Gγ1* gene, it is clear that the first 3′-splice site within intron I is utilized in a ovary-specific manner. Although the significance of this extra 187 nt sequence in 1.3-kb RNA is not known, it is tempting to speculate that this sequence may play some role in the translational ability of the 1.3-kb mRNA. Many maternal transcripts are stored in the oocyte and do not undergo translation before fertilization. Polysomal recruitment of these mRNAs happens only after fertilization in early embryos (Mahowald & Kambysellis, 1970). Various mechanisms have been proposed or documented to explain how maternal mRNAs are masked and prevented from translation in oocyte. For example, it has been suggested that the 3′-UTR sequences, and the length of the poly(A)

tail may regulate this process (Jackson & Standart, 1990). Some evidence also indicates that the 5′-UTR may have a regulatory role on the translatability of mRNAs. For example, a region within the 5′-UTR has been identified which is involved in the translational regulation of the ribosomal proteins in *Xenopus* embryo (Mariottini & Amaldi, 1990). It has also been reported that in a chimeric construct consisting of the 5′-UTR of murine c-myc mRNA, fused to the coding region of a CAT reporter gene, microinjected into Xenopus oocytes, the CAT mRNA came under translational regulation by the *c-myc* 5′-UTR. The chimeric message was not translated in unstimulated oocytes, but was recruited into polysomes when they were induced to mature with progesterone (Lazarus et al., 1988). It is possible that the extra 187 nt 5′-UTR sequence of the 1.3-kb D-Gγ1 mRNA may play a similar role in *Drosophila* oocyte. Binding of a specific protein with this unique sequence may keep 1.3-kb RNA in a cytoplasmic RNP compartment and inhibit its translation. Release of this factor after fertilization may confer polysomal recruitment of this RNA.

CHAPTER 5

CONCLUDING REMARKS

The present study has identified for the first time a *Drosophila melanogaster* G protein γ subunit gene (*D-Gγ1*) and shown that this gene produces three differentially expressed transcripts. The *D-Gγ1* gene is strikingly similar to the mammalian γ-subunits with respect to the size and amino acid sequence. Like the mammalian the γ subunit genes, the D-Gγ1 gene is predominantly expressed in the central nervous system (CNS).

In mammals, different forms of α, β, and γ subunits of G proteins have been identified (review: Birnbaumer et al., 1990, Simon et al., 1991). It has been proposed that these different subunit forms may associate in various combinations to produce many functionally distinct forms of G proteins (Simon et al., 1991; Cali et al., 1992). In *Drosophila*, however, only one form of γ subunit has been identified so far. The present investigation has indicated that other locus with high nucleotide sequence homology is not present in *Drosophila*. Moreover, the expression pattern of the D-Gγ1 gene shows that at least one of the D-Gγ1 mRNA is expressed ubiquitously in all cell types. On this basis one can speculate that there is only one form of γ subunit

in *Drosophila*. It is possible that the diversity of the *Drosophila* G proteins may not be as wide as in mammals and it may primarily depend on different forms of α and β subunits but not on the γ subunits. However, it is not clear from the present investigation whether other divergent forms of γ subunits can be found in *Drosophila* or not.

Although the structural aspects of the generation of the three mRNAs of different lengths are well-understood from the results, the biological significance of this regulation is not clear. A most interesting question has emerged from the present study is: why do three different D-Gγ1 mRNAs with the same coding region show differential tissue-specific expression patterns? While the 2.6-kb D-Gγ1 RNA is expressed only in the CNS, the 1.1-kb RNA is expressed ubiquitously and at highest level in the CNS. The 1.3-kb RNA, on the other hand, is expressed only in the ovary.

From the structural point of view, the only difference between 2.6- and 1.1-kb RNAs is the presence of a extra 1661 nt in the 3′-UTR of the 2.6-kb RNA not found in the 1.1-kb RNA. It can be speculated that due to the presence of destabilizing sequences in this long 3′-UTR, the 2.6-kb RNA may be synthesized but rapidly degraded in non-neuronal cells. These destabilizing sequences are not present in the shorter 3′-UTR of 1.1-kb RNA. In neuronal cells, however, the 2.6-kb RNA is not degraded. It is possible that an RNA

binding factor binds the 3′-UTR destabilizing sequences and prevents degradation of 2.6-kb RNA. If this is the case, the proposed RNA binding protein should be present only in the CNS. The reason for such a mechanism could be that the CNS is heavily loaded with G-protein-coupled signal transduction machinery and thus high levels of G protein subunits are needed to be produced. The proposed mechanism would provide elevated level of D-Gγ1 mRNAs (1.1- and 2.6-kb RNAs) and D-Gγ1 protein. In non-neuronal cells, on the other hand, where a high level of G proteins may not be needed, extra amount of D-Gγ1 mRNA may be unnecessary, and for this reason, the 2.6-kb RNA is not protected in non-neuronal cells.

The ovary-specific expression of the 1.3-kb RNA is intriguing. Although, the 1.3- and 1.1-kb RNAs have the same 3′-UTRs and use the same promoter, the latter is not found in the ovary. The only difference between these two RNAs is the presence of an extra 188 nt in the 5′-UTR sequence of the 1.3-kb RNA. If it is assumed that translatability of D-Gγ1 RNA needs to be adjusted in relation to the physiological condition of the oocytes, a possible role of the extra 188 nt sequence in the 5′-UTR in this regard can be speculated. The extra 188 nt sequence may be the site where a repressor protein binds to prevent translation of the 1.3-kb RNA. Removal of this protein in early embryos may allow translation of the 1.3-kb RNA. A

similar mechanism of translational control by 5'-UTR sequence has been observed in the case of globin and ferritin mRNAs (reviewed in Kozak, 1987). Alternatively, it is also possible that like maternal mRNAs in sea urchin egg, the 1.3-kb RNA may attach to the cytoskeletal framework via the 188 nt sequence and be prevented from translation (Moon et al., 1983).

The present study has indicated that the expression of D-Gγ1 is subjected to transcriptional and post-transcriptional regulatory controls. All these control mechanisms are regulated in a tissue and stage-specific manner. The physiological significance of these await further studies.

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APPENDIX

METHODS

I. Nucleic Acids Isolation Techniques

A. Isolation of genomic DNA

Genomic DNA was isolated from adult flies by the method described previously (Ganguly et al., 1985). Adult flies were collected, frozen in -80°C. Frozen flies were homogenized in nuclei isolation buffer, NIB (50 mM Tris-HCl pH 7.5, 25 mM KCl, 5mM magnesium acetate, and 350 mM sucrose), using a Dounce homogenizer. Nuclear pellets were recovered by centrifugation at 10K and resuspended in NIB again. The final nuclear pellet after two resuspension was then dissolved in 1X DNA extraction buffer, DEB (containing 0.15 M NaCl, 100 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 7.5). Sodium perchlorate was added to a final concentration of 0.5 M and incubated for 5 minutes on ice. SDS was added to a final concentration of 0.1% and reincubated for another 5 minutes. The resulting solution was extracted twice with an equal volume of an 1:1 mixture of DEB-saturated phenol and chloroform and once with chloroform. The aqueous phase was precipitated with 1/10th volume of 2M sodium acetate (pH 7.5) and 2 volumes of 95% ethanol at -20°C, for atleast 2 hours. DNA was precipitated by centrifugation and dissolved in TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and quantitated spectrophotometrically at 260 nm.

B. Isolation of phage DNA

Phage DNA was isolated by a modification of a method originally described by Helms et al., (1985). 1X 10^8 pfu of phage were incubated with 100 ml of host bacteria for 20 minutes at room temperature and plated on 100 mm LD plates (5% tryptone, 2.5% yeast extract, 2.5% NaCl, 1.5% bactoagar) with 3 ml LD top agar (same as LD except 0.7% bactoagar). The incubation was allowed to continue at 37°C incubator for 6-8 hours for complete lysis of host cells. After lysis, the plates were cooled down at 4°C regrigarator and overlayed with 6 ml of lamda diluent (10 mM Tris-HCl, pH 8.0, 50 mM magnesium acetate, 60 mM sodium acetate) and kept overnight at 4°C. The lysate from each plate was loaded onto a DE-52 cellulose column and washed with 3 ml of chase buffer (10 mM Tris-HCl pH 8.0, 10 mM magnesium acetate, 60 mM sodium acetate). For the extraction of phage, 0.9 ml elution buffer (10 mM Tris-HCl, pH 8.0, 50 mM magnesium acetate) was passed through the column without collection, and the phage was eluted with 1.2 ml of elution buffer.

Phage DNA was isolated by adding proteinase-K and SDS to the effluent to a concentration of 0.8 mg/ml and 0.4% respectively and incubating at room temperature for 5 minutes. 3M potassium acetate (pH 8.8) was added to a final concentration of 1M and the suspension was incubated at 88°C for 20 minutes. The suspension was cooled in ice for another 20 minutes and by centrifugation at 10K the resulting precipitate was removed. The supernatent with the DNA was then precipitated with equal volume of isopropyl alcohol, overnight, at -20°C. The DNA was collected by centrifugation at 10K, dissolved in TE, and quantitated spectrophotometrically at 260 nm.

C. Isolation of plasmid DNA

Plasmid DNA was isolated by a modified alkali lysis method, described originally by Birnhoim and Doly (1979). Bacterial cells with plasmids were grown in Luria broth (1% tryptone, 0.5% yeast extract, 1% NaCl, 0.2% Magnesium sulfate.7H2O and 1% Casamino acid) containing 50mg/ml ampicilin. Cells were harvested by centrifugation at 10K, resuspended in solution-I (containing 50 mM glucose, 10 mM EDTA and 25 mM Tris, pH 8.0), and incubated at room temperature for 5 minutes. Two volumes of solution-II (0.2 N NaoH and 1% SDS) were added and the mixture was incubated on ice for 5 minutes. To the lysate one and half volume of solution-III (Glacial acetic acid titrated to pH 4.5 with 10 N KOH) was added and incubated again on ice for 5 minutes. After centrifugation, the supernatant was collected and plasmid DNA was quick precipitated by added equal volume of iso-propanol. The plasmid DNA was treated with DNase-free RNase at a concentration of 10 mg/ml at 37°C for 30 minutes. The sample was then extracted twice with an equal volume of 1:1 phenol:chloroform, and once with chloroform. The aquous phase was reprecipitated with two volumes of ethanol and by adding 1/10th volume of 2M sodium acetate. Quantitation of DNA was done in spectrophotometer at 260 nm.

D. Isolation of total RNA

Total RNA was isolated by using the hot phenol method as described by Jowett (1986). Frozen tissues were homogenized in hot phenol saturated with 0.2 M sodium acetate (pH 5.0) at 70°C waterbath. An equal volume of 0.2 M sodium acetate (pH 5.0) and 1/10th volume of 10% SDS were added to the homogenate and incubated at 70°C for 10 minutes. The homogenate was then cooled at room temperature and 1/2 volume of chloroform was added. The mixture was vigorously shaken and centrifuged at 10K for 2 minutes. The aqueous phase was extracted twice with equal volume of 1.1 phenol:chloroform (phenol was saturated with 0.2 M sodium acetate pH 5.0) and once with chloroform only. The RNA in the final aqueous phase after extraction was precipitated with 1/10th volume of 2M sodium acetate and 2.5 volume of ethanol.

E. Isolation of PolyA$^+$ RNA

PolyA$^+$ RNA was isolated by oligo-dT column-chromatography as described by Aviv and Leder (1972). The oligo-dT column (1 ml) was generated by treating the column with 0.1N NaOH and equilibrating the column with 10 volumes of 1X TES (10 mM Tris-HCL pH 7.5, 1 mM EDTA, 0.1% SDS) until the column effluent measured a pH of 7.0. Total cellular RNA was diluted to 0.5 mg/ml, 1/10th volume of 10X TES added and the sample incubated at 68° C for 2 mins, placde in ice for a few seconds. The column was then washed with 10 ml of 1X TES and 0.5M NaCl and then with 10 ml of 1X TES. The eluent was collected in six fractions and RNA in each fraction was quantitated by spectrophotomentry at 260 nm. Peak fractions were pooled and RNA precipitated by adding 1/10th volume of 2M sodium acetate and 2.5 volume ethanol at -20° C.

II. Screening of Libraries

A. Isolation of genomic clones

The genomic library of the Canton-S strain of *Drosophila melanogaster* was made at the BamHI site of EMBL3 vector as described previously (Krishnan et al., 1991). KH802 cells were incubated with 1 x 10^5 pfu of recombinant phages per 100 mm plate and grown to near confluence. Plaques were blotted on the nitrocellulose filters and the filters were then placed in denaturation buffer (0.1M NaOH and 1.5M NaCl) for 5 minutes. The filters were then neutralized in neutralizing buffer (0.2M Tris.HCl pH 8.0 and 2X SSCP; 10X SSCP is 1.2M NaCl, 0.15M sodium citrate, 0.13M K2HPO4 and 10 mM EDTA). The blots were air dried and baked in a speedvac at 80°C for 2 hours. Blots were prehybridized in 6X SSC, 5X Denhardt's solution with 200 μg/ml denatured fish sperm DNA for 3 hours at 68°C. Hybridization was carried out under same high stringent condition with 20X 10^6 cpm of probe. Final pure plaques were selected after three rounds, titered and stored in SM (100 mM NaCl, 8 mM MgSO4, 50 mM Tris.HCl pH 7.5, 0.01 % gelatin) at 4°C.

B. Isolation of cDNA clones

cDNA clones were isolated by using the method of Benton and Davis (1977) as described earlier. Four different cDNA libraries were screened to isolate clones specific for different mRNAs expressing in a tissue and stage-specific manner. A Drosophila head-specific cDNA library, made in a SWAJ2 vector (Palazzolo and Meyerowitz, 1987), was screened to isolate cDNA clones complementary to mRNAs expressed abundantly in head tissues. In this library, the 5'- and 3'- ends of the inserts were directionally cloned in EcoRI and XbaI cloning sites, respectively. Two other cDNA libraries, a 0-3 hour embryonic and a 3-12 hour embryonic cDNA library, were oligo(dt)-primed libraries obtained from Tom Kornberg. The fourth cDNA libaray, a ovary-specific cDNA library in λZAPII, cloned in EcoRI site by random and oligo(dt) priming, was obtained from Tulle Hazelrigg. After isolation of pure cDNA clones, after appropriate restriction digestion, the cDNA inserts were ligated in either PBSK$^+$ (Stratagene) or pGEM (Promega) vectors. Transformation of the recombinant plasmids in JM 83 or DHα5 cells was done by the method described by Sambrook et al., (1989). The recombinant plasmid DNA was incubated with 50 μl of competent cells and incubated on ice for 30 minutes. The cells were heatshocked for 30 seconds at 37° C. The cells were then grown for 1 hour after adding 1 ml of Luria-broth (LB). Cells were then plated on LB plates containing 50 μg/ml ampicillin and 0.2 % X-gal and grown overnight for blue-white selection.

III. Radiolabeling of Nucleic Acid Probes

A. Nick translation

DNA was nick translated using nick translation kit purchased from BRL. The following components were added in a 50λ reaction mixture: 1.8 μM each of dNTPs (-dCTP), 50 μCi α^{32}P-dCTP, 0.5-1 μg template DNA, 2 units DNA PolymeraseI and 100 ng/ml DNaseI. The reaction was incubated at 150 C for 1 hour, and the reaction was terminated by extracting the mix with 1:1 phenol-chloroform. The DNA probe was separated from the unincorporated nucleotides by exclusion chromatography using sephadex G-50 column.

B. Random priming method

DNA was labeled using random priming method as described by Feinberg and Vogelstein, 1984. DNA was denatured in boiling water bath with 3 μg/ml randomly generated hexanucleotide primers. The mixture was cooled on ice and 1X oligo labeling buffer (200 mM HEPES pH 6.6, 50 mM γ-mercaptoethanol, 50 mM Tris pH 8.0, 5 mM MgCl2, 0.6 mM EDTA), 50 μCi [^{32}P] dATP or [^{35}S] dATP, 2.5 mg/ml BSA, 0.5 mM each of dGTP, dTTP, dCTP, and 5 units of the Klenow fragment of DNA polymerasel was added and incubated at room temperature for three hours for extension reaction. The probe was fractionated over Sephadex G-50 column for purification.

C. Synthesis of heterogenous cDNA probe

Single-stranded cDNA probe was made against polyA$^+$ RNA by a method described by Krishnan et al., (1991). PolyA$^+$ RNA (1-2 μg) was mixed with 50 μCi ^{32}P dCTP, 2.4 μg random hexamer primers and 10 mM methyl mercury hydroxide and incubated for 10 minutes at room temperature. β-mercaptoethanol to 100 mM and 60 units of RNasin were added for further incubation for 5 minutes at room temperature. Reverse transcriptase reaction buffer (50 mM Tris.HCl pH 7.5, 75 mM KCl, 6 mM MgCl2, 200 μm dATP, 200 μm dGTP, 20 μm dCTP, 20 mM DTT) and 30 units of AMV reverse transcriptase were then added to the reaction mixture for extension reaction at 42° C for 1 hour. The probe was finally purified by column chromatography using sephadex G-50.

IV. *In Situ* Hybridization Techniques

A. *In situ* hybridization to polytene chromosomes.

Third instar larvae were placed in 45% acetic acid on gelatinized slide and salivary glands were dissected out. A siliconized cover slip was placed on the top of the salivary glands in acetic acid, and the glands were squashed firmly for spreading of polytene chromosomes. The cover slips were removed by immediately dipping the slides in liquid nitrogen and flipping the coverslips off. Slides were passed through a graded ethanol series and dehydraded by incubating finally in 95% ethanol and drying in air.

Prior to hybridization, slides were incubated in 2X SSC at 68°C for 10 minutes. The slides were treated in 0.1 M triethanolamine (pH 8.0) at room temperature for 5 mins and acetic anhydride was added to a final concentration of 5ml/liter of triethanolamine, incubated for 10 mins. The slides were then washed twice in 2x SSC at room temperature for 5 mins each, dehydrated thru graded ethanol and air dried. The chromosomes on the slides were denatured by incubating in 0.07N NaoH for 3 mins at room temperature and again washed twice in 2x SSC for 5 mins each wash and dehydraded, as before. Probes were denatured by incubating in boiling water for 5 mins and added in hybridization solution, containing 50% deionized formamide, 0.6 M NaCl, 13 mM PIPES pH 7.5. The chromosomes were hybridized in a hybridization solution volume of 20 μl/slide and 200,000 cpm (^{3}H-labeled) count/slide. Chromosomes were hybridized for 24 hours at 37°C.

After hybridization, slides were washed in 50% formamide, 10 mM PIPES, and 0.4M NaCl solution at 37°C. Three washes were given with a change in every 15 mins. The slides were then transferred in 2x SSC for three washes for 10 mins each at room temperature. The slides were passed thru graded ethanol, dried and covered with autoradiographic emulsion. After developing, chromosomes were stained

in Geimsa.

B. *In situ* hybridization to head, thorax and ovary sections

Head, thorax and ovaries of freshly anaesthetized flies were manually dissected, embedded in OCT (Tissue-Tek), and frozen in liquid nitrogen. Sectioning of the frozen blocks were performed on a Reichert cryostat at -20° C. The sections (8 μm) were placed serially on gelatinized subbed slides. Fixation and hybridization steps were done as described by Ashburner, 1989 with a few modifications. The sections were air dried, incubated in 0.2 M HCl for 20 minutes and 2X SSC for 5 minutes at 68°C. The sections were then fixed in 4 % paraformaldehyde for 20 minutes. The slides were washed five minutes each in 3X PBS and 1X PBS and dehydrated through 50%, 70% and 95% graded alcohol.

For hybridization, either ^{35}S or ^{3}H-labeled DNA probes, with specific activities of 1-2 X 10^{9} cpm/μg of DNA, were generated using random priming method (Cobianchi & Wilson, 1987) as discussed earlier. Denatured probe was added to the hybridization mixture containing 50% deionized formamide, 0.6% NaCl, 10 mM Tris.HCl pH 7.6, 1 mM EDTA, 1X Denhardt's solution and 25 μg/ml tRNA. 20 μl of the hybridization mixture containing probe (200,000 cpm/slide) was added to each slide and the slides were hybridized in a humid chamber for 18 hours at 37° C. After hybridization, the slides were washed with wash solution (50% formamide, 0.6 M NaCl, 10 mM Tris.HCl pH 7.6, 1 mM EDTA) four times at 37° C for 2 hours followed by 3 more changes at every four hour intervals. Hybridization signals were detected by autoradiography. The slides were covered with Kodak (NTB2) X-ray emulsion and stored at 4° C for 2-6 days. After developing, the sections were stained with Giemsa.

C. *In situ* hybridization to embryo and larval sections

In situ hybridization to embryo sections was performed as described by Ashburner, 1989. Staged embryos were collected from rapidly laying cultures and removed from the population cage to mature for additional hours until required age. The embryos were dechorionated in 3% sodium hypochlorite with agitation and washed thoroughly in distilled water. The embryos were fixed in a 1:1 mixture of n-heptane and 4% paraformaldehyde (in PBS) and incubated with frequent agitation for fifteen minutes. The embryos were were drawn from the n-heptane/buffer interface and expelled into a 1:1 mixture of n-heptane/90% methanol and 50 mM EGTA pH 8, which was precooled on dry ice. The embryos were then devitallinated by periodic vortexing and incubation in dry ice for 5-10 minutes and quickly brying them in room temperature. The devitallinated embryos were collected from the bottom of the tube and gradually rehydrated through graded methanol containing 50 mM EGTA and 4% paraformaldehyde solution. The rehydrated embryos were then fixed in 4% paraformadehyde in PBS for an additional 20 minutes and dehydrated again by passing through 30%, 50%, 70% and 95% ethanol.

The larvae of the required age were collected and washed well to free from food. The larvae were fixed in 3% paraformaldehyde in PBS for 1 hour at room temperature and dehydrated in 30%. 50%. 70%, 95% and absolute ethanol (10 min each).

The dehydrated embryos or larvae were transferred to OCT (Tissue-Tek) and frozen in liquid nitrogen. The resulting blocks were sectioned on microtome precooled at -200 C and 5-6 μm sections were placed on gelatinized slides and allowed to air dry. The slides were incubated in 200 mM HCl for 20 min at room temperature and incubated in 2X SSC for 20 min at 680 C. The slides were rinsed in PBS for 3 secs twice and fixed in 4% paraformaldehyde in PBS (freshly made) for 20 min at room temperature. Acetylation was done by incubating

slides at room temperature in 66 ml 100 mM TEA pH 8, to which 3 ml of acetic anhydride was added with the slides in place. Slides were then rinsed in PBS for 1 min twice and dehydrated in ethanol series. Making probe and hybridization essentially followed the same method used for *in situ* hybridization to head, thorax or ovary sections described earlier.

V. Blot Hybdridization Techniques

A. Southern blotting

Southern blotting was performed according to Southern, 1975. Genomic or subcloned recombinant plasmid DNA was digested with appropriate restriction endonucleases and electrophoresed at 50V on 0.8-1.0% agarose gels in 1X TBE buffer (90 mM Tris.HCl pH 8, boric acid and 2 mM EDTA). After electrophoresis, the DNA was denatured by treating the gel with denaturing buffer (0.5 N NaOH and 1.5 M NaCl) for 1 hour and neutralized in neutralizing buffer (3 M NaCl and 0.5 M Tris.HCl pH 7.6) for 3-4 hours. The DNA was blot transferred onto nitran membrane using 6X SSC (1X SSC is 150 mM NaCl, 15 mM sodium citrate) as transfer medium. After 12 hours of blot transfer, the blot was baked in vacuo at 80° C for two hours to fix the DNA irreversably on the blot. The blot was then prehybridized in 6X SSC and 5X Denhardt's (1X Denhardt's is 0.02% ficoll, 0.02% Polyvinyl pyrolidine, 0.02% Bovine Serum Albumin fraction V) and 100 μg/ml fish sperm DNA for 2-3 hours at 68° C and hybridized for 16-20 hours under the same condition using appropriate probe (20X 10^6 cpm count/per hybridization). After hybridization, the blots were washed in 0.01X SSC at 60° C for four times with changes at exposed to X-ray film (Kodak) to detect autoradiographic signals.

B. Northern blot hybridization

Total or PolyA + RNA was electrophoresed in 1.2% agarose gel containing 1X northern gel buffer (40 mM 3-(N-Morpholino) propanesulfonic acid, 10 mM sodium acetate, 1 mM EDTA) and 6.6% formaldehyde. The tank buffer used was 1X northern gel buffer. The gel was blotted onto nitran membrane for 16-20 hours using 20X SSC as transfer buffer and the blot was baked for 2 hours at 800 C in vacuo. Before hybridization, the blots were prehybridized in 0.8M NaCl, 50% formamide, 0.1M PIPES pH 6.5, 5X Denhardt,s solution and 100 μl/ml denatured fish sperm DNA. The blots were hybridized using the same solution using 20-25X 10^6 cpm probe/hybridization reaction for 18-20 hours at 42° C. Following hybridization, the blots were washed twice in 2X SSC, 0.1% SDS, and 0.05% sodium pyrophosphate at room temperature for one hour and in 0.2X SSC at 60° C for one more hour. The signals were detected by autoradiography as described earlier.

VI. Nucleic Acid Sequencing Technique

A. Generation of deletions

Deletions for sequencing were generated using the Erase-a-base system from Promega. This system provides the protocol for the construction of unidirectional nested deletion sets from recombinant plasmid clones using the ExonucleaseIII-Si nuclease procedure developed by Henikoff (1984). Nested deletion sets were generated with 150 base pair overlaps between each deletion fragments. The multistep protocol i.e. restriction digestion, ExonucleaseIII digestion, S1 nuclease cleavage, Klenow-fill-in and religation, was performed in a same eppendorf tube using the erase-a-base protocol without intermediate extraction or precipitation steps.

B. Dideoxy method of sequencing DNA

This sequencing method was performed using Sequenase Version 2.0 sequencing kit (USB) based on a method originally described by Sanger et al., 1977. The DNA fragments to be sequenced were subcloned in PBSK$^+$ vector. Sequencing was done by using either T7 promoter sequence, puc/M13 reverse promoter sequence or custom synthesized primers. Template DNA isolated by alkali lysis preparation was denatured in 2 mM NaOH, 2 mM EDTA for 20 minutes at room temperature. The DNA was precipitated by adding 3M sodium acetate pH 5.2 and 2 volumes 95% ethanol at -80° C for 15 minutes. The DNA was washed with 70% ethanol and pelleted. The denatured DNA was dissolved in 7 μl H_2O in which 2 μl sequenase reaction buffer (200 mM Tris.HCl pH 7.5, 100 mM MgCl2, and 250 mM NaCl) and 1 μl (10 ng/μl) of appropriate primer was added. The primer was allowed to anneal to the template DNA by in cubating the DNA and primer at 68° C for 3 minutes and gradually cooling the solution down to room temperature for 30 minutes. After annealing, 1 μl of DDT, 2 μl labeling mixture (1.5 μM each of dGTP, dTTP and dCTP), 1 μl ^{35}S-dATP (1 μCi) and three units sequenase version 2.0 enzyme were added. The labeling reaction was carried out at 15° C for 5 minutes. After 5 minutes 3.5 μl of the reaction mix was transferred to each of the four termination mixes (80 μM of each dNTPs with 8 μM of the corresponding ddNTPs) and incubated at 37° C for five minutes. The termination reaction was stopped by adding 5 μl of stop buffer (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue, 0.05% Xylene Cyanol FF). Sequencing reaction products were denatured by incubated at 100° C for 3 minutes and then subjected to electrophoresis on 6% Urea-0polyacrylamide gel (50% Urea, 6% acrylamide, 0.4% bisacrylamide, 1X TBE) at 80 watts using an IBI sequencing gel apparatus. After electrophoresis, the gel was dried for two hours at 80° C using a BioRad model 583 gel dryer. Fixation of the gel prior to drying was excluding by covering the gel with myarin paper. Dried sequencing gel was exposed for 16-24 hours using a X-omat RP X-ray film.

VI. RNase Protection Technique

The nuclease protection procedure of Zinn, Dimaio and Maniatis (1983) was initially used to map the transcription start site. Total RNA (15 mg) or Poly(A$^+$) RNA (1-2 μg) precipitated with ethanol was redissolved in 28 μl of hybridization buffer (80% (v/v) formamide, 400 mM NaCl, 40 mM PIPES, pH 6.4). cRNA probe was synthesized using 50 μCi of [α-^{32}P]UTP and 1 μg of DNA template. After synthesis of cRNA probe, the DNA template was removed by digestion with DNase I, the reactioon extracted with phenol and chloroform, and the probe was recovered

by ethanol precipitation. The probe was redissolved in 10-15 μl of hybridization buffer and 2 μl (5x 10^5 cpm) was then added to the RNA solution. The hybridization mixture was heated to 80°C for 3 min, then hybridized overnight at 45°C. The hybrids were then digested in 300 μl of an RNase solution (40 μg/ml RNase A, 2 μg/ml RNase T1, 33 mM NaCl, 10 mM Tris pH 7.5, 5 mM EDTA) for 1 hour at 32°C. The digestion was stopped by phenol/chloroform extraction and the hybrids precipitated by the addition of 5 μg of carrier tRNA and 2 volumes of cold ethanol. The samples were electrophoresed on sequencing gels for maximum resolution.

VII. Primer Extension Technique

The method used for primer extension experiments was modified from a protocol by Sambrook et al., 1989. 16 mer Oligonucleotides primer was 5'-end-labeled using [γ-^{32}P]ATP (Maniatis, Fritsch and Sambrook, 1982). Poly(A$^+$) RNA (10-12 ng) was mixed with 5 pmol of 5'-end-labeled primer(=5x10^5 cpm) in a total volume of 10 μl hybridization solution (80% formamide, 50 mM NaCl, mM EDTA, 10 mM Tris pH 7.5). The mixture was heated 3 mins at 65°C and then incubated for 16 hours at 42° C. An equal volume of 2X reaction buffer containing 100 mM Tris (pH 8.3), 20 mM DTT, 20 mM MgCl2, 2mM of each of the four NTPs, 100 units/ml placental RNase inhibitor and 1000u/ml AMV reverse transcriptase. The reaction was allowed to proceed for 2 hours at 42°C, then stopped by adding EDTA to a final concentration of 20 mM. Sodium acetate was added to a final concentration of 0.3 M and the reaction products were precipitated with cold ethanol. The redissolved samples were electrophoresed on sequencing gels.

VITA

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