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Jack Preiss
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# MOLECULAR CHARACTERIZATION OF BOVINE AND HUMAN RETINAL ROD cGMP PHOSPHODIESTERASE AND CHROMOSOMAL LOCALIZATION OF THE HUMAN GENE

By

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## ABSTRACT

**MOLECULAR CHARACTERIZATION OF BOVINE AND HUMAN RETINAL ROD cGMP PHOSPHODIESTERASE AND CHROMOSOMAL LOCALIZATION OF THE HUMAN GENE**

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Retinal rod cGMP phosphodiesterase (cGMP PDE) is a heterotrimeric enzyme that functions as a signal amplifier in photo-transduction. This work was initiated to study the molecular biology of the genes encoding the cGMP PDE subunits.

Characterization of overlapping cDNA clones encoding the α-subunit of cGMP PDE revealed an open reading frame that encodes a protein of 859 amino acids. Four differing cDNA clones were also identified that are highly homologous to the α-subunit sequence. All of the isolated cDNAs contain an identical segment in their predicted protein coding regions of ~1.6 kb. The sequence of one of these cDNAs differs from the α-subunit sequence only at the 5' end encoding a predicted polypeptide of 500 residues. Two other cDNAs differ in sequence from the α-subunit only at their C-termini, and the fourth cDNA shows sequence divergence in the 3' untranslated region. Predominant retinal RNAs of 4.6 and 4.0 kb were identified with cDNA probes representative

of the common segment, or with probes specific to the α-subunit sequence. Using probes specific to the α-subunit-like cDNAs, a number of lower abundance RNAs were observed. Strong evidence from DNA blotting experiments suggests that a single bovine gene encodes all of the identified transcripts. The size of this gene is estimated to be at least 160 kb.

Several cDNA clones, including one full length clone were isolated encoding the human cGMP PDE α-subunit. Overall, the predicted primary structures of the human and bovine α-subunits showed 94% identity. Transcripts of ~5.9 and 5.4 kb are identified in human retinal poly (A)$^+$ RNA. Probes specific for the two bovine cDNAs that differ at their C-termini do detect human retinal RNAs of similar size and abundance to that found in bovine retinal RNA. The human α-subunit gene has been mapped to the long arm of chromosome 5 using somatic cell hybrid DNA panels.

Similarities were found between the predicted α-subunit amino acid sequence and that of nonretinal cyclic nucleotide phosphodiesterases (CN PDEs), indicating that the α-subunit gene is a member of a conserved gene family of CN PDEs spanning diverse species from yeast to man. The complexity of the bovine cGMP PDE α-subunit gene is comparable to other PDE genes of higher eukaryotes and may be a general feature of the members of this gene family.

I dedicate this work to my family, especially my parents, Floris and Carl Pittler, who believed in me and supported me even when I gave them every reason not to. And to my sister, Jean Webb, who I am very proud of for believing in herself. I also am compelled to extol the contributions of my grandparents, the memories of grandpas Mickey Pittler and Leonard Finkelstein, and to Mildred and Joseph Loundy, and Ida Pittler, whom I hope will live forever. I apologize to the rest of my family that there is not sufficient space to mention all of you for the cornerstone of Judaism is the camaraderie of the family which has served to establish the ethics and values that I live by.

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## ABBREVIATIONS

Abbreviations used without definition were taken from the Proceedings of the National Academy of Sciences, U.S.A., Information for contributors: Volume 85, p.p. iii-v, 1988.

Additional abbreviations used are as follows:

RPE: retinal pigment epithelium

IPM: interphotoreceptor matrix

ROS: rod outer segment

cGMP PDE: bovine rod outer segment 3',5'-cyclic guanosine monophosphate phosphodiesterase (EC 3.1.4.17)

CN PDE or PDE: denotes any 3',5'-cyclic nucleotide phosphodiesterase that hydrolyzes cAMP or cGMP

TPCK: tosylphenylchloroketone

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

MW: molecular weight

PTH: phenylthiohydantoin

DEPC: diethylpyrocarbonate

PEG: polyethylene glycol (molecular weight 8,000)

TMAC: tetramethyl ammonium chloride

DOS: disk operating system

VMS: virtual memory system

pfu: plaque forming unit

# INTRODUCTION

Phototransduction in rod cells is initiated by the absorption of light. Compelling data from a number of laboratories (25) have shown that three proteins; rhodopsin, transducin, and cGMP PDE, are central in the pathway of information flow from the light stimulus to a reduction in intracellular cGMP concentration. Upon absorption of a photon, the receptor, rhodopsin, activates a specific G-protein, transducin, which then stimulates a marked increase in cGMP PDE activity. The ensuing decrease in cGMP levels produces a hyperpolarization of the plasma membrane, caused by the closure of cGMP gated ion channels.

In bovine, cGMP PDE is a three subunit enzyme that is disk membrane associated, with stoichiometric composition $\alpha$(88 kD), $\beta$(84 kD), and $\gamma_2$(10 kD) (26,27). The $\gamma$-subunit functions as an inhibitor of cGMP PDE maintaining its activity at a basal level in the dark. Although the enzyme activity purifies as a heterotrimer, it is not clear that the enzyme is composed this way *in vivo*. Hurwitz *et al.* have shown that both the $\alpha$ and $\beta$ subunits have activity that is separately inhibited by the $\gamma$-subunit (28). Unfortunately, the inability to separate and isolate active $\alpha$ and $\beta$ subunits in amounts amenable for reconstitution experiments has made it impossible to investigate their potential independent regulation.

Recently, Ovchinnikov *et al.* reported on the primary structure of the $\alpha$-subunit deduced from overlapping cDNA clones (29). A portion of

this sequence is homologous to an ~275 amino acid segment found in many nonretinal cyclic nucleotide PDEs (CN PDEs) in diverse species (50). This region, which has been termed the "conserved domain", is thought to form part of the catalytic domain of this enzyme family.

We initiated a molecular dissection of bovine cGMP PDE to study the interactions of the α and β subunits and the regulation of the enzyme in the phototransduction cascade. The analysis was extended to the study of the human α-subunit to probe the potential clinical signifigance of the enzyme in hereditary retinal dystrophies. In these studies we confirm the sequence of the bovine cGMP PDE α-subunit by sequence analysis of several cDNA clones. Additionally, during this analysis we have surprisingly identified four strikingly similar α-subunit like variants. These variants, as well as the α-subunit, are encoded by multiple RNAs from a gene extending over more than 160 kilobases. A full length human α-subunit cDNA encodes a protein molecule that is 94% identical to the bovine α-subunit. The gene encoding the α-subunit has been mapped to the long arm of human chromosome 5.

## LITERATURE REVIEW

Retinal Morphology. In the eye, light focused onto the retina is absorbed and then converted to an electrical impulse; a process called phototransduction (1). The initial transducing events are mediated by highly specialized cells termed photoreceptors. Most mammalian retinas contain two morphologically distinct photoreceptor cell types, cones and rods. There are typically three types of cone cells characterized by the wavelength of light absorbed by their receptor molecules. The cone cells function in bright light and mediate color vision (2). Rod cells, on the other hand, function at lower levels of illumination (3). Rod cells out-number cone cells in higher mammals by approximately 15 to 1, and because of their abundance and ease of isolation, phototransduction in the rod cells is better understood.

Rod cells are elongated neural cells that can be subdivided into four distinct regions (Figure 1). Phototransduction occurs in the outer segment which contains about 2000 membranous discs that are formed when an invagination of the plasma membrane is pinched off (4). Old discs are shed at the distal end of the rod cell as new discs are formed. The shed discs are destroyed by the phagocytic activity of the RPE, a layer of cells contiguous with the distal part of ROS that extends between rod and cone cells. The space between rods and cones that is not filled

by the RPE is made up of a filamentous mesh of glycosaminoglycans, proteins, and glycoproteins termed the IPM (5).

A Model for Phototransduction. The biochemical basis of signal transduction in the rod cell has been subjected to intensive study (6-9). Three proteins, rhodopsin, transducin, and cGMP PDE make up greater than 80% of the total protein found in ROS. It has been proposed that the interaction of these three proteins, in large part, mediates the transducing process (10, summarized in Figure 1 and Table 1). In its activated form, rhodopsin interacts with transducin in an undefined manner to cause the exchange of GDP for GTP on the α-subunit of the transducin molecule (18). The GDP-GTP exchange brought about by the interaction of transducin with rhodopsin causes the transducin α-subunit to dissociate from a complex of the β and γ subunits. The dissociated α-subunit is then free to interact with and activate the cGMP PDE. In the dark, cGMP directly interacts with $Na^+$ ion conductance channels to keep them open (76). Impinging light on the retina leads to the hydrolysis of cytoplasmic cGMP to 5' GMP catalyzed by the cGMP PDE. This is accompanied by the subsequent closure of many $Na^+$ channels which produces a transient hyperpolarization in the rod cell. Thus, a light stimulus has been converted into an electrical signal that will be transmitted through other retinal layers finally arriving at the visual centers of the brain.

Rhodopsin. The seminal work by Wald uncovered the important role of rhodopsin in the signal transduction process (85), and set the direction for future experiments. It is now known that rhodopsin is an integral transmembrane protein that has been localized within the disc

Figure 1: The cGMP Cascade of Vision in Retinal Rods. Shown at the bottom of the figure is a schematic of a rod cell. Rod cells are differentiated into inner and outer segments that are connected by a cilium. The inner segment contains the nucleus, most of the cell's biosynthetic machinery, and are continuous with the synaptic terminals. The membranous discs in the outer segment that form by an invagination of the plasma membrane are pinched off to become autonomous. It is within these discs that the light receptor, rhodopsin, resides. The primary biochemical events that initiate phototransduction are shown at the top. A model to explain these events has been proposed by Stryer (10). Table 1 identifies the symbols used in this model.

1) In the dark, nearly all of the transducin is in the T-GDP form, which does not activate the cGMP PDE.

2) The chromophore of R, 11-cis retinal absorbs a photon which results in the activation of R to $R^*$.

3) $R^*$ encounters T-GDP and forms an $R^*$-T-GDP complex.

4) GTP exchanges for GDP in the $R^*$-T-GDP complex. In essence, the role of $R^*$ is to activate transducin.

5) The exchange of GTP for GDP markedly diminishes the affinity of $R^*$ for transducin. In addition, the affinity of $T_\alpha$ for $T_{\beta\gamma}$ is very much decreased. These changes in binding affinities are crucial for the efficient operation of the cycle. The released $R^*$ is free to interact with another T-GDP, enabling $R^*$ to act catalytically.

6) $T_\alpha$-GTP activates the cGMP-PDE by overcoming an inhibitory constraint (i) imposed by its $\gamma$-subunit. Activated cGMP PDE hydrolyzes cGMP at a very rapid rate. This leads to the closure of many $Na^+$ ion channels and results in a transient hyperpolarization of the plasma membrane.

7) The GTPase activity inherent in the $\alpha$-subunit of tranducin converts $T_\alpha$-GTP to $T_\alpha$-GDP in times of seconds to a minute; this in turn results in the deactivation of the cGMP PDE. $T_\alpha$-GDP then rejoins $T_{\beta\gamma}$.

One activated rhodopsin molecule can activate as many as 500 transducins, which in turn activate cGMP PDE molecules that can hydrolyze as many as 2000 cGMP molecules per second, resulting in an amplification cascade that yields a $10^6$ gain from a single activated rhodopsin (1).

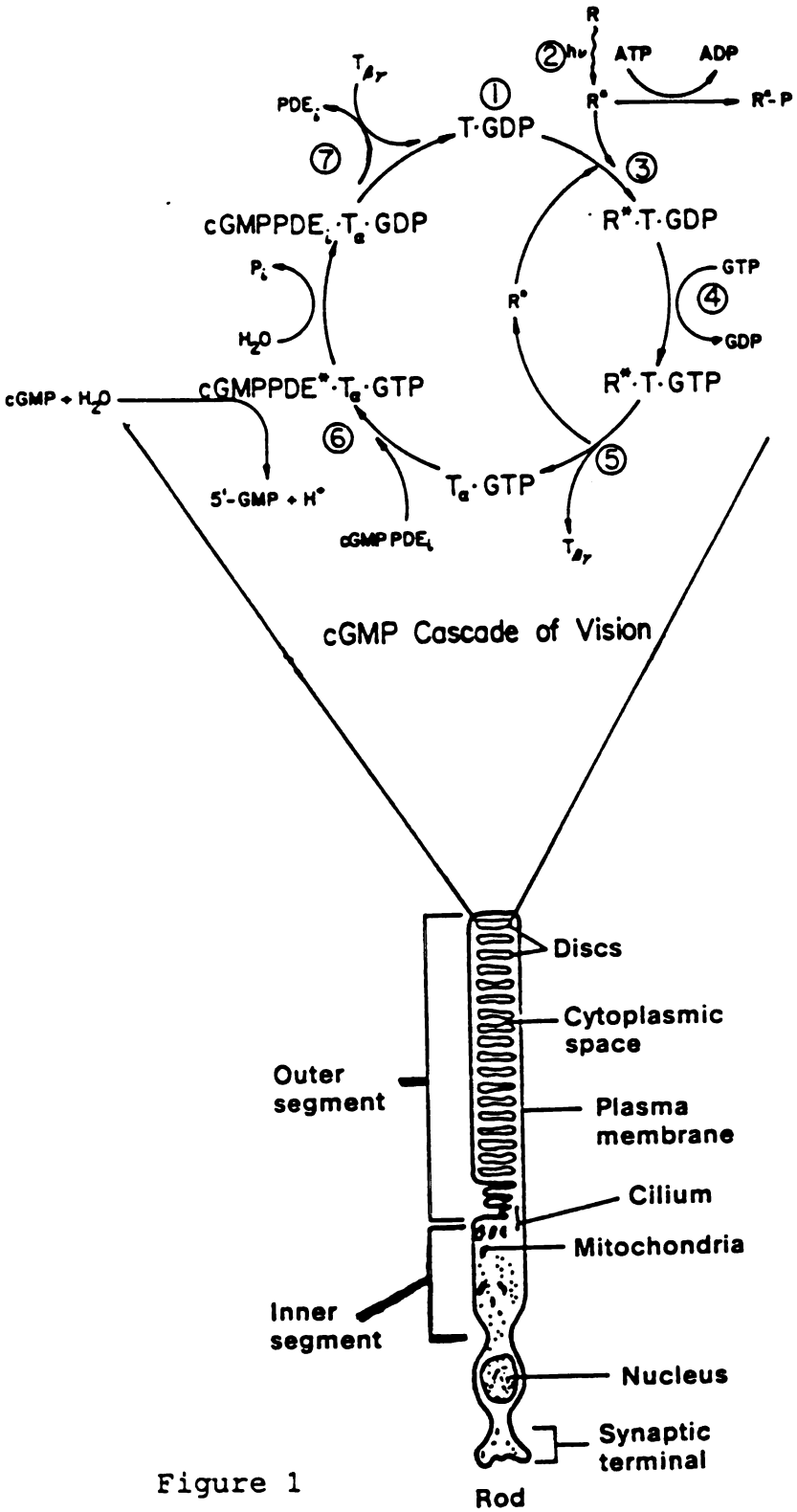

R
hν
ATP
ADP
R*
R*-P
T·GDP
PDE
cGMPPDE·T·GDP
R*·T·GDP
GTP
GDP
cGMPPDE*·T·GTP
R*·T·GTP
cGMP + H2O
5'-GMP + H+
T·GTP
cGMP PDE
cGMP Cascade of Vision
Discs
Cytoplasmic space
Outer segment
Plasma membrane
Cilium
Mitochondria
Inner segment
Nucleus
Synaptic terminal
Rod

Figure 1

Table 1: Proteins of the cGMP Cascade of Vision. Some of the major constituent proteins of the ROS are shown. These proteins comprise greater than 80% of the total protein of the ROS. A model proposed by Stryer (10) that integrates the biochemical properties of these proteins is shown in Figure 1. The sizes of the proteins are based on that observed from SDS-PAGE analysis.

Table 1: Proteins of the cGMP Cascade of Vision.

| Protein[a] | Mass (kD) | Role |
|---|---|---|
| Rhodopsin (R) | 40 | Photoexcited rhodopsin ($R^*$) activates transducin by catalyzing GTP-GDP exchange. |
| Transducin (T) | α 39 | $T_\alpha$-GTP is the amplified intermediate in the activation of the phosphodiesterase. |
| | β 36<br>γ 8 | Transducin interconverts between an inactive $T_{\alpha\beta\gamma}$-GDP form and an active $T_\alpha$-GTP form. $T_{\beta\gamma}$ is required for GTP-GDP exchange. $T_\alpha$ hydrolyzes bound GTP to GDP to return to the dark state. |
| Phosphodiesterase (cGMP PDE) | α 88<br>β 84<br>γ 10 | Hydrolyzes cGMP when activated by $T_\alpha$-GTP. The catalytic activity of αβ is inhibited by the γ-subunit of the PDE in the dark state. |
| Rhodopsin kinase | 68 | Phosphorylates $R^*$ at multiple serine and threonine residues, enabling it to bind arrestin. Involved in the deactivation of $R^*$. |
| Arrestin (S-antigen) | 48 | Binds to phosphorylated $R^*$ and blocks its capacity to activate transducin. May also bind directly to cGMP PDE to regulate its activity. |

[a] The molar amounts of these proteins in intact rod outer segments are estimated to be rhodopsin, 1000; transducin, 100; phosphodiesterase, 14; rhodopsin kinase, 1; arrestin, 100.

and plasma membranes of ROS. It consists of a chromophore, 11-cis retinal, as the prosthetic group, that is covalently attached (lysine 296) to the apoprotein, opsin (11). The molecule contains seven hydrophobic regions proposed to loop through the disc membrane with the C-terminus on the cytoplasmic face and the N-terminus within the lumen of the disc (12). Several potential sites of phosphorylation are located near the C-terminus that are thought to be involved in the regulation of rhodopsin activity (13). The initial event of phototransduction is produced through the chromophore, 11-cis retinal. Light absorbed by the chromophore causes it to be isomerized to all trans retinal, a process called bleaching (16). Spectroscopic studies have shown that at one of the transition states (metarhodopsin II) in the isomerization process, the rhodopsin is converted to an activated form (17). The basis for the structural changes causing the activation are not understood.

The genes for both human and bovine rhodopsin have been cloned and their nucleotide sequences determined (14,15). There is marked conservation in both gene structure and nucleotide sequence. The coding region in both genes is interrupted by four introns. Both proteins have the same number of amino acid residues and exhibit 94% overall homology. Most importantly, three loops thought to be on the cytoplasmic face of the disc are perfectly conserved. This is consistent with the suggestion that the three loops are involved in the binding of transducin, arrestin, and rhodopsin kinase; however, the stoichiometry has not been established. Additionally, the 11-cis retinal attachment site and all but one of the phosphorylation sites are identical in the two proteins. The human rhodopsin gene has been mapped to chromosome 3 (101).

Transducin. Transducin was so named because of its role as an intermediate in the transduction process (18). It is composed of three subunits, α (39 kD), β (36 kD), and γ (8 kD), and is found peripherally on the disc membrane, following illumination. Transducin is a member of the well-characterized family of G-proteins which have been shown, or are implicated, to be signal mediators in a variety of transduction processes (19). Characteristic of all G-proteins is at least one guanyl nucleotide binding site and intrinsic GTPase activity. These functions reside on the α-subunit of transducin (10).

Recently, a transducin α-subunit cGMP PDE γ-subunit complex was identified (98), consistent with the hypothesis that activation of cGMP PDE occurs by removal of the inhibitory constraint imposed by the γ-subunit. A three dimensional model has been proposed for the α-subunit based on crystallography data from other related proteins (93). The region of interaction with the cGMP PDE γ-subunit is suggested to be delimited to amino acids 49-175. This region is predicted to be composed of eight antiparallel β strands.

Corresponding cDNA clones have been isolated for the α, β and γ subunits of transducin (78-80). Of the several groups that reported the sequence of the α-subunit, one group reported a sequence (81) that differed slightly from the others. Subsequently, it was shown that rod and cone specific transducin molecules are the basis for this difference (82). The gene encoding the α-subunit has been mapped to human chromosome 3 (94) and mouse chromosome 9 (95). The two genes encoding the β-subunits that are thought to be common to all G-proteins (99) have also been mapped. $G_{\beta}1$ is on human chromosome 1 (94) and mouse 19

(94). $G_\beta 2$ is on human chromosome 7 (94), its mouse location has not been determined.

cGMP PDE. The cGMP PDE also consists of three subunits: α (88 kD), β (84 kD), and $\gamma_2$ (11 kD) (20,26,27). In the dark, the enzyme exhibits a very low catalytic activity (50 moles cGMP hydrolyzed\second\mol), which is increased markedly (3700 moles cGMP hydrolyzed\second\mol) by light. The observation that limited tryptic digestion also greatly enhanced the activity suggested that activation might occur by removal of an inhibitory constraint. Subsequent studies showed that this constraint is levied by the γ-subunit of the cGMP PDE (21). Three observations led to this conclusion (21). First, it was shown that limited trypsinolysis rapidly degraded the γ-subunit, while α and β and their association remained unaltered. Second, when purified γ-subunit was added back to trypsin activated PDE, 99% of the activity was inhibited. The dissociation constant for the binding of γ to α-β is 0.13 nM. Third, during purification of the enzyme, the inhibitory activity copurified with the catalytic activity.

Complementary DNA clones representing bovine and mouse cGMP PDE γ-subunits have been isolated (22,92). Thirteen of the 87 amino acids of the γ-subunit are tryptic cleavage sites. Interestingly, 10 of these residues are concentrated in a stretch of 22 amino acids that contains no acidic residues. This is consistent with the observation that trypsinolysis increases the activity of the cGMP PDE by removing the inhibitory constraint imposed by its γ-subunit. The γ-subunit gene has been localized to mouse chromosome 11 (97).

Purified cGMP PDE has a measured $k_m$ for cGMP of ~50-70 μM and 2200 μM for cAMP. Recently, the stoichiometry was determined by

two groups, using markedly different approaches, to be $\alpha\beta\gamma_2$ (27,96). Although the enzyme purifies as a heterotrimer of the three subunits, it has not been unambiguously demonstrated that the enzyme is composed this way *in vivo*. Based on the results of biochemical and immunological analysis, Beavo has suggested that minor isozymic forms of cGMP PDE exist in the rod outer segment (28, 120). In the report of this analysis, it was shown that the α and β subunits can be separated by HPLC. It was then discovered that the subunits retain activity independent of their presumed association and that the activity could be inhibited by addition of the γ-subunit. Moreover, the inhibition could be removed by addition of activated transducin, leaving open the possibility that an αγ and a βγ subunit association may exist *in vivo* representing two distinct enzymes.

Ovchinnikov *et al.* have also reported on the analysis of cDNA clones encoding the α-subunit and a portion of the β-subunit (86). A composite sequence spliced together from overlapping cDNA clones predicts an α-subunit with a MW of ~99 kD, significantly higher than the 88 kD value predicted from SDS-PAGE analysis (20). The sequence of two peptides was identified from an α-subunit tryptic hydrolysate, that are not found in a β-subunit hydrolysate in support of the cDNA sequence. Additionally, the first 12 amino acids at the N-terminus of the α-subunit were determined by mass spectrometry. However, only one cDNA clone was isolated covering a portion of the C-terminus of which no peptide sequence was presented. Preliminary findings of C-terminal heterogeneity in retinal cGMP PDE (87) left doubt as to the authenticity of the reported sequence. It was possible that the overlapping cDNA clone covering the C-terminus represented a closely related PDE and not the α-subunit. A portion of the β-subunit sequence was also presented,

as deduced from cDNA clones. No peptide sequence was provided in support of the sequence presented leaving open the possibility that a PDE polypeptide other than the β-subunit had been cloned. The identity of their postulated β-subunit still remains unclear. The question of the number of genes encoding the α and β subunits also remains unanswered. Even if the reported β-subunit sequence is correct, the differences in sequence between the subunits can be accounted for either by alternative splicing of transcripts from a single gene, or two separate genes.

Characterization of Other Retinal PDEs. While the number of cGMP specific PDE isozymes in the retina has not been firmly established, it is clear that multiple forms do exist. A high affinity form identified only by histological methods has been found in the inner plexiform layer (100). A cone outer segment PDE has been characterized that is similar to rod cGMP PDE, but appears to have only one large subunit of 94 kD and three small subunits of 11,13, and 15 kD (31) . The 11 kD subunit has been shown to be very similar if not identical to the rod cGMP PDE γ-subunit. Other properties of the cone enzyme further distinguish it from the rod enzyme. The cone enzyme binds 10 fold more cGMP/mole than the rod enzyme. This binding occurs at a noncatalytic site with very high affinity (kD=11nM). The dissociation of cGMP from this site is slow with a halflife of 10-20 minutes at 37°C. Rod transducin will activate the cone enzyme with half maximal activation requiring 50 fold less (15 nM) transducin than that required for the same activation of rod cGMP PDE.

A PDE in the IPM, termed IPM PDE, has also been characterized which has subunit sizes of 47 and 45 kD (102). The apparent native MW

is 350,000 by gel filtration suggesting a multi subunit assembly. The kinetic properties for this PDE are essentially the same as that observed for the rod enzyme. The enzyme could be immunoprecipitated with a monoclonal antibody that blocks activity of the cGMP PDE (28). The possibility that this PDE is simply a proteolytic product of the rod PDE is ruled out by the procedure used to isolate the enzyme, which was shown not to release the cGMP PDE from the disk membrane. The function of this PDE is unknown, however it may act as a scavenger to mop up excess cGMP, which has been shown to be toxic in high dosage to the rod cell (84).

Other Rod Cell Proteins of Importance in Phototransduction. Several other proteins have been shown or are thought to participate in the regulation of phototransduction. Rhodopsin kinase has been shown to phosphorylate rhodopsin on accessible sites near the C-terminus, thereby decreasing its activity (23). The enzyme has recently been purified to near homogeneity (88). It exists as a single polypeptide chain of MW ~70 kD. Similarities were observed in comparison to the β-adrenergic receptor kinase (89), suggesting that the two kinases may be members of a kinase family that also includes the muscarinic acetylcholine receptor kinase.

The decrease in activity of rhodopsin requires another protein called arrestin (24) or S-antigen which will bind to phosphorylated active rhodopsin but not to unphosphorylated active rhodopsin, thus inhibiting the binding of transducin (25). S-antigen is a 48 kD protein that appears to be cytosolic in the absence of light, and associated with the disk membrane following illumination (90). A cDNA clone encoding arrestin has been isolated (91). Comparison of deduced amino acid sequence for

arrestin and the α-subunit of transducin revealed striking similarities (83), consistent with the idea that arrestin competes with transducin for binding sites on activated rhodopsin, thereby inhibiting rhodopsin's ability to bind transducin and activate it. It has also been suggested, based on the results of crosslinking analysis that arrestin binds directly to cGMP PDE to inhibit its activity (103). Perhaps arrestin is responsible for the reassociation of the cGMP PDE γ-subunits and αβ, facillitating the return to the dark state.

Guanylate cyclase catalyzes the conversion of GTP to cGMP, and therefore is an essential component of the recovery process following a light stimulus. The instability of the enzyme has made its purification difficult, and thus its characterization is based on crude or partially purified preparations (104). It has been shown, based on microdissection studies of histologically defined retinal regions that much of the enzyme activity present in the retina is concentrated at the axoneme of the rod cell. Only in the plexiform layers has a significant amount of activity been found outside of the photoreceptor cell (105). More recently, Koch and Stryer (106) have shown that the enzyme activity is increased markedly (5-20 fold) in response to very small changes (200nM-50nM) in the intracellular calcium concentration. It was also suggested that a new type of calcium binding protein mediates this effect.

Additional proteins important for guanine nucleotide metabolism have also been identified and partially characterized in ROS. A 5' nucleotidase which converts 5'-GMP to guanosine exists in ROS as a membrane bound enzyme of ~75 kD (107). The $k_m$ for 5'-AMP was measured at 1.3 μM and for 5'-GMP a $k_m$ value of 2.3 μM was observed.

A guanylate kinase which produces GDP from 5'-GMP and a nucleoside diphosphate kinase which can convert GDP to GTP has been characterized in frozen retina and from isolated rods (108,109). The guanylate kinase has been purified from ROS and it was determined to have a $k_m$ of 13 μM for GMP and 430 μM for AMP. It was suggested that the enzyme exists in one copy per 800 rhodopsins. These results establish that the enzymes capable of recycling 5'-GMP to cGMP, namely, guanylate kinase, nucleoside diphosphate kinase, and guanylate cyclase are present in ROS, but it is unclear whether the anabolic or catabolic pathways for the regeneration of 5'-GMP predominate.

The major phosphoprotein identified in ROS has been termed 33k (110). This protein is thought to be specific to visual cells (111), and phosphorylated in a cyclic nucleotide dependent manner (112). Interestingly, phosphorylation levels of the protein are highest in dark adapted retinas and dephosphorylated upon illumination (113), in direct contrast to rhodopsin phosphorylation. The recent finding that 33k exists as a complex with the β and γ subunits of transducin (52) suggests an important role in the regulation of phototransduction, which is yet to be determined.

Post Translational Modifications. The post translational addition of palmitate is thought to occur only through a covalent thioester linkage to cysteine residues. Thioester linked lipidation has been shown to occur on two cysteines in bovine rhodopsin, but its importance has not been established (114,115). However, palmitylation at a cysteine in two N-ras and a closely related yeast YPT1 protein has been shown to be essential for normal enzyme function (116). Palmitylation has also been observed in a number of other proteins in which methylation at the C-terminus

was also identified, including the ras and ras related proteins (117). Intriguingly, potential palmitylation sites are located near the C-terminus of the predicted cGMP PDE α-subunit sequences in both bovine and human (118), and methylation has previously been reported to occur on the bovine α-subunit (159). Indeed, it has been suggested that methylation of the α-subunit, and potential palmitylation are coupled and may serve as a membrane anchor by increasing the hydrophobicity at the C-terminus (117).

Classification of Eukaryotic Cyclic Nucleotide PDEs. Characterization of CN PDE was reported as early as 1961 by Drummond and Perrot-Yee (119). At that time it was thought that only one form of PDE existed that hydrolyzed cyclic nucleotides. Currently it is known that multiple PDEs exist in most, if not all, cell types that are regulated by very differing mechanisms. It is expected that many more isozymes are yet to be identified (120).

A pioneer in the study of mammalian PDEs, J. Beavo, has recently undertaken the effort to classify the numerous isozymes of PDEs that have been identified (120). This task has proven formidable due to the large number of recent studies on PDEs, and the problems associated with studying the biochemistry of the enzymes. Even though many forms of PDE clearly exist, separation and characterization have proven difficult because many of the isozymes (even within a single cell type) are very closely related. Additionally, the problem is compounded by the relatively low levels of PDE that are present for most isoforms, and partial proteolysis leading to false predictions of heterogeneity.

Nonetheless, certain properties of the various PDEs allow them to be distinguished and thus separated into separate classes. Shown in

Table 2 are the six classes of mammalian PDEs adapted from the review by Beavo (120). The photoreceptor PDEs exhibit a marked preference for cGMP PDE and therefore are grouped into the cGMP specific class. The "low $k_m$" PDEs in general are more selective for cAMP as substrate. Ro-20-1724 is a pharmacological agent that has been shown to selectively inhibit PDEs in this class. The cCMP and cUMP PDEs are thought to be very minor species and have not been well characterized.

Several non-mammalian PDEs have also been characterized, most notably a yeast and a Drosophila cAMP PDE, both of which are most closely related to the low $k_m$ class of mammalian PDEs (121,122). Comparison of the primary structures of these enzymes deduced from cDNA clones to mammalian PDEs in classes 1-4 for which sequences were available has uncovered a significant similarity (50, see also Figure 17). This establishes the concept that at least some, and perhaps all, members of the four classes of mammalian PDEs comprise a conserved enzyme family that has endured several hundred million years of evolution.

Molecular Genetics of Eukaryotic Cyclic Nucleotide PDEs. The best studied PDE gene to date is the Drosophila *dunce* gene, which encodes a low $k_m$ cAMP PDE. The locus resides at chromomere 3D4 on the X-chromosome (132). While the complete gene structure is yet to be determined, it is clear that the locus is unusually complex. The gene spans more than 100 kb of DNA and encodes at least six related RNAs ranging in size from 9.5 to 4.5 kb (132,133,134). Surprisingly, all of the RNAs appear to encode an identical protein. The upstream untranslated region of the gene is split by multiple introns that are spliced differentially to account for some of the RNA complexity (133,134). The

Table 2: Classification of Mammalian Cyclic Nucleotide PDEs. Cyclic nucleotide PDEs have been grouped according to enzymylogical, biochemical, and pharmacological properties adapted from Beavo (120). The tissue source is noted with its appropriate reference in parentheses. The term "low $k_m$" has been designated arbitrarily to signify any PDE with a $k_m$ less than 1 μM. The "low $k_m$" forms present in classes 3 and 4 represent distinct isozymes. Subclasses of brain $Ca^{++}$-calmodulin activated PDEs have been identified (39,41).

Table 2: Classification of Mammalian Cyclic Nucleotide PDEs.

| Class of PDE | Source or Property of Enzymes | Comments |
|---|---|---|
| 1. cGMP Specific | a. rod (30) cone (31)<br>b. lung (50) platelet (51) | These PDEs define the two major classes identified within this subgroup. Isozymes are expected in both subgroups. |
| 2. cGMP Stimulated | liver (32) adrenal medulla (33)<br>platelet (34) adipose tissue (35)<br>heart (36) thymocyte extract (37) | Appears to be one major form, however a smaller enzyme that exhibits similar kinetic properties has been reported (48). |
| 3. $Ca^{++}$-Calmodulin activated | lung (38) brain (39)<br>heart (40) brain (41)<br>"low $K_m$" (42) | It is thought that as many as six subclasses exist within this class. |
| 4. "low $K_m$" | a. cGMP inhibited (43)<br>b. RO 20-1724 sensitive (44) | The cGMP inhibited subclass has also been referred to as insulin sensitive (49). The RO 20-1724 sensitive class exhibits a 100 fold selectivity for cAMP as substrate. Both subclasses are thought to contain many isozymes. |

Table 2: (Cont'd.).

| Class of PDE | Source or Property of Enzymes | Comments |
|---|---|---|
| 5. Nonspecific | intestine (45) | This enzyme hydrolyzes cAMP, but is not specific for cyclic nucleotides. |
| 6. Other Cyclic Nucleotide PDEs | cCMP (46) cUMP (120) | These PDEs will hydrolyze other cyclic nucleotides (i.e. cCMP and cUMP. They are not as well characterized as the other PDEs. |

most unusual feature of the dunce locus is the presence of multiple seemingly independent transcription units identified in a 79 kb intron in the upstream region of the gene (134). At least three genes have been identified within the locus. The significance of this novel organization has yet to be determined.

Clinical Significance of a Defective cGMP PDE. A great deal of evidence has been reported that suggests a link between retinal degeneration and defects in phototransduction metabolism. Hollyfield and coworkers (84) demonstrated that elevated levels of cGMP are toxic to photoreceptor cells in *Xenopus laevis* eye rudiments, and in the adult human retina maintained in culture. The specificity of the degeneration for rod cells is quite remarkable. Several animal retinal degeneration mutants have been identified which exhibit very high levels of photoreceptor cGMP (82, 127, 128, 129), prior to the onset of any morphological disruption. In one of these mutants, the *rd* mouse (86), cGMP PDE has been shown to exhibit abnormal subunit assembly (122), and to display altered kinetic properties (82). The retinas of these mice undergo complete photoreceptor layer degeneration within 20 days postnatal (123), however other retinal layers remain essentially intact.

High cGMP levels were also reported in rod photoreceptors of a 17 year old male with autosomal dominant retinitis pigmentosa (ADRP). The authors suggested a strong correlation between morphological and biochemical characteristics in this patient's retina, and those observed in the *rd* mouse (124). Indeed, the cGMP PDE gave rise to abnormal kinetic parameters similar to the *rd* mouse enzyme. Moreover, the morphological degeneration observed was shown to be mostly limited to areas of the photoreceptor layer that are predominantly composed of rod

cells. ADRP is one of a group of clinically related retinal dystrophies of which there is presently no effective treatment and the cause is unknown (125).

The general objectives of the dissertation research presented here were to carry out a molecular dissection of rod cGMP PDE. The availability of the molecular probes provided from this work allows the opportunity to further analyze the enzyme in ways that have not, up to now, been possible. A detailed description and analysis of the work is presented in the pages ahead.

## MATERIALS AND METHODS

### Materials

Bovine eyes for retinal nucleic acid isolation were obtained from Murco, Inc. of Plainwell, MI. D. G. McConnell in the Department of Biochemistry, Michigan State University graciously provided purified cGMP PDE prepared according to Kohnken *et al.* (26). Further purification to near homogeneity was achieved by rechromatography of the final product on Sephacryl S-200 (Pharmacia). $\gamma$-[$^{32}$P] ATP and $\alpha$-[$^{32}$P] dCTP were obtained from New England Nuclear. Oligo dT cellulose type III was purchased from Collaborative Research, Inc. HPLC grade acetonitrile, trifluoroacetic acid, ammonium acetate and TMAC were obtained from Aldrich. Proteinase K, DNA polymerase I and Klenow fragment were purchased from Boehringer Manheim Biochemicals. Restriction endonucleases were obtained from New England Biolabs, International Biotechnologies, Inc., or United States Biochemicals. T4 polynucleotide kinase was purchased from Pharmacia. Sodium dodecyl sulfate was obtained from Pierce. All other biochemicals were purchased from Sigmal Chemical Co. Reagents for media preparation were obtained from Difco, except NZ-amine which was from Sheffield Products. Bacterial strains used in this study were C600 *hfl*, JM101, JM103 and JM109 (51). Cloning vectors used were pUC8, pUC19, M13mp8, M13mp9, M13mp18, M13mp19 (51), pBS (Stratagene) and $\lambda$ EMBL-3 (51).

**Methods**

Denaturing Polyacrylamide Gels. SDS-PAGE analysis was performed in 15% acrylamide/0.08% bis-acrylamide gels as described (20). Electrophoresis was carried out at 100 V until tracking dye neared the bottom of the gel. The protein bands were visualized by staining the gels with Coomassie brilliant blue (53) or with a more sensitive silver staining method (74).

Modification of the cGMP PDE. The enzyme was modified by S-carboxymethylation for sequence analysis as described by Gracy (54).

Tryptic Digestion of Modified Protein. Ten μL of TPCK treated trypsin (100 μg/ml) was added to the modified protein in an Eppendorf tube. Digestion was allowed to proceed for 3 hours at 37°C on a roller drum incubator. An additional ten μL of trypsin solution was added and the incubation continued overnight. After digestion, the solution was dried in a speed vac concentrator (Savant SVC 100). The protein was redissolved in 500 μL of water. A 20 μL aliquot was removed for SDS-PAGE analysis and the remainder was redried and stored at -20°C.

High Performance Liquid Chromatography of Trypsin Cleaved cGMP PDE. Fractionation of the trypsin cleaved protein was performed on Waters HPLC equipment utilizing reverse phase chromatography. Initial separation of peptides was achieved by elution from $C_4$ or $C_{18}$ reverse phase columns using a gradient of $H_2O$/0.075% TFA and acetonitrile/0.075% TFA as the solvent system. A linear gradient of 0-70% acetonitrile/0.075% TFA was generated over 60 minutes and approximately one mL fractions were collected manually at timed intervals throughout the gradient. Most of the collected fractions containing peptides were purified in two additional chromatography

steps. First, the peptides were eluted a second time from a reverse phase column using the same solvent system as previously described, but with a narrowed gradient range. Second, each sample was dried, resuspended and rechromatographed using 25 mM ammonium acetate, pH 6.0 (buffer A), and 50 mM ammonium acetate, pH 6.0/60% acetonitrile (buffer B). Elution was achieved with a linear gradient of 0-70% buffer B generated over 60 minutes. Samples that appeared to be homogeneous after the first rechromatographic step were not further purified.

Determination of Amino Acid Sequence of Peptide Fragments. Peptide fragments that appeared to be homogeneous after the first rechromatography step were dried in a speed vac concentrator before being sequenced. Peptides that were chromatographed in the ammonium acetate gradients were dried, redissolved in water and dried again to remove excess buffer before analysis. Sequencing reactions were carried out on a Beckman System 890 M sequencer, and the resulting PTH amino acids were determined by two independent HPLC chromatographs. All sequence analysis was carried out in the Macromolecular Facility in the Department of Biochemistry, Michigan State University under the direction of Dr. Y. M. Lee.

Molecular Cloning Protocols. Restriction enzyme digestions were carried out as previously described (55). Standard ligations into m13 replicative form DNA or plasmid DNA were as described (51). Ligations directly in nusieve agarose (FMC Bioproducts) were performed according to Dumais and Nochumson (56). Appropriate strains of E. coli were transformed as described (55).

RNA Isolation. RNA for sequencing was isolated from bovine retinas as described by Labarca and Paigen (57) with modification. Briefly, retinal tissue was homogenized (2-3 mL/gm retina) in ice-cold buffer (8 M guanidine HCl, 0.01 M EDTA in DEPC treated water). One-tenth volume of 2 M NaAc in DEPC water and one-half volume of cold ethanol were added and the solution incubated for 3 hours at -20°C. The solution was centrifuged at 8000 rpm for 20 minutes in an HB-4 rotor to pellet the RNA. The pellet was redissolved in one-half original volume of homogenization buffer. The solution was then reprecipitated as above and two more rounds of resuspension and reprecipitation were performed. RNA was extracted by adding one-twentieth of the original volume of water and heating to 70°C for 10 minutes. The solution was centrifuged for 10 minutes at 10,000 rpm in the HB-4 rotor, the supernatant recovered and the RNA content determined spectrophotometrically. Water extractions were repeated until no RNA remained in the pellet. Yields were typically 0.6 mg/gm retina (wet weight). RNA, for northern analysis, was isolated according to Chirgwin *et al.* (58).

Isolation of mRNA. Isolation of poly $(A)^+$ retinal RNA was accomplished by oligo dT cellulose chromatography as described (55), except that the SDS concentration was reduced to 0.1% and the total RNA was passed through the column three times. In addition, recovered polyadenylated RNA was rebound to the column and eluted a second time, resulting in a higher recovery of poly $(A)^+$ RNA and less contaminating nonpolyadenylated RNA.

RNA Blot Analysis. Five to ten μg of twice selected poly $(A)^+$ retinal RNA was fractionated on 0.8-1.2% formaldehyde containing agarose gels

and directly blotted onto nitrocellulose as described (59). Radiolabeled DNA probes were prepared by nick translation (55) or by random priming directly from low melting agarose gels (56). Hybridizations with cDNA probes were performed at 42°C in a buffer containing 50% formamide, 0.1 M pipes, 0.8 M NaCl, 5x Denhardts solution (59), 100 μg/ml denatured salmon sperm DNA and 10% dextran sulfate as described (59). For oligonucleotide probes, prehybridization was done in 12 mL of a buffer consisting of 40% formamide, 5x Denhardt's, 5x SSC, 50 mM sodium phosphate pH 6.8, 0.1% SDS and 250 μg/mL alkali denatured salmon sperm DNA for 2-4 hours at 42°C. Hybridization was performed in eight mL prehybridization solution, 2 mL 50% dextran sulfate, and 100 ng unpurified end labelled oligomer for 12-16 hours at 42°C. Washes were one hour with three changes in 2x SSC at room temperature with agitation, 30 minutes in 0.5x SSC at 50°C, and five minutes in 0.1x SSC at 50°C.

DNA Isolation.

a) Bacteriophage. Cesium chloride purified and miniprep bacteriophage DNAs were isolated according to Botstein and Davis (60).

b) Plasmid. Cesium chloride purified plasmid DNA was isolated according to a scaled up miniprep procedure. Briefly, transformed cells were grown to saturation, pelleted at 3500 rpm, washed in 50 mL of 50 mM tris-EDTA pH 8.5. After resuspension by gentle pipetting the cells were repelleted and resuspended in 18 mL of solution A (15% sucrose, 50 mM tris pH 8.5, 75 mM EDTA). On ice, 4.5 mL of 5 mg/mL lysozyme in solution A was added, and incubated for 10 minutes with occasional gentle agitation. Thirteen and one half mL of solution B (Solution A + 0.1% triton x-100) was added and the mixture

incubated at 37°C for 15 minutes with gentle and occasional mixing. The majority of the very viscous solution that resulted was transferred to a hard walled SW28 tube and spun at 24,000 rpm for 45 minutes at 4°C. The supernatant (cleared lysate) was then purified by two subsequent cesium chloride equilibrium gradient centrifugations (55) in vertical rotors. Cesium chloride was removed by dialysis in DSB (10 mM Tris, 1 mM EDTA, 10 mM NaCl, pH 8.0). Yields were typically 500-1500 µg/500 mL culture. Miniprep isolation of plasmid DNA was essentially as described (61), except that lysozyme treatment was performed in the absence of triton X-100, which was added just prior to boiling.

c) Genomic. Genomic DNA from a single bovine brain and from human placenta was isolated according to Hogan *et al.* (62).

Isolation of Nucleic Acids from Gels. DNA fragments were isolated from agarose gels by lining, with dialysis tubing, a trough cut in front of the band in the gel. The trough was then filled with buffer and electrophoresis was continued until the DNA was immobilized against the membrane. Then, the current was reversed for 30 seconds and the DNA was removed with a pasteur pipette. Agarose contaminants were removed by passing the solution through glass wool, and ethidium bromide was removed by extraction with n-butanol. The DNA was then precipitated and redissolved at a concentration of 250 µg/mL. Yields were from 60-90% and the DNA was suitable for all commonly used molecular biological techniques. In some experiments DNA was isolated using geneclean milkbeads (Bio 101) according to the manufacturers protocol.

Synthesis, Purification, and Radiolabelling of Oligonucleotide Probes. Some oligonucleotides were synthesized on an Applied

Biosystems 380B DNA synthesizer in the Macromolecular Facility at Michigan State University under the direction of Dr. Y. M. Lee. Additionally, oligonucleotides were made on an Applied Biosystems 381A synthesizer in the lab. Oligonucleotides were purified for use as described (63), or by a method of M. Zoller. Briefly, 150μg of oligomer was fractionated in 14-20% polyacrylamide gels. Products were identified with short wave UV illumination onto fluorescent TLC plates behind the gel. Bands were excised, crushed and eluted into a buffer containing 0.5 M ammonium acetate, 1 mM EDTA, and allowed to diffuse overnight at 37°C. Gel pieces were removed from the DNA by passage through millex-HV filters (Waters). Further purification of the oligomer was achieved by Sep Pak $C_{18}$ (Waters) chromotography.

Labelling of Nucleic Acids.

Kination. Oligomers were radiolabeled with γ-[$^{32}$P] ATP using T4 polynucleotide kinase according to the manufacturer's protocol. Unincorporated radionucleotide was removed by two rounds of precipitation using denatured salmon sperm DNA as carrier.

Nick Translation. Labelling was performed as previously described (59).

Oligolabelling. Random hexamers (pharmacia) were used with the Klenow fragment of DNA polymerase to incorporate α-[$^{32}$P] dCTP essentially as described by Feinberg and Vogelstein (130).

Screening Human and Bovine cDNA Libraries. The human and bovine retinal cDNA libraries were constructed in the bacteriophage lambda cloning vector λgt10 (14,15). The human cDNA library was screened with an oligomer and a cDNA probe. Hybridizations with the oligomer were performed at 42°C in a buffer containing 0.9 M NaCl, 0.09

M NaCitrate, 50 mM sodium phosphate, pH 6.8, 5x Denhardts solution, 100 μg/mL denatured salmon sperm DNA and 10% dextran sulfate as described (59). Hybridizations with the cDNA probe were done at 42°C in a buffer containing 50% formamide, 0.1 M pipes (Ultrol), 0.8 M NaCl, 5x Denhardts solution, 100 μg/mL denatured salmon sperm DNA and 10% dextran sulfate as described (59). The bovine library was screened according to a method developed by E.F. Fritsch, Genetics Institute, Boston, MA. Briefly, filters containing ~20,000 p.f.u. were prehybridized at 65°C in a buffer consisting of 3.0 M TMAC, 50 mM sodium phosphate pH 6.8, 1mM EDTA, 0.5% SDS, 5x Denhardt's solution and 100 μg/mL alkali denatured salmon sperm DNA. After 1-2 hours filters were washed with kimwipes (Kimberly-Clark Corp.), and then placed into fresh prehybridization solution for 12-16 hours at 65°C. Hybridization was carried out in a large volume (10-15 mLs per filter) of prehybridization solution with 0.1 picomoles of kinased oligomer probe added per mL of solution. Incubation was done at 35-45°C for at least 48 hours, or 72 hours for highly redundant probes. Washes were done in a large volume of 2x SSC, 0.2% SDS at room temperature for two hours with one change of solution. Filters were then air dryed and placed at -70°C for autoradigraphy.

Screening and Analysis of a Bovine Genomic DNA Library. A bovine genomic DNA library constructed with λ EMBL-3 was purchased from Clontech. The library was screened as described for the cDNA library with cDNA probes. An isolated genomic clone was mapped by restriction analysis, and according to the labelling and partial digestion method of Baker and Board (64).

Determination of Gene Copy Number. The fraction of DNA that a 6.2 kb fragment represents in 10 μg of genomic DNA was calculated by assuming a genome size of $3x10^9$ base pairs, and 660 g/mole/base pair. Amounts of the isolated genomic fragment equivalent to 1-6 copies of the fragment were loaded into an agarose gel adjacent to 10 μg of genomic DNA digested with HindIII or EcoRI. Comparison of hybridization intensities allows an estimate of gene dosage (see Fig. 10).

Nucleic Acid Sequence Analysis.

M13 Single Stranded DNA. M13 single stranded DNA was isolated as previously described. Nucleotide sequences from inserts cloned into M13 vectors were determined using the dideoxy chain termination method as described (65) with the following modifications. Buffer gradients (66) were employed using 60 cm gels, α-thio-[$^{35}$S] dCTP was used in place of α-[$^{32}$P] dCTP and reactions were performed with a sequenase kit (U. S. Biochemicals). These modifications allowed up to 500 nucleotides to be determined on a single 60 cm gel.

Double Stranded Plasmid DNA. Plasmid DNAs were sequenced exactly as described by Zhang *et al.* (67).

λ DNA. Inserts in λ EMBL-3 were sequenced according to Manfioletti and Schneider (68) except that DNA isolated from cesium chloride purified λ phage was used as a template.

mRNA. RNA was sequenced essentially as described by Geliebter (69) except that all dideoxynucleotides were added from a 0.5 mM stock.

Chromosomal Localization. The initial location was determined using fourteen human-rodent somatic cell hybrids. Construction, karyotyping, and DNA blot analysis of these hybrids has been previously

described (70). Confirmation of the assignment and regional localization was obtained with a panel of 11 human-hamster hybrids that contain only subsets of human chromosome 5 (71,131). DNA blotting, hybridizations, and washings at high stringency were as described (59).

Computer Analysis. RNA and DNA sequences were analyzed with a number of sequence analysis packages run on microcomputers under DOS, or on a minicomputer under the VMS operating system. Programs written by R. Staden (72), D. Mount (73), and Bucholtz and Reisner (74), were used to align and analyze all of the sequence described in this work. Sequences from autoradiographs were read directly into microcomputers using a graphbar digitizer (Scientific Accesories Corp.).

# RESULTS

## Section A Bovine cGMP PDE α-Subunit.

Trypsinolysis and Peptide Sequence Analysis of Bovine cGMP PDE. One mg of highly purified (see Materials) cGMP PDE was graciously provided by D.G. McConnell, Department of Biochemistry, Michigan State University. After SDS-PAGE, cGMP PDE gives rise to three subunits, α (88 kd), β (84 kd), and γ (11 kd) which does not stain well (Figure 2A, lane 1). After modification and tryptic digestion, the sample appeared as a low molecular weight smear (Figure 2A, lane 2), on a 20% acrylamide gel. One-half (250 μg) of the total modified and digested sample was fractionated by reverse phase HPLC. The initial sample was divided into three aliquots, each of which was fractionated separately. Figure 2B, profile a, illustrates a representative chromatographic profile. For two of these fractionations, fractions of one ml were collected at thirty second intervals. Fractions indicated on the figure were pooled for rechromatography. However, fractionation of one aliquot gave very high absorbance readings and so all the material from this final fractionation were combined with the selected pooled fractions from previous chromatography steps and rechromatographed. The resulting profile is shown in Figure 2B, profile b. Fractions were collected at thirty second intervals and regions were selected for rechromatography. Material

Figure 2: Analysis of Tryptic Peptides of Bovine cGMP PDE. One mg of highly purified protein was modified and digested as stated in Methods. **A.** Lane 1: SDS-PAGE analysis of 3 μg purified cGMP PDE protein. Lane 2: 1/25 of the total sample after modification and trypsinolysis. **B.** Initial fractionation of 250 μg of modified trypsin digested sample. Profile a: Tryptic fragments were eluted from a reverse phase HPLC column. Peaks designated by letters were pooled for subsequent purification. Profile b: Pooled fractions from initial chromatographs were recombined and rechromatographed. Peaks corresponding to peptides which yielded sequence data are indicated. Profile c: Samples remaining from all previous chromatographs were pooled and rerun as described. Peaks corresponding to peptides that yielded sequence data are indicated. **C.** Purification of pooled samples. Profile a: Peaks E and F (panel B, profile b) were combined and fractionated under the same conditions as previously to yield peptide cPDE6. Profile b: A peak (not shown) purified from peak D (panel B, profile c) was rechromatographed under the same conditions as previously. Profile c: Peaks A and B (panel C, profile b) were fractionated using a linear gradient of ammonium acetate/acetonitrile as described in Methods. **D.** Sequence analysis of peptides. Seven purified peptides were subjected to automated Edman degradation and analyzed by reverse phase HPLC. Yields of PTH amino acids are shown for two peptides, PDE3 and PDE6, that proved to be crucial to this study.

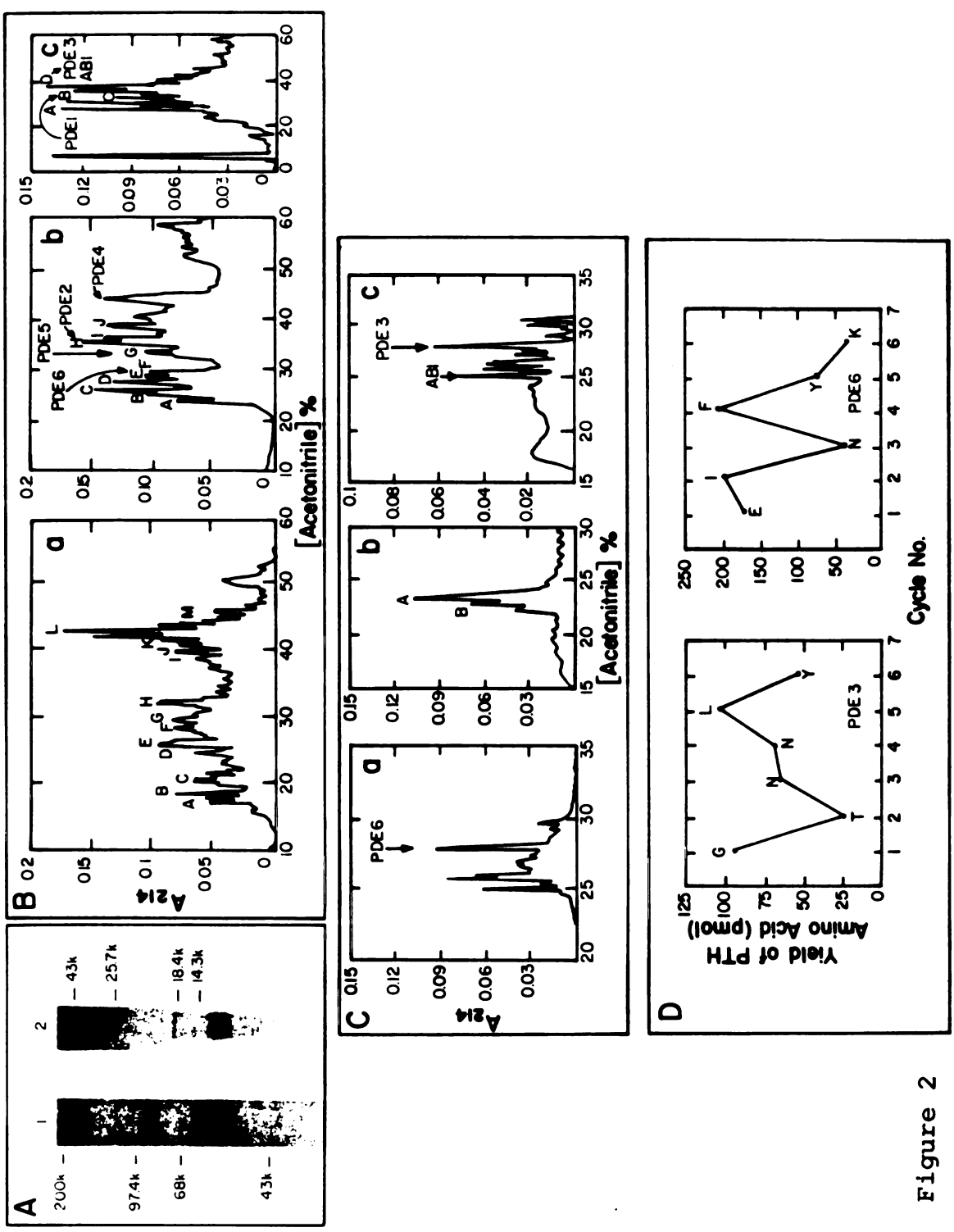

A
200k
97.4k
68k
43k
1
2
43k
25.7k
18.4k
14.3k
B
a
b
c
A214
[Acetonitrile] %
PDE1
PDE2
PDE3
PDE4
PDE5
PDE6
ABI
C
D
Yield of PTH
Amino Acid (pmol)
Cycle No.

Figure 2

remaining from the previous fractionations and any material not used from the experiment shown in profile b was combined and again chromatographed with the resulting profile shown in Figure 2B, profile c. Fractions corresponding to regions A-D were pooled separately. Each combined fraction was rechromatographed to obtain homogeneous peptide. Figure 2C, profile a shows the result of rechromatographing peaks E and F (Figure 2B, profile b), from which peptide PDE6 was obtained. Similarly, peptides AB1 and PDE3 were obtained after further purification of peak D (Figure 2B, profile c). In all, eighteen peptides were purified in sufficient yield to obtain amino acid sequence. Eight peptides were chosen for sequence analysis in the Department of Biochemistry, Michigan State University macromolecular facility. Amino acid sequences were obtained for seven of the peptides, but the eighth could not be analyzed. No trace of PTH derivative was observed for the eighth sample indicating the absence of protein. Figure 2D shows the yield of PTH amino acid for peptides PDE3 and PDE6.

Synthesis of Oligonucleotide Probes. A unique oligonucleotide unambiguously reflecting the sequence of each peptide cannot be fashioned due to the degeneracy of the genetic code. Several alternative approaches have been used to circumvent this problem. First, unique oligonucleotides of length greater than 32 bases have been synthesized using information such as codon preferences and relative instability of an A:C mismatch compared to a G:T mismatch to create a sequence that is most likely to correspond to the *in vivo* sequence (75). Second, mixtures of oligonucleotides have been synthesized that reflect all possible sequences of a peptide (77). In this case, peptides containing serine, leucine or arginine residues which have degeneracies at more

than one position are avoided. And third, deoxyinosine has been used at the *wobble* position (78), since preliminary evidence suggested that the hypoxanthine base of deoxyinosine acted in an inert fashion, neither contributing to, nor destabilizing the DNA hybrid (79). Again, peptides containing amino acids with greater than four degeneracies are not desirable with this approach. Elements of all three of these approaches were incorporated into the design of the oligonucleotides described in this section. A codon usage table was compiled (not shown) from the deduced amino acid sequences of 12 bovine genes extracted from the Genbank nucleic acid database (80). From this table, it was found that leucine residues are encoded by C,T at the first and second positions, respectively, greater than 75% of the time. Therefore, for the leucines encountered in the seven peptides, the codon C T I was used. For amino acids where there were only two possible codons, both were included in the oligonucleotide mixture, and any amino acid where three or four codons were possible, a deoxyinosine was incorporated as the *wobble* base. Figure 3 presents the amino acid sequence for each peptide and the corresponding oligonucleotides.

Screening the Bovine Retinal cDNA Library. One hundred and twenty thousand plaques from a bovine retinal cDNA library (15) were screened with each oligonucleotide on duplicate filters. A novel method of plaque hybridization was used, taking advantage of the properties of TMAC (see Methods). From the initial library screen, 285 hybridizing clones were identified on both the original and duplicate filters. These clones were rescreened by scoring, into sixteen sections (Figure 4), an 85 mm petri dish plated with the appropriate bacterial host strain. A drop of phage suspension was placed in a section and allowed to drip down

Figure 3: Design of Oligonucleotide Probes. **A.** Sequence of seven tryptic peptides from amino to carboxyl terminus. Peptides PDE3 and AB1 terminate with a tyrosine residue, presumably due to contaminating chymotryptic activity not quenched by TPCK. Peptide PDE4 is truncated due to an insufficient amount of sample. No PTH amino acid was observable for residue 7 in peptide 5. **B.** Sequence of the oligonucleotides. Oligonucleotide mixtures containing deoxyinosine residues and reflecting some codon degeneracy were synthesized (see text). I is used as the single letter designation for deoxyinosine. An oligonucleotide was not synthesized reflecting the sequence of peptide AB1.

**A**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| PDE1 | ser | gln | asn | pro | leu | ala | lys | | |
| PDE2 | glu | ile | leu | phe | tyr | lys | | | |
| PDE3 | gly | thr | asn | asn | leu | tyr | | | |
| PDE4 | phe | leu | asp | gln | asn | | | | |
| PDE5 | val | asp | ile | tyr | ile | leu | - | gly | lys |
| PDE6 | glu | ile | asn | phe | tyr | lys | | | |
| AB1 | glu | leu | ala | gln | tyr | | | | |

**B**

| | |
|---|---|
| cPDE1 | 3' G T $\frac{T}{C}$ T T $\frac{A}{G}$ G G I G A I C G I T T 5' |
| cPDE2 | 3' C T $\frac{T}{C}$ T A I G A I A A $\frac{A}{G}$ A T $\frac{A}{G}$ T T 5' |
| cPDE3 | 3' C C I T G I T T $\frac{A}{G}$ T T $\frac{A}{G}$ G A I A T 5' |
| cPDE4 | 3' A A $\frac{G}{A}$ G A I C T $\frac{A}{G}$ G T $\frac{T}{C}$ T T 5' |
| cPDE5 | 3' C A I C T $\frac{A}{G}$ T A I A T $\frac{A}{G}$ T A I G A 5' |
| cPDE6 | 3' C T $\frac{T}{C}$ T A I T T $\frac{A}{G}$ A A $\frac{A}{G}$ A T $\frac{A}{G}$ T T 5' |

Figure 3

Figure 4: Screening of the Bovine Retinal cDNA Library. The primary screening of the bovine cDNA library resulted in the recovery of 285 positively hybridizing recombinant phages. **A.** In order to rapidly rescreen the clones obtained, an 85 mm petri dish was scored into sixteen sections. Undiluted phage representing each clone was allowed to drip down one scored slot as described in Methods. **B.** Number of positively hybridizing phage for each oligonucleotide probe obtained in the original screen. **C.** Number of positively hybridizing phage after rescreening each clone obtained in the original screen with each oligonucleotide probe. For example, of the thirty-two clones identified with cPDE1, four of them hybridized to cPDE3. Three more rounds of hybridization reduced the number of clones to seventeen, all of which hybridized to cPDE3 and cPDE6.

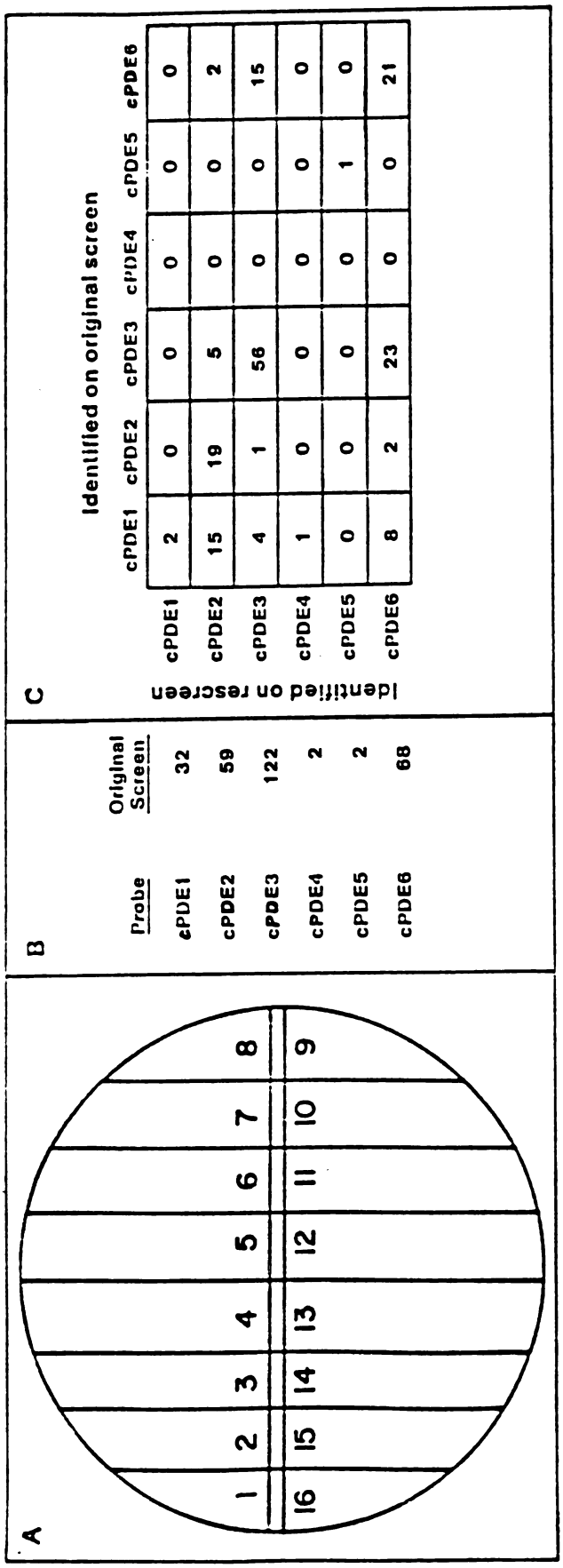

A
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
B
Probe
Original Screen
cPDE1 32
cPDE2 59
cPDE3 122
cPDE4 2
cPDE5 2
cPDE6 68
C
Identified on rescreen
Identified on original screen
cPDE1 cPDE2 cPDE3 cPDE4 cPDE5 cPDE6
cPDE1 2 0 0 0 0 0
cPDE2 15 19 5 0 0 2
cPDE3 4 1 56 0 0 15
cPDE4 1 0 0 0 0 0
cPDE5 0 0 0 0 1 0
cPDE6 8 2 23 0 0 21

Figure 4

the agarose surface. Six filters were lifted from each plate and hybridized as described in Methods. In this manner, all of the initially identified clones could be rescreened with each oligonucleotide probe. More than 200 of the original clones gave a hybridization signal using this procedure. Therefore, it was necessary to find a means of limiting the subsequent analysis. The criteria used to limit further analysis were strength of hybridization signal and hybridization to more than one oligonucleotide. Twenty-five clones met these criteria and were rescreened with the oligonucleotide probes. Of the 25 clones, 20 showed hybridization with both cPDE3 and cPDE6, three hybridized to cPDE2 only, one hybridized to cPDE5, and one showed no apparent hybridization. Further characterization focused on 17 of the 20 clones that hybridized to both cPDE3 and cPDE6 oligonucleotide probes. The remaining three clones were omitted from further analysis because they failed to hybridize in subsequent rescreens. A summary of the results of the first two screening procedures is shown in Figure 4.

Characterization of Bovine cDNA Clones. DNA isolated from the seventeen clones described above was digested with EcoRI, fractionated on a 0.8% agarose gel, and visualized by staining with ethidium bromide (not shown). All of the clones share 662 and 144 bp fragments, except one clone where no insert was apparent. Six of the clones designated 8, 15, Pα-1, Pα-2, Pα-3, and Pα-4 (Figure 5) contain more than two EcoRI fragments. Subsequent analyses concentrated on these clones since it was thought that they would represent longer cDNAs. Additionally, another clone designated 7 (Figure 5) was isolated by rescreening the library with cDNA probe 3 (Figure 7). A schematic map constructed based on sequence analysis of the cDNA clones is shown in Figure 5.

Figure 5: Schematic Map of Bovine cDNA Clones. The approximate positions of four oligonucleotides (designated 1,3,5 and 6) that were identified from a cGMP PDE tryptic hydrolysate are shown on the restriction map at the bottom. Wider rectangles represent protein coding segments and more condensed rectangles depict upstream or downstream untranslated regions. A segment that is essentially identical in all of the clones is shown as an unfilled rectangle. Included in this region is the "PDE HOMOLOGY" domain that defines a segment conserved in a number of CN PDEs (see text). Regions of sequence divergence are represented by different fill patterns. Arrows at the top mark the positions of serine (Ser) and glycine (Gly) residues that define the endpoints of the region common to all of the clones. Predicted amino and carboxy termini are designated $NH_2$ and $COO^-$ respectively. The three cDNAs (numbered 8,7, and 15) that overlap to define the cGMP PDE α-subunit sequence are shown. Shown at the bottom are the restriction sites that are common to the clones, and other EcoRI sites that are clone specific are shown below that clone with the corresponding size of the unique restriction fragment.

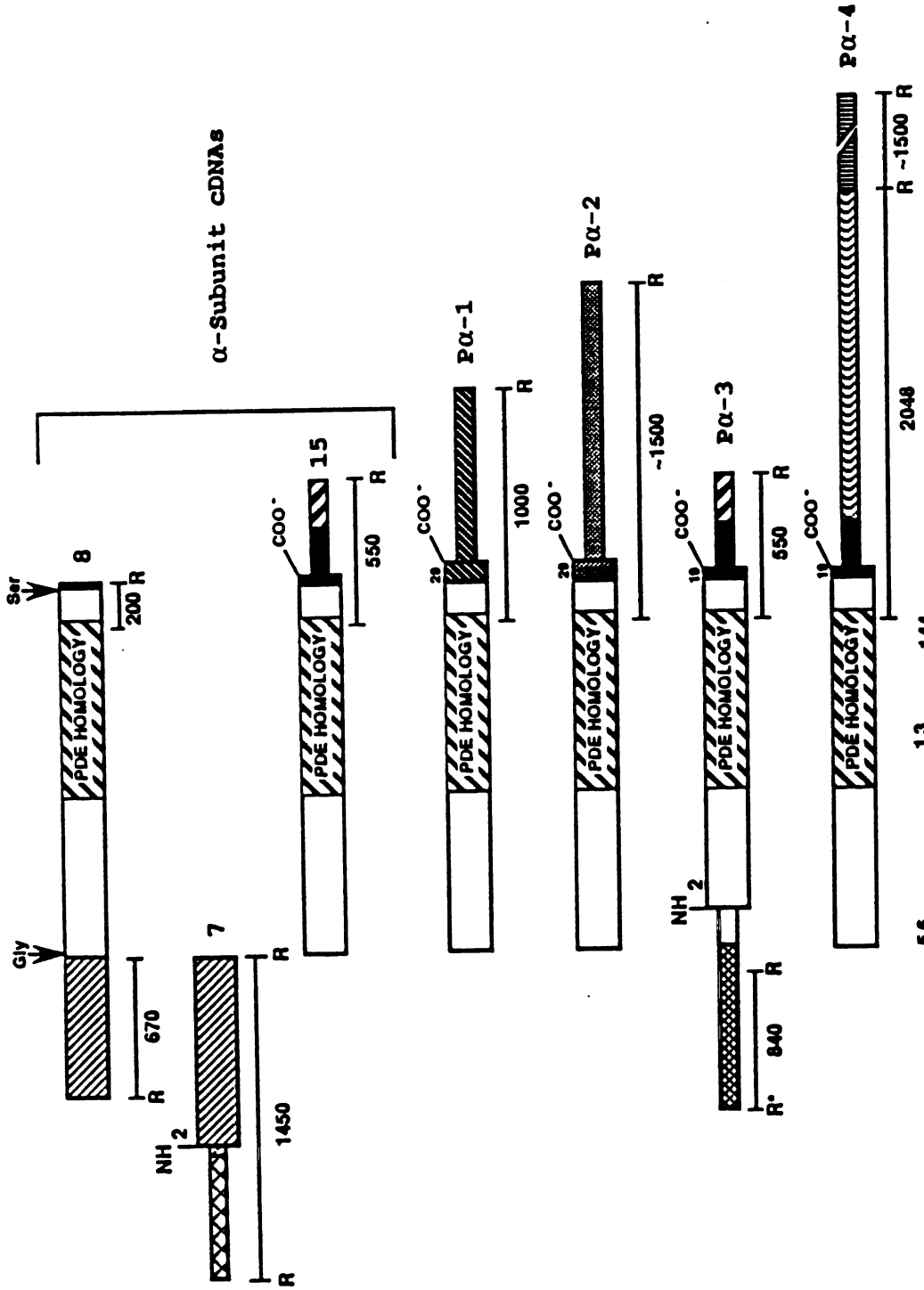

α-Subunit cDNAs
Pα-1
Pα-2
Pα-3
Pα-4
PDE HOMOLOGY
COO⁻
NH2
Gly
Ser
R
R*
670
1450
200
550
1000
-1500
2048
R -1500
840
705
662
56
144
200 bp

Figure 5

The sequences of specific oligonucleotide primers used to complete all of the sequence analysis described in this study are shown in Table 3.

Three clones (7,8,15, Figure 5) within one cDNA class overlap to define the cGMP PDE α-subunit. The composite sequence from these cDNAs contains a 2577 nucleotide open reading frame that encodes a sequence of 859 amino acids (Figure 6A). Although the nucleotide sequence of this cDNA is 99.3% identical to the cDNA sequence of the α-subunit reported by Ovchinnikov *et al.* (6), a number of differences were determined. Discrepancies were identified at fourteen positions of which three result in amino acid changes (summarized in Table A1).

Four closely related α-subunit like variant cDNAs (designated Pα-1, Pα-2, Pα-3, and Pα-4) were also identified (Figure 5). Nucleotide sequences representative of important residue changes in these cDNAs are shown (Figure 6B,C,D, Figure A4). Pα-1 and Pα-2 are identical in sequence with the α-subunit from the start of each clone at nucleotide 914 (see Figure 6A,B,C) through to position 2575. Both cDNA clones predict 29 additional amino acids following this position before an in frame stop codon is encountered (Figure 6B,C). These 29 residues show no obvious relationship to each other or any similarity to the 19 residues at the C-terminus of the α-subunit.

The nucleotide sequence of Pα-3 is identical to the sequence of the α-subunit from nucleotide 941 to the end of the clone at nucleotide 2958 (see Figure 6A,D). The divergent sequence in Pα-3 converts the methionine codon at amino acid position 296 (Figure 6A) to a valine codon and introduces an in frame stop codon at nucleotides -5 to -7 (Figure 6D). The methionine at amino acid position 360 (Figure 2A) would then define the N-terminus yielding a protein of molecular weight

Table 3: Primers Used for Sequence and RNA Blot Analysis. The sequence of all oligonucleotides useful to this study is shown. Base positions are according to the designated figures. Figure numbers preceeded by an A signify the Appendix. Position numbers are ascending for sense primers and descending for antisense primers. Nucleotides shown in lower case represent mismatches in primers used for human cDNA sequencing. For example, for sequencing primer sp-35 the human cDNA contains the sequence CCACAGAGGCAC**CAATA** (mismatches in bold letters). Primer sp-7 was synthesized according to a published sequence of a portion of λ gt10 (126). The letter designations of oligonucleotide probes mapped in Figure 7 is shown next to the primer name.

Table 3: Primers Used for Sequence and RNA Blot Analysis.

| Name | Sequence | Figure | Position | Name | Sequence | Figure | Position |
|---|---|---|---|---|---|---|---|
| sp-1 | CATACATGTGGTAGA | A4 | 1935-1921 | sp-2 | TCTACCACATGTATG | A4 | 1921-1935 |
| sp-3 | TTGGCCAGTGGGTTC | 6 | 1913-1898 | sp-4 | GCGaATCATTGCTcACA | 6 | 2047-2061 |
| sp-5 | CACATGACAACTGTA | A4 | 3105-3091 | sp-6 | TACAGTTGTCATGTG | A4 | 3091-3105 |
| sp-7 | GACTGCTGGGTAGTC | - | λ left | sp-15 | GGCTCTAAACCTTCA | A4 | 2276-2290 |
| sp-16 | GACCTTGATGTAGCT | A4 | 2741-2727 | sp-17 | CATAGTGATAAGAAT | A4 | 3920-3906 |
| sp-20 | GCGTAGGGTGGAGCC | 6 | 962-948 | sp-22 | GGCTCCACCCTACGC | 6 | 948-962 |
| sp-23 | GTCCTCTGAAGGCGC | 6 | 1153-1138 | sp-24 | TGAAGGTTTAGAGCC | A4 | 2290-2276 |
| sp-27 | AGCTACATCAAGGTC | A4 | 2727-2741 | sp-28 | GTAGCCCTTACTCAGGG | 6 | 1711-1697 |
| sp-29 | GCCTGATTTGCAACATC | 6 | 1114-1130 | sp-30 | AGACCACAACAGTATCTG | 6 | 765-748 |
| sp-33 | CTGACACTcTATGAGTTcGa | 6 | 1355-1371 | sp-34 | CtAgACTCATAAgGTGTCAG | 6 | 1371-1355 |
| sp-35 | CCACAGAGGCACTcAACtA | 6 | 1860-1876 | sp-36 | GTCTTAGACTGATCCAC | 6 | 2123-2107 |
| sp-37 | CTCACAGAGTACCAGAC | 6 | 559-575 | sp-38 | CCAGATCATATTCCATG | A3 | 597-613 |
| sp-39 | TTCATGACATTGAAGACtGC | 6 | 302-284 | sp-40 | GaATTTCCtCTTCCATCCGG | 6 | 993-976 |
| sp-50 | CATGATTATGGCCACCACATC | 6 | 633-613 | sp-51 | CCTGGGTCCCAGCAGGTCTGA | 6 | 174-153 |

Table 3: (Cont'd.).

| Name | Sequence | Figure | Position |
|---|---|---|---|
| sp-54 | GcGGCtCCTgGTACATGAATcAGGCT | 6 | 360-339 |
| sp-55 (A) | AAACTTCTCTACTTCCTCTGCCGTCACCTCGCCCAT | 6 | 90-55 |
| sp-56 | GACTTCTCTGAGTCTTGTTTGTGAACCTCGTTGGTGTGGC | 9 | 4- -36 |
| sp-57 (B) | ACTTCCTGCGGCTGCTTGGTTGGCTGC | 6 | 2574-2548 |
| sp-58 (C) | TTGGACGCAGCAAGACTTGGATGCAGGCCCGCCCCCAGGTTGCTTTCCGC | 6 | 2631-2582 |
| sp-59 (D) | GGAAAGATGTAAACTAAATTCTCTTTCAGTTTTCAAACCCCACAACTC | 6 | 2777-2730 |
| sp-60 (G) | CTTCTTACATGTTCACTGCTGGTTATATCTATAACGCACAGTGGCATAG | 6 | -1- -49 |
| sp-61 (E) | AACAAGGCTCTTCCATATGCATCACAAGCAGCTCCATAAGAGTGTATTCT | 6 | +85- +36 |
| sp-62 (F) | AGGTGAGGTGGTGTCACCTCCTATATCTTGGCCCTCAGGCTTTGGGCTTG | 6 | +86- +37 |

Figure 6: Nucleotide and Deduced Amino Acid Sequences of Bovine cDNA Clones. Nucleotide numbering is shown on each side of the sequence and amino acids are numbered above each tenth residue. The complement of oligonucleotides synthesized for RNA blotting experiments (see Table 3) are underlined. In frame stop codons that define the open reading frame are denoted by asterisks. **A.** Sequence of the cGMP PDE α-subunit. Peptide sequences obtained from cGMP PDE (see Figure 3) are boxed. Internal EcoRI sites and the EcoRI* site are underlined. A solid box is present above residues at the C-terminus that are potential sites of palmitylation (see text). **B.** Sequence of cDNA Pα-1 and **C.** Pα-2. Serine 840 corresponds to the α-subunit sequence. Nucleotide sequence divergent from the α-subunit is shown in capital letters and numbering is preceeded with a plus sign. **D.** Sequence of cDNA Pα-3. Nucleotide sequence of Pα-3 that differs from the α-subunit sequence is shown in capital letters and numbering is preceeded with a minus sign. The proposed amino terminal methionine corresponds to amino acid 360 of the α-subunit sequence.

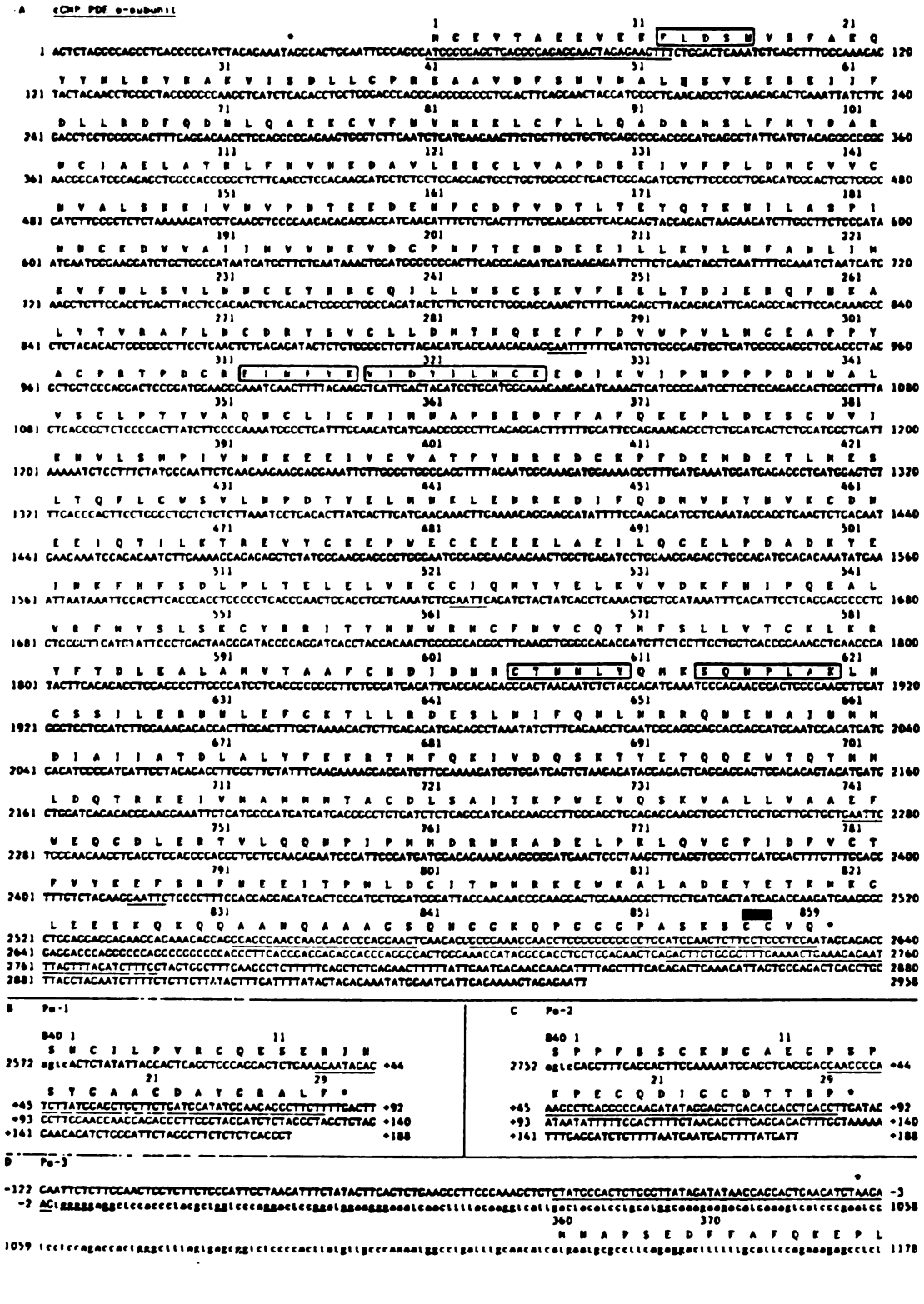


Figure 6

58,262. Clone Pα-4 differs from the α-subunit sequence only in its 3' untranslated region. The sequence divergence begins at residue 1816 (Figure A4). An additional EcoRI fragment of ~1500 bp is also present in the Pα-4 cDNA clone. An artifactual organization caused by the ligation of two unrelated cDNAs cannot be ruled out. Thus, cDNA sequence analysis predicts that three proteins exist in the bovine retina with a striking similarity to the cGMP PDE α-subunit, and one additional cDNA clone that may differ only in its 3' untranslated sequence.

Blot Analysis of Bovine Retinal RNA. In order to understand the relationships amongst the cDNAs specific oligonucleotides were used as probes for bovine and human retinal poly $(A)^+$ RNA. Several oligonucleotide probes specific to the α-subunit sequence, and a bovine cDNA probe containing sequence common to all of the clones hybridize predominantly to two relatively abundant bovine retinal RNAs of 4.6 and 4.0 kb (Figure 8, lanes 1-6). Oligonucleotide probes specific to the C-termini of Pα-1 and Pα-2 hybridize to bovine RNAs of ~4.3 and 4.8 kb respectively (Figure 8, lanes 7,8). Additionally, a Pα-3 specific oligonucleotide hybridizes to low abundance transcripts of ~5.8, 4.4, and 3.7 kb (Figure 8, lane 9).

Using oligonucleotide probes specific for the C-termini of Pα-1 and Pα-2, transcripts of ~5.3 and 3.5 kb are detected in poly $(A)^+$ from human retina, respectively (Figure 8, Lanes 10,11). A smear of hybridization to high molecular weight RNAs was observed (not shown) with cDNA probe 9 (Figure 7) that is specific for the 2048 bp EcoRI fragment of Pα-4. A similar but nonidentical smear was obtained (not shown) using a probe specific to the 1500 bp EcoRI fragment from Pα-4 (Figure 7). It is possible that these probes are hybridizing to a common

Figure 7: Location of cDNA and Oligonucleotide Probes. The cDNA clones are shown in schematic form as in Figure 5. The α-subunit cDNAs have been combined to show their composite organization. Maps for each cDNA clone (7, 8 and 15) that make up the α-subunit are also shown. Positions of oligonucleotide probes are designated by small boxes (labelled A-G) that are sized to roughly represent the length of the oligomer (see Table 3). Lines labelled with arabic numerals represent cDNA probes used in RNA and DNA blotting experiments.

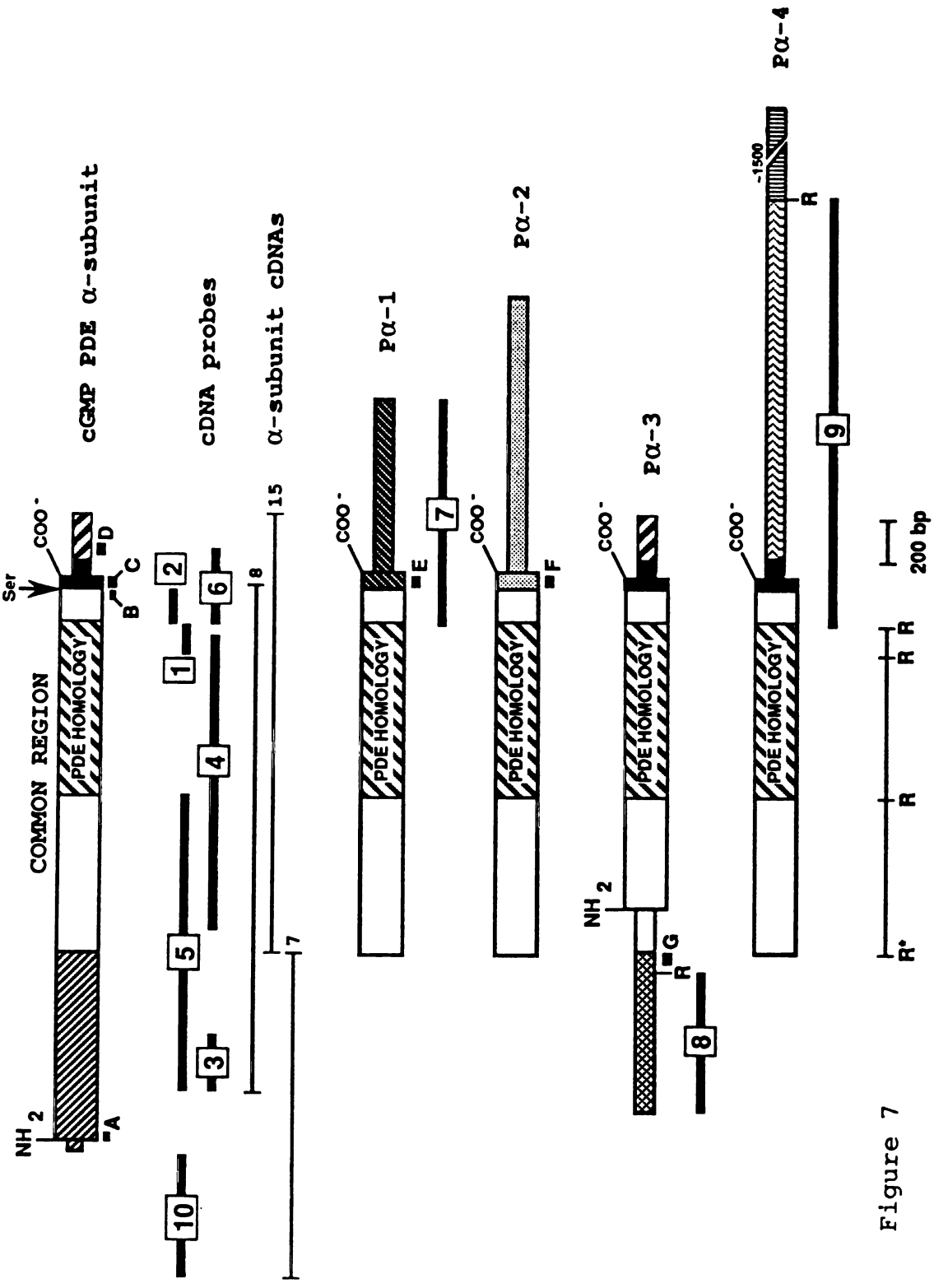

cGMP PDE α-subunit
COMMON REGION
PDE HOMOLOGY
Ser
COO⁻
NH 2
A
B
C
D
cDNA probes
α-subunit cDNAs
1
2
3
4
5
6
7
8
10
15
Pα-1
Pα-2
Pα-3
Pα-4
E
F
G
R
-1500
9
200 bp

Figure 7

Figure 8: Analysis of Bovine Retinal RNA. Approximately 2 μg poly $(A)^+$ RNA (lanes 1-9, bovine RNA; lanes 10-11, human RNA) was fractionated on a 1.0% agarose, formaldehyde containing gel and transferred to nitrocellulose (22). Nitrocellulose strips were hybridized as described in Methods. The probes used for each blot strip shown are as follows: lane 1, probe 5; lane 2, pos. -35-+6 (Figure 9); lane 3, probe A; lane 4, probe B; lane 5, probe C; lane 6, probe D; lanes 7 and 10, probe E; lanes 8 and 11, probe F; lane 9; probe G. All probe locations are shown in Figure 7 unless otherwise noted. Exposure times were three hours without an intensifying screen for lanes 1-6 and 54 hours with two intensifying screens for all other lanes. Lane 1 blot strip was hybridized once prior with probe 10 (Figure 7).

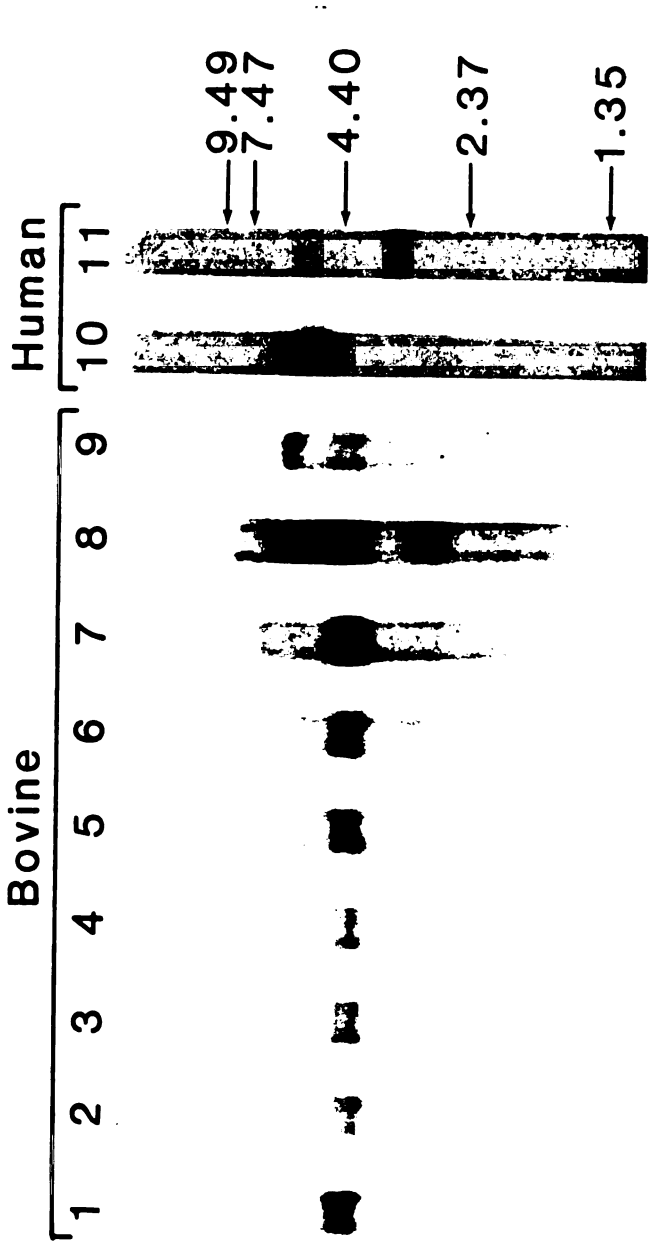



Figure 8

set of RNAs, but the data do not unambiguously support such a conclusion. However it does appear that at least seven related RNAs are identified using clone specific probes.

Sequence Analysis of Bovine Retinal RNA. The identification of the a-subunit sequence was based on comparison of the cDNA sequences described in this study to that reported by Ovchinnikov *et al.* (29). In that report the α-subunit sequence was also determined from a number of overlapping cDNA clones. Further support for the authenticity of the sequence was provided by the presentation of several peptide sequences obtained from purified α-subunit. Two of the identified peptides were found only in the α-subunit hydrolysate and not in the β-subunit hydrolysate. However, two regions of the reported sequence were not supported by sufficient overlap of cDNA clones, nor by peptide sequence. These regions are roughly defined from nucleotide 175-592 and 2512-3' end (Figure 6A). In order to confirm the sequence in these regions direct dideoxy sequencing of RNA was performed. The results of this analysis is shown in Figure 9. Several specific oligonucleotides (see Table 3) were used to prime specific regions of interest in the RNA. The sequence obtained shows complete agreement with the reported sequence (29) and the sequence identified in this study from cDNAs 7,8, and 15 (Figure 6A). Therefore, the peptide sequences reported for the α-subunit (29), and those reported here, together with the RNA sequencing data provides strong support for the entire predicted α-subunit primary structure.

Isolation of a Bovine Genomic Clone. A genomic library was screened with bovine cDNA probe 4 (Figure 7) resulting in the recovery of a ~13.5 kb clone. A restriction map of this clone is shown in Figure 10A. The EcoRI digestion pattern is somewhat tentative because of the small

Figure 9: Direct Chain Termination Sequencing of Bovine Retinal RNA. The results of sequence analysis of bovine retinal poly (A)+ RNA is shown. The location of the sequencing primers used are underlined with the primers name below the sequence. Numbering is according to Figure 6A. Nucleotides shown in bold uppercase letters represent the extent of the sequence determined with confidence. A 1340 nucleotide portion of the sequence designated "1340 nt" has been omitted because no RNA sequence analysis was necessary in this region.

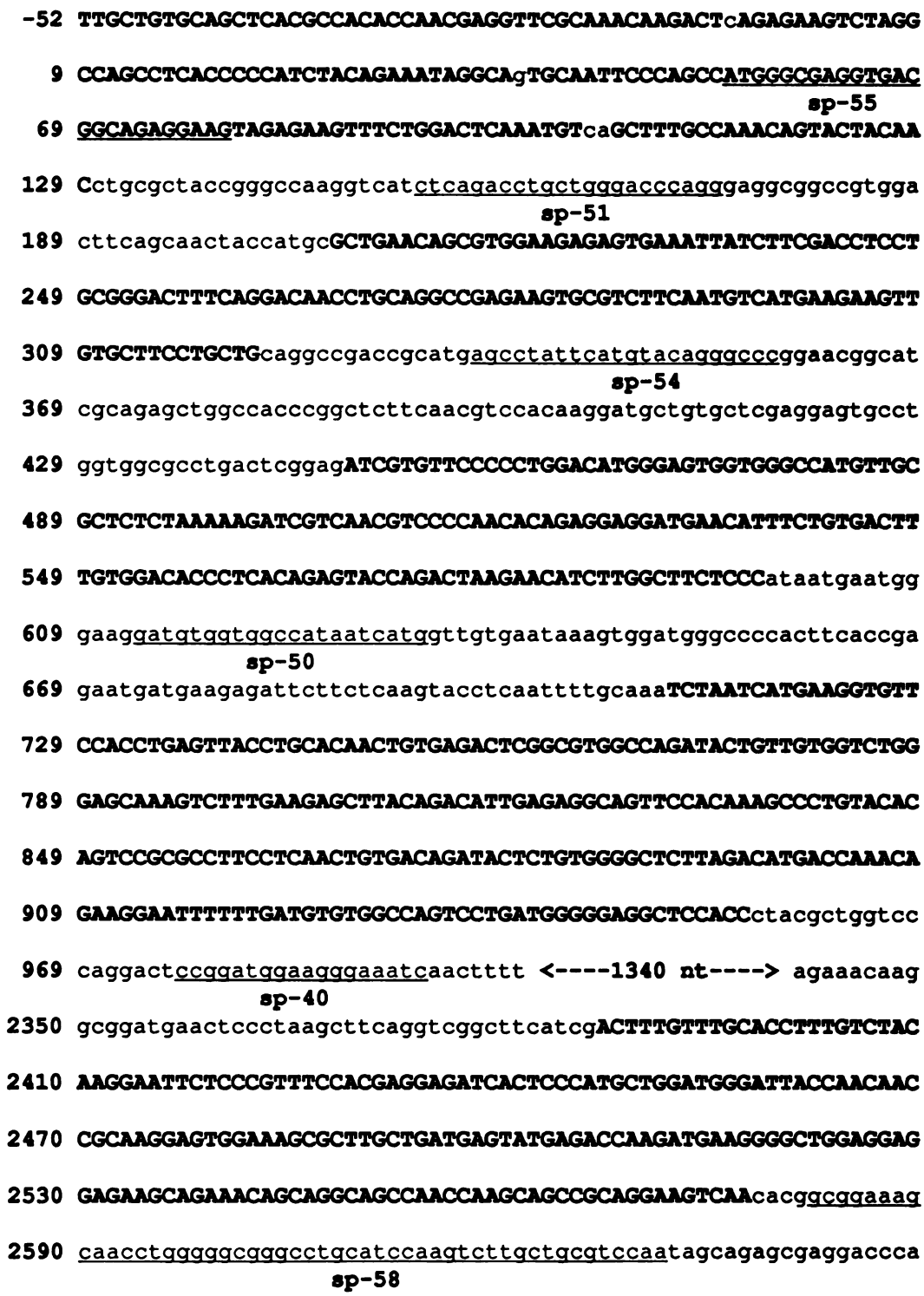

-52 TTGCTGTGCAGCTCACGCCACACCAACGAGGTTCGCAAACAAGACTcAGAGAAGTCTAGG
9 CCAGCCTCACCCCCATCTACAGAAATAGGCAgTGCAATTCCCAGCCATGGGCGAGGTGAC
sp-55
69 GGCAGAGGAAGTAGAGAAGTTTCTGGACTCAAATGTcaGCTTTGCCAAACAGTACTACAA
129 Cctgcgctaccgggccaaggtcatctcagacctgctgggacccagggaggcggccgtgga
sp-51
189 cttcagcaactaccatgcGCTGAACAGCGTGGAAGAGAGTGAAATTATCTTCGACCTCCT
249 GCGGGACTTTCAGGACAACCTGCAGGCCGAGAAGTGCGTCTTCAATGTCATGAAGAAGTT
309 GTGCTTCCTGCTGcaggccgaccgcatgagcctattcatgtacagggcccggaacggcat
sp-54
369 cgcagagctggccacccggctcttcaacgtccacaaggatgctgtgctcgaggagtgcct
429 ggtggcgcctgactcggagATCGTGTTCCCCCTGGACATGGGAGTGGTGGGCCATGTTGC
489 GCTCTCTAAAAAGATCGTCAACGTCCCCAACACAGAGGAGGATGAACATTTCTGTGACTT
549 TGTGGACACCCTCACAGAGTACCAGACTAAGAACATCTTGGCTTCTCCCataatgaatgg
609 gaaggatgtggtggccataatcatggttgtgaataaagtggatgggccccacttcaccga
sp-50
669 gaatgatgaagagattcttctcaagtacctcaattttgcaaaTCTAATCATGAAGGTGTT
729 CCACCTGAGTTACCTGCACAACTGTGAGACTCGGCGTGGCCAGATACTGTTGTGGTCTGG
789 GAGCAAAGTCTTTGAAGAGCTTACAGACATTGAGAGGCAGTTCCACAAAGCCCTGTACAC
849 AGTCCGCGCCTTCCTCAACTGTGACAGATACTCTGTGGGGCTCTTAGACATGACCAAACA
909 GAAGGAATTTTTTGATGTGTGGCCAGTCCTGATGGGGGAGGCTCCACCctacgctggtcc
969 caggactccggatggaagggaaatcaactttt <----1340 nt----> agaaacaag
sp-40
2350 gcggatgaactccctaagcttcaggtcggcttcatcgACTTTGTTTGCACCTTTGTCTAC
2410 AAGGAATTCTCCCGTTTCCACGAGGAGATCACTCCCATGCTGGATGGGATTACCAACAAC
2470 CGCAAGGAGTGGAAAGCGCTTGCTGATGAGTATGAGACCAAGATGAAGGGGCTGGAGGAG
2530 GAGAAGCAGAAACAGCAGGCAGCCAACCAAGCAGCCGCAGGAAGTCAAcacggcggaaag
2590 caacctgggggcgggcctgcatccaagtcttgctgcgtccaatagcagagcgaggaccca
sp-58

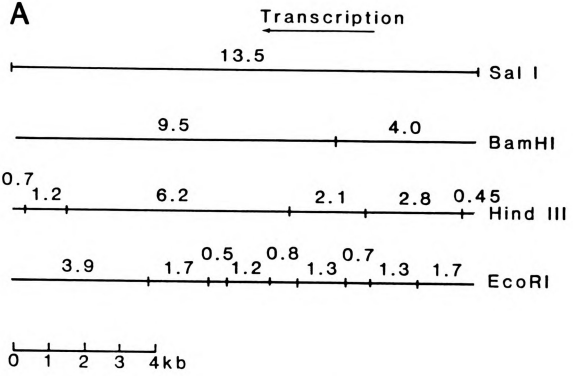
Figure 9

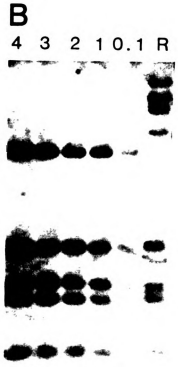
size and large number of inserts. However, the HindIII and BamHI maps were determined both by double digestion, and using a method of cos site labelling (64). This analysis confirms that the 6.2 kb HindIII fragment identified in the bovine genome is wholly represented in the isolated genomic clone (see Figure 10C).

Analysis of the Bovine cGMP PDE α-Subunit Gene. To assess the number of genes responsible for these RNAs we carried out several DNA blotting experiments. The intensity of hybridization of a 1.3 kb cDNA probe (probe 4, Fig 7) to EcoRI and HindIII fragments of genomic DNA, compared to EcoRI and HindIII fragments of varying amounts of an isolated genomic clone representing 0.1 to 4 genome equivalents, suggests that the gene is present in single copy (Figure 10B, C, respectively). Additionally, three cDNA probes representative of N-terminal and C-terminal sequences (probes 1, 3, 6, Figure 7) hybridize to single genomic DNA fragments in EcoRI, BamHI, or PstI digested DNA (only the BamHI digests are shown, Figure 11, lanes 1, 5, 7), consistent with the gene copy number data. Therefore, a single locus in the bovine genome encodes all of the identified transcripts.

A minimum size for the gene was obtained by summing the sizes of BamHI fragments identified with representative cDNA probes (Figure 11). Fragments that are identified by more than one cDNA probe due to overlap were only counted once. From such analysis we estimate the bovine gene to be at least 160 kb and is likely to be much larger. This estimate is likely a minimum value since any restriction fragments that contain only intron sequence would not be detected, and the cDNA clones do not represent the entirety of the RNA molecules identified. However, we cannot formally rule out the possibility that some of the

Figure 10: Copy Number of the Bovine α-Subunit Gene. **A.** A restriction map of the bovine genomic clone used for the copy number determination is shown. The SalI restrictions sites are provided from the cloning vector λ EMBL-3. All other sites shown represent sites found in the isolated genomic clone. The order of the EcoRI restriction fragments is tentative due to the number of small fragments. **B.** Genomic DNA [lanes R (EcoRI) and H (HindIII)] and the λ genomic clone (lanes 0.1-4) were digested with EcoRI and HindIII, electrophoresed into a 0.8% agarose gel and transferred to a nylon membrane. Hybridizations were performed with cDNA probe 4 (Figure 7). Lanes 0.1-4 contain amounts of the λ genomic clone DNA equivalent to 0.1-4 copies, respectively of a 6.2 kb fragment that would be present in the 10 μg of genomic DNA loaded onto the gel.


A
Transcription
13.5
Sal I
9.5
4.0
BamHI
0.7
1.2
6.2
2.1
2.8
0.45
Hind III
3.9
1.7
0.5
1.2
0.8
1.3
0.7
1.3
1.7
EcoRI
0 1 2 3 4kb


B
4 3 2 1 0.1 R

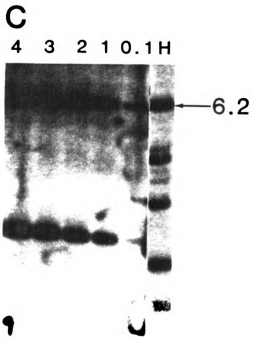

C
4 3 2 1 0.1 H
6.2

Figure 10

Figure 11: Size of the Bovine α-Subunit Gene. Bovine genomic DNA isolated from a single brain was digested with BamHI, EcoRI, PstI or HindIII, electrophoresed into a 0.8% agarose gel and transferred to a nylon membrane. Blot strips containing the DNAs were hybridized with oligo labelled cDNAs. The results of hybridization with the BamHI digested DNAs are shown. The probes used for each blot strip were as follows: lane 1, probe 3; lane 2, probe 5; lane 3, probe 4; lane 4, probe 8; lane 5, probe 1; lane 6, probe 2; lane 7, probe 6; lane 8, probe 7; lane 9, probe 9. All probe designations are according to Figure 7.

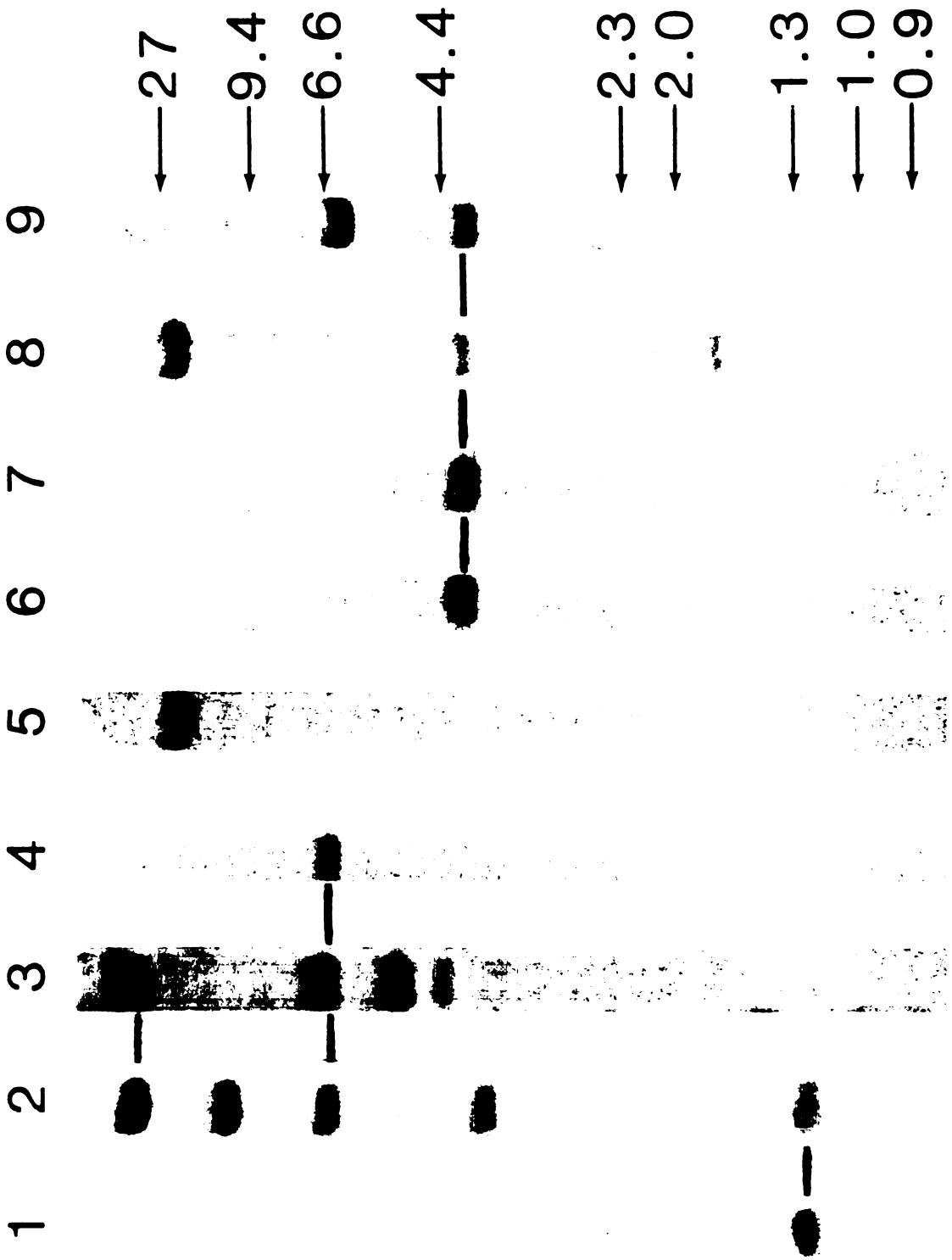



Figure 11

probes are detecting fragments that represent closely related genes.

**Section B Human cGMP PDE α-Subunit.**

Isolation of Human cGMP PDE α-Subunit cDNAs. A human retinal cDNA library constructed in λ gt10 was screened with a bovine α-subunit cDNA probe (probe 4, Figure 7 and 12) representing about one half of the protein coding region. Five cDNA clones designated HPA-2 through HPA-6, were recovered that contained only partial sequence for the α-subunit (Figure 12). Additional small EcoRI fragments in clones HPA-5 and HPA-6, which presumably hybridized to the probe were not analyzed, since it was clear that these clones were not full length. In order to isolate a full length cDNA clone, the library was rescreened with an oligonucleotide representing the first 12 amino acids of the bovine α-subunit sequence (Figure 6A). Twenty clones were recovered from a screen of $2 \times 10^4$ recombinants. The complete sequence was determined for all of the cDNAs shown in Figure 12. The sequence of specific primers used for sequence analysis is shown in Table 3. No sequence differences were observed in any of the overlapping regions of the cDNAs.

Characterization of Human α-Subunit cDNAs. One of the clones, designated HPA-1 (Figure 12), contains the entire open reading frame of human cGMP PDE α-subunit. The sequence of this 2,577 nucleotide open reading frame and the 78 nucleotides of 5' noncoding sequence and 249 nucleotides of 3' untranslated sequence is shown in Figure 13. A poly (A) stretch of 17 nucleotides is present at the 3' end of the clone, however no consensus poly (A) site was found. The underlined sequence near the 3' end of the clone (Figure 13) may serve as a poly (A) site or it is

Figure 12: Restriction Map of Human cDNA Clones. A schematic map of the full length human cDNA clone is shown at the top. Wider rectangles represent protein coding segments and more condensed rectangles depict upstream or downstream untranslated regions. Included within the protein coding region is the "PDE HOMOLOGY" domain that defines a segment conserved in a number of CN PDEs (see text). Predicted amino and carboxy termini are designated $NH_2$ and $COO^-$ respectively. Bovine cDNA fragments (probes 4 and 5, Figure 7) that were used as probes to isolate some of the cDNAs and for chromosomal localization are shown as thick darkened lines. An EcoRI restriction map (sites designated R) is shown with the full length clone designated HPA-1. Other partial cDNA clones are shown as line segments.

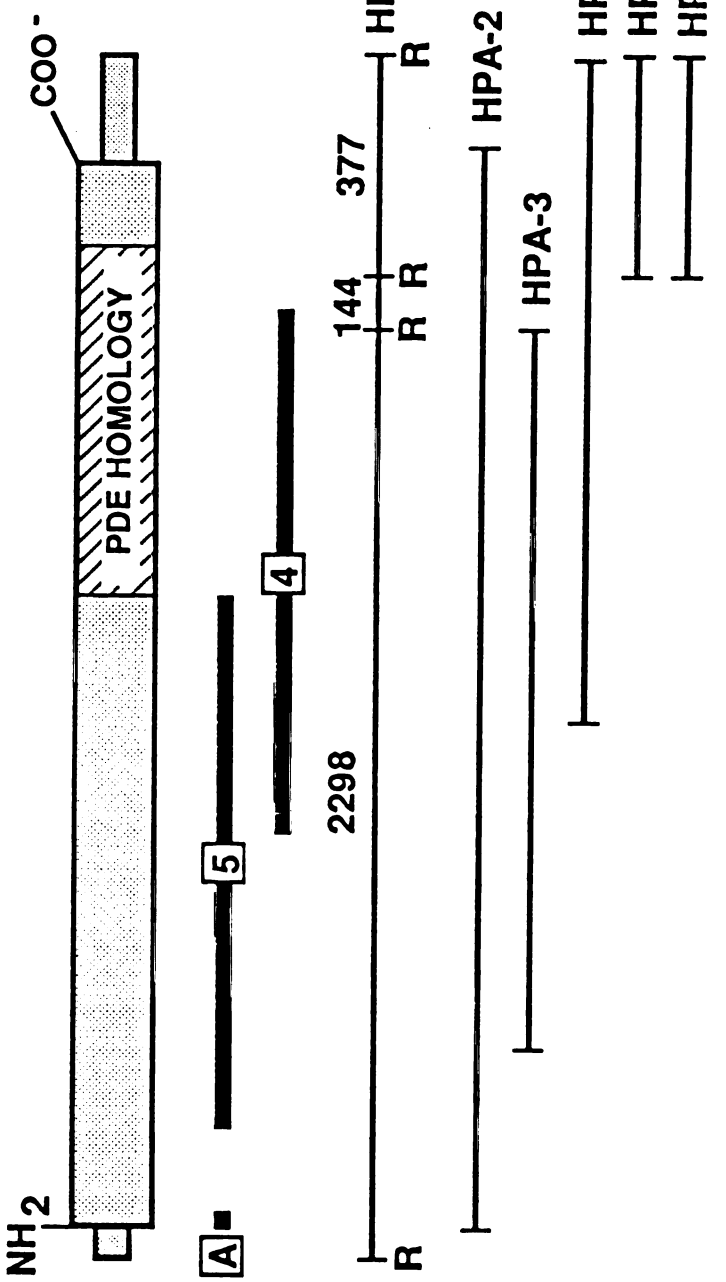

COO-
PDE HOMOLOGY
NH2
A
5
4
R
2298
144
377
R R
R
HPA-1
HPA-2
HPA-3
HPA-4
HPA-5
HPA-6

Figure 12

possible that the poly (A) stretch represents an internal sequence. An identical poly (A) segment is found at the ends of clones HPA-5 and HPA-6.

The cDNA sequence predicts a polypeptide chain of 859 residues (MW ~100,000), significantly larger than that predicted from its mobility on SDS gels (20). Negative charges are in excess and appear to be uniformly distributed (calculated pI=5.0). No apparent leader peptide was detected in the predicted amino acid sequence, consistent with the existence of the PDE alpha subunit as an unsecreted, soluble protein. It is possible that the N-terminal Met is cleaved in the mature protein, and Gly (nucleotide 2, Figure 13) is acetylated, as has been shown for the bovine α-subunit (29).

Blot Analysis of Human Retinal RNA. To evaluate the transcription pattern of the gene in the adult retina we used a 1.4 kb human cDNA probe (HPA-3, Figure 12) to hybridize to human poly $(A)^+$ RNA. Transcripts of 5.3 and 4.9 kb were identified consistent with the size of the predicted open reading frame (Figure 14). The size and relative abundance of these RNAs is similar to the two predominant RNAs seen in bovine retinal poly $(A)^+$ with α-subunit probes (Figure 8, lanes 1-6). The basis for the two transcripts and their differences in abundance in either organism has not been determined.

Chromosomal Localization of the Gene. A 1.5 kb cDNA probe was hybridized to a panel of human-rodent somatic cell hybrid DNAs digested with EcoRI to determine the location of the human cGMP PDE α-subunit gene. A representative blot is shown in figure 15, and the results of all blot data are summarized in table 4. We observed 100% concordancy with chromosome 5 and at least three discordancies were found for all

Figure 13: Nucleotide and Deduced Amino Acid Sequence of Human cGMP PDE α-subunit. Nucleotide numbering is shown on the left of the sequence and amino acids are numbered above each tenth residue. In frame stop codons that define the open reading frame are denoted by asterisks. Internal EcoRI sites and a putative nonconsensus polyadenylation signal are underlined. A solid box is present above residues at the C-terminus that are potential sites of palmitylation (see text).

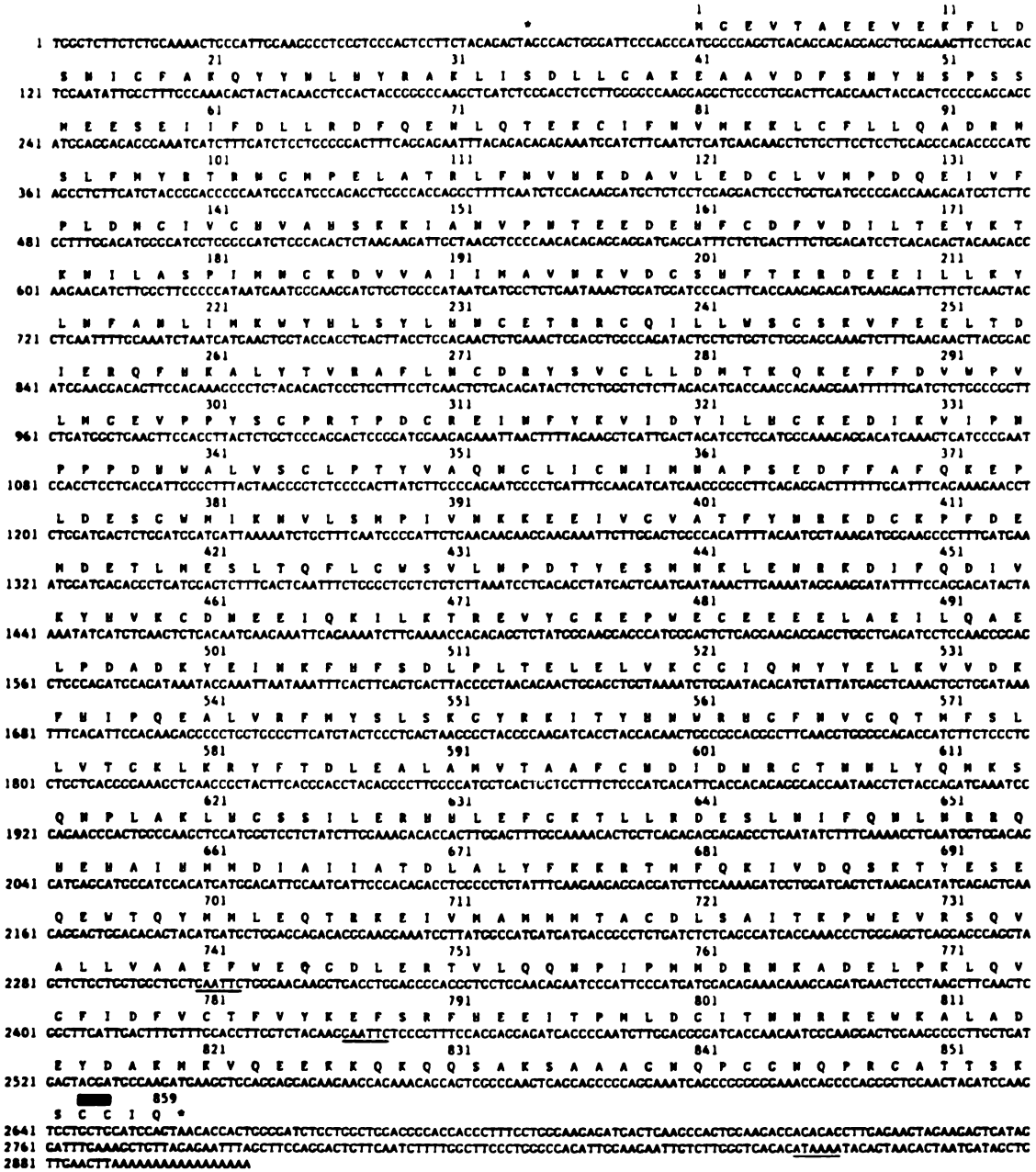


Figure 13

Figure 14: Analysis of Human Retinal RNA. Approximately 2 μg human poly $(A)^+$ RNA was fractionated on a 1.0% agarose formaldehyde containing gel and transferred to nitrocellulose. The results of hybridization with radiolabelled human cDNA clone HPA-3 (Figure 12) is shown.

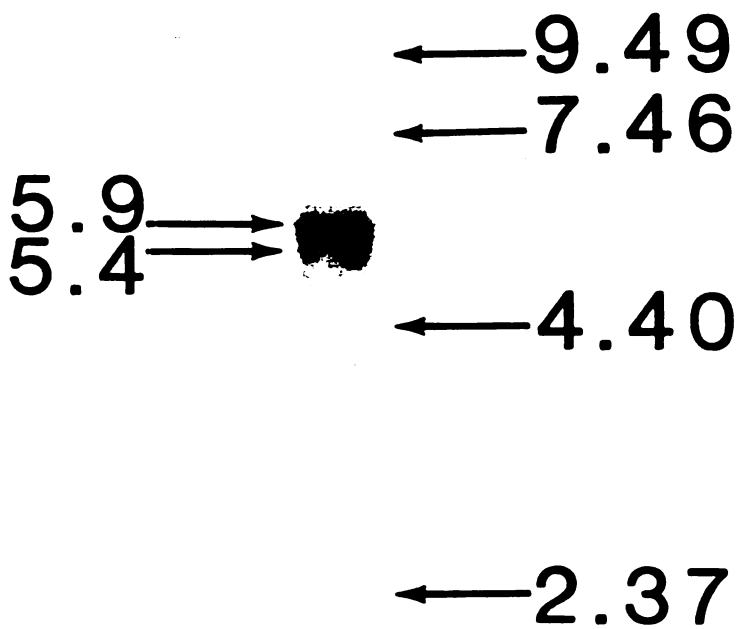



Figure 14

Figure 15: Blot Analysis of Human-Rodent Somatic Cell Hybrid DNA. Approximately 15 μg of genomic was digested with EcoRI, fractionated on a 0.8% agarose gel, and transferred to a nylon membrane. The blot was hybridized with radiolabelled probe 5 (Figure 7). Lanes designated human and mouse are from control cell lines that contain only human or mouse chromosomes respectively. Lanes designated hybrids (see Table 4) contain from left to right the following human-mouse somatic cell hybrids: MH-74, MH-18, P-12, MH-41. Arrows mark the positions of human DNA fragments that segregate with the cGMP PDE α-subunit gene.

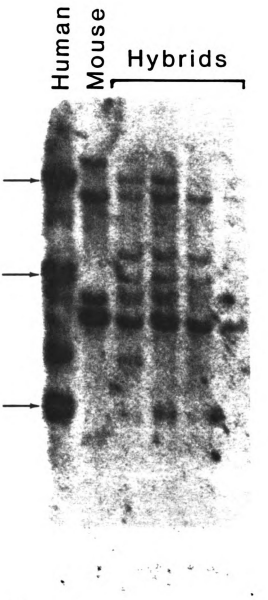



Figure 15

Table 4: Segregation of cGMP PDE α-Subunit Gene in Human-Rodent Somatic Cell Hybrids. The complement of chromosomes present in each somatic cell hybrid versus the segregation of the cGMP PDE α-subunit gene is shown. A plus sign designates the presence of the chromosome and the minus sign its absence. Abnormal karyotypes identified in some of the hybrids are explained below the table. The presence of hybridizing human DNA for each hybrid is denoted by a plus sign in the column labelled $PDE_{\alpha}$, and by a minus sign to indicate the absence of hybridization to human DNA.

Table 4: Segregation of cGMP PDE α-Subunit Gene in Human-Rodent Somatic Cell Hybrids.

| Hybrids[a] | $PDE_\alpha$[b] | Human Chromosomes | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | X | Y |
| 1) 1.11H2 | - | - | - | - | - | - | + | - | + | - | - | + | - | - | - | - | - | - | - | - | - | - | - | + | - |
| 2) 1.21 | - | - | - | - | - | - | + | + | - | - | - | - | + | - | + | - | - | - | - | - | - | - | - | + | - |
| 3) MR3.31 | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | + | - |
| 4) 1.4 | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | + | - | - | + | - | + | - | + | - |
| 5) MR7.11 | - | - | - | - | - | - | - | - | - | - | - | + | - | + | - | - | - | - | + | - | - | - | + | + | - |
| 6) 8.2 | - | - | + | + | + | - | - | + | + | - | + | + | - | + | + | + | + | - | - | - | + | - | - | + | - |
| 7) 15.2 | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | + | - | - | - | - | - | - | - | + | - |
| 8) SA-5 | - | - | - | - | - | - | - | + | - | - | - | - | + | - | - | *c* | - | *c* | - | - | - | + | - | + | - |
| 9) MH-7 | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - | - | - | - | - | - |
| 10) MH-18 | + | - | - | + | - | + | + | + | + | *d* | - | + | + | + | + | + | - | *d* | - | - | + | + | - | - | - |
| 11) P-12 | + | + | - | + | - | + | + | + | - | - | - | + | - | + | - | + | + | + | + | + | + | + | - | + | - |
| 12) MH-74 | + | - | + | + | - | + | - | + | + | - | - | - | + | + | + | + | - | *e* | + | + | + | + | + | + | - |
| 13) MH-41 | + | + | + | - | - | + | - | + | - | - | - | - | - | - | - | - | - | *f* | - | *f* | - | - | - | - | - |
| 14) MH-82 | - | + | - | + | - | - | + | + | + | - | - | + | - | - | + | - | + | *e* | + | + | + | + | + | - | - |
| % Discordancy | | 21 | 29 | 36 | 43 | 0 | 36 | 36 | 43 | 29 | 36 | 43 | 29 | 21 | 36 | 21 | 43 | 21 | 36 | 21 | 29 | 29 | 36 | 71 | 29 |

*a* Hybrids 1-7 are human-Chinese hamster hybrids and the rest are human-mouse hybrids.
*b* cDNA probe 5 (Figure 7) was used for all hybrids as described in the text.
*c* t(15;17)(q15;p11.2), retains 17p11.2-qter and 15q15-qter.
*d* t(9;17)q12;p11), retains 17p11-qter and 9q12-qter.
*e* ring(17)(p13.3;q25.3), retains 17p13.3-q25.3.
*f* t(17:19)(q23;p13), retains 17q23-qter and 19p13-qter.

other chromosomes. In order to delimit the assignment further we probed 11 human-hamster hybrid DNAs (not shown) that contain various deletions of chromosome 5. Two of these hybrids are deleted for the majority of 5q. The other nine hybrids contain intact long arms and various deletions of 5p. Hybridization signals were observed in all of the hybrid DNAs except the two hybrids deleted for 5q. Therefore the gene resides on the long arm of chromosome 5 within the region 5q11-qter.

Since this is the first CN PDE gene to be localized, the locus designation CGPR-A is suggested. This designation takes into account the classification system of Beavo (120) for CN PDEs and is in agreement with the recommendations of the committee on cytogenetics nomenclature (47). The locus name denotes the gene encoding the cGMP phosphodiesterase α-subunit of the rod photoreceptor cell.

## Section C Similarities Between Bovine and Human α-Subunits.

Comparison of Human and Bovine α-Subunits. Shown in Figure 16 is a comparison of the predicted amino acid sequences for the human and bovine α-subunits. The human and bovine cDNA sequences are 88% identical over their matched lengths, and 93% identity was found at the protein level. The majority of the nonhomologies appear to be concentrated in the first ~240 amino acids at the N-terminus, and the last 43 amino acids at the C-terminus of the proteins. The region identified as the "conserved domain" (see below) in several eukaryotic CN PDEs (bracketed in Figure 16) is contained within the region that is the most highly conserved. Interestingly, two cysteine residues at the C-terminus (residues 856 and 857) are present in both species, suggesting

Figure 16: Comparison of Human and Bovine α-Subunit Primary Structures. Amino acid alignment of the predicted primary structures of the bovine and human cGMP PDE α-subunits is shown. The bracketed region (522-780) roughly defines the region that is conserved in many CN PDEs (see Figure 17). Dicysteine residues (856-857) that are conserved in the two sequences and are potential sites of palmitylation are boxed.

```
human     MGEVTAEEVEKFLDSNICFAKQYYNLHYRAKLISDLLGAKEAAVDFSNYHSPSSMEESEI 60
bovine                    VS         R    V       PR           ALN V

human     IFDLLRDFQENLQTEKCIFNVMKKLCFLLQADRMSLFMYRTRNCMPELATRLFNVHKDAV 120
bovine              D   A   V                         A   IA

human     LEDCLVMPDQEIVFPLDMCIVGHVAHSKKIANVPNTEEDEHFCDFVDILTEYKTKNILAS 180
bovine      E   A  S          V     L    V                T    Q

human     PIMNGKDVVAIIMAVNKVDGSHFTKRDEEILLKYLNFANLIMKWYHLSYLHNCETRRGQI 240
bovine                  V       P   EN                   VF

human     LLWSGSKVFEELTDIERQFHKALYTVRAFLNCDRYSVGLLDMTKQKEFFDVWPVLMGEVP 300
bovine                                                              A

human     PYSGPRTPDGREINFYKVIDYILHGKEDIKVIPNPPPDHWALVSGLPTYVAQNGLICNIM 360
bovine      A

human     NAPSEDFFAFQKEPLDESGWMIKNVLSMPIVNKKEEIVGVATFYNRKDGKPFDEMDETLM 420
bovine                        V

human     ESLTQFLGWSVLNPDTYESMNKLENRKDIFQDIVKYHVKGDNEEIQKILKTREVYGKEPW 480
bovine                       L             M            T

human     ECEEEELAEILQAELPDADKYEINKFHFSDLPLTELELVKCGIQMYYELKVVDKFHIPQE 540
bovine                C

human     ALVRFMYSLSKGYRKITYHNWRHGFNVGQTMFSLLVTGKLKRYFTDLEALAMVTAAFCHD 600
bovine                  R

human     IDHRGTNNLYQMKSQNPLAKLHGSSILERHHLEFGKTLLRDESLNIFQNLNRRQHEHAIH 660
bovine

human     MMDIAIIATDLALYFKKRTMFQKIVDQSKTYESEQEWTQYMMLEQTRKEIVMAMMMTACD 720
bovine                                      TQ          D

human     LSAITKPWEVRSQVALLVAAEFWEQGDLERTVLQQNPIPMMDRNKADELPKLQVGFIDFV 780
bovine              Q K

human     CTFVYKEFSRFHEEITPMLDGITNNRKEWKALADEYDAKMKVQEEKKQKQQSAKSAAAGN 840
bovine                                            ET   GL  E     A NQ    S

human     QPGGNQPRGATTSKSCGIQ 859
bovine     H  K  G GPA     V
```

Figure 16

that there may be some selective pressure to maintain this sequence (see Discussion).

Homologies to Nonretinal Cyclic Nucleotide PDEs. Beavo and collaborators (50) have identified a significant region of similarity that is present in several CN PDEs from diverse species. This region has been termed the "conserved domain" and is thought to represent at least part of the catalytic region of the enzymes. This work establishes that a number of CN PDEs with very differing properties are likely to have arisen from a common primordial ancestor. The cGMP PDE α-subunit also contains the conserved domain, and therefore is included as a member of the CN PDE gene family. A comparison of amino acid sequences of the identified CN PDEs is shown in Figure 17. While the average percentage identity is only about 35% between enzymes ofdifferent classes, there are a number of amino acids that have been maintained through several hundred million years of evolution. Additionally, there is a high degree of conservative substitutions.

Figure 17: Similarities Amongst cGMP PDE α-Subunit and Nonretinal CN PDEs. Manually optimized alignments for many of the eukaryotic cyclic nucleotide phosphodiesterase primary structures reported to date showing only the region of homology and some flanking sequence. Some of the sequnces have not been determined in their entirety within this region. Asterisks denote stop codons that define the C-termini in some of the sequences. Bov Ret α and Human Ret α represent conceptualized amino acid sequence from the bovine and human cGMP PDE α-subunits determined in this study, respectively. Bov cG-S, Bov CaM, Yst cA and Dro cA sequences are from Charbonneau *et al.* (120). Bov cG-S is from a cGMP stimulated PDE identified in bovine brain. Bov CaM is from a $Ca^{++}$/calmodulin dependent PDE isolated from bovine heart. Rat dnc sequence is from a homologue of the Drosophila *dunce* PDE (Dro cA) deduced from cDNA (R. L. Davis, Y. Takayasu, M. Eberwine, and J. Myres, unpublished results). Yst cA is from a yeast cAMP PDE deduced from cDNA. Mouse and Human partial sequences were deduced from genomic clones isolated by virtue of homology to the *dunce* locus (Y. Qiu and R. L. Davis, unpublished results). Numbering is according to the bovine α-subunit sequence (Figure 6A). Gaps denoted by "-" have been introduced to optimize alignments.

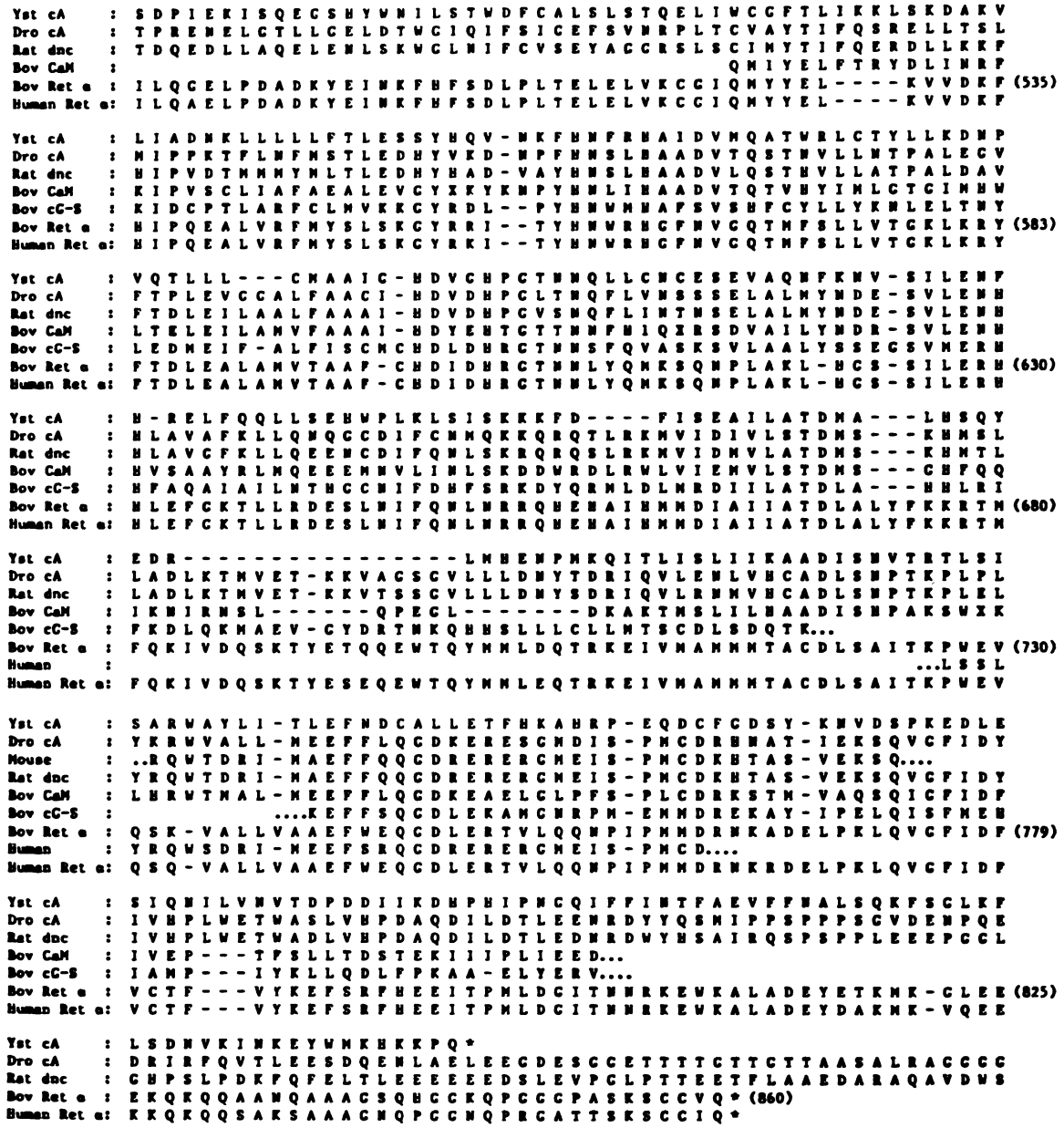


Figure 17

## DISCUSSION

Sequence of Tryptic Peptides. Seventeen peptide sequences were obtained from a tryptic hydrolysate of intact purified cGMP PDE. Since the α and β subunits of the cGMP PDE comprise 94% of the total mass of the protein, most, if not all, of the peptides were likely to represent sequences from these subunits. In fact, none of the peptide sequences could be found in the reported amino acid sequence of the γ-subunit (22), indicating that the sequence of the tryptic peptides originates from either the α or β subunit. The entire protein, rather than isolated subunits, was cleaved with trypsin because it was not possible to isolate sufficient quantities of well separated α and β subunits. Of the seven peptide sequences obtained (Figure 3A), only three were found (PDE1, PDE3, PDE6) that showed identity to sequences present in the α-subunit (Figure 6A).

Peptide PDE5 sequence is found in the α-subunit sequence, however residues 2 and 3 are juxtaposed. It is possible that this anomaly represents a true strain variation reflecting the difference in the source of cattle used to isolate the protein (26), and the source of cattle used to isolate the RNA for cDNA library construction (14), but technical error is also a possible explanation. This is supported by the fact that the sequence presented in this study predicted from the cDNA is identical to that published for the α-subunit (29). Additionally, the same sequence is found in the predicted human α-subunit sequence (Figure

13). The sequence difference explains the failure to isolate cDNA clones (Figure 4C) with the corresponding oligomer (cPDE5, Figure 7B).

No amino acid determination could be made for residue 7 in this peptide, however residues 8 and 9 yielded amounts of PTH derivatives comparable to that obtained for residue 6. The determination of amino acid sequence depends on the consistency of expected elution profiles for each amino acid in an HPLC chromatograph. Each PTH derivative is mixed with a standard amino acid solution. The residue determination is made essentially by comparison of the HPLC profile of the mixture to a profile of standards only. For a homogeneous peptide the chromatograph should show only one difference, corresponding to the elution time of the determined residue. The failure to identify residue 7 in peptide PDE5 suggests that it is modified in some manner, which affected the normal elution profile for a histidine residue. This may be of significance to enzyme function. The participation of specific active site histidines in catalysis is well documented (135,136). Moreover, post-translational modification of a specific histidine involved in catalysis has been shown to occur (137).

Peptides PDE2 and PDE4 sequences also are closely related to sequences found in the α-subunit, differing only at residues 3 and 4, respectively (see Figures 3A, 6A). No closely related sequence of peptide AB1, however, could be found in the α-subunit sequence. It is possible that these peptides represent sequence corresponding to the β-subunit.

Design of Oligonucleotide Probes. Six oligonucleotides were synthesized reflecting the amino acid sequence of the tryptic peptides (Figure 3B). There are several possible codons for many of the amino acids in the peptide sequence. Therefore, in order to reduce the number

of oligonucleotides necessary to reflect all possible combinations of codon sequences, deoxyinosine was incorporated into the third position of every codon with three or four degeneracies. Initial reports suggested that deoxyinosine behaved inertly as a base analog (79), and reports emerged that used oligonucleotide probes containing deoxyinosine at the "wobble" position of each codon in the oligonucleotide (78,138). The melting temperature of the hybrid formed with these probes was empirically determined to be equal to the melting temperature of a sequence without the deoxyinosine. However, recent thermodynamic studies using model oligomers, conducted after the oligonucleotides reported here were synthesized, unambiguously demonstrated that deoxyinosine does not behave in an inert fashion, and that surrounding sequence greatly contributes to the overall stability of a deoxyinosine base pair (157,158). This suggests that the melting temperature cannot be predicted for an oligonucleotide probe containing deoxyinosine even in the presence of TMAC as the hybridizing salt. Therefore, when screening the cDNA library with oligonucleotides containing dI, it had to be assumed that the deoxyinosines would behave in a relatively inert fashion and would not reduce the stability of the hybrid by more than 5°C. The low number of clones identified with oligonucleotide probe cPDE4 (Figure 4C) may have resulted if the hybrid formed was unstable, or more likely it was due to the difference in amino acid sequence observed for the corresponding peptide compared to that deduced from α-subunit cDNAs. While this study provides additional evidence that oligonucleotide probes containing deoxyinosine residues can be used successfully to obtain clones of interest from λ libraries, their use should be restricted until continuing studies on their stability (157) are completed.

Oligonucleotide Screening and Sequence Analysis of Bovine Retinal cDNAs. Radiolabelled oligonucleotides, described above, were used in conjunction with the TMAC hybridization method to screen a bovine retinal cDNA library. Two of the oligonucleotides, cPDE3 and cPDE6, were successful in isolating cDNAs that encode PDEs. Seventeen clones were isolated that show hybridization to both oligonucleotide probes (not shown). All but one of these clones contains 662 and 144 bp EcoRI fragments. Additionally, six of these clones contain other EcoRI fragments. This result can be explained in several ways. A number of cDNA fragments that derive from the same transcript may have been isolated. Alternatively, the similar DNA fragments may be the result of alternative processing events (142,143) from a single gene. It is also possible that a number of transcripts derived from a family of related genes may be represented. Several experiments were conducted to distinguish these possibilities.

Sequence Analysis of cDNA Clones. Subsequent restriction analysis of the six cDNAs demonstrated heterogeneity, suggesting that sequence analysis would be necessary to uncover the basis of the heterogeneity. To facilitate rapid sequence analysis the use of specific oligonucleotide primers was employed using primers that were evenly spaced (~300 bp) on both strands of the cDNA (Table 3). Additional primers were synthesized as needed to sequence through troublesome areas. The use of sequence specific primers proved to be a very elegant and expeditious method to generate nucleotide sequence. Over 20 kb of DNA sequence was determined manually in less than nine months. Another major advantage of this strategy is that primers can be used, not only for closely related bovine cDNAs produced by a single gene, as

demonstrated here, but also to analyze genes from other mammals. The primers were successfully used to sequence the human counterpart of the bovine cGMP PDE α-subunit (Figure 13). Surprisingly, 17 base oligomers with as many as three mismatches were used successfully (Table 3, sp33, and sp34).

Identification of cGMP PDE α-subunit cDNAs. The sequence analysis of the bovine cDNA clones predicts the existence of five distinct classes depicted in Figure 5. The long stretch of identity in the isolated cDNAs (~1.6 kb), coupled with the fact that the CN PDE conserved domain (see Figure 17) is contained within this sequence establishes that all of the isolated cDNAs do represent PDEs. Comparison of cDNA sequences with a published sequence for the α-subunit (29) suggested that one of the classes represented the cGMP PDE α-subunit.

However, the results reported did not provide sufficient evidence to support that the identified overlapping clones did indeed represent the entire molecule. Only one cDNA clone was found that contained the C-terminal coding region of the predicted polypeptide. Based on the results presented here it was possible that the predicted C-terminal region represented an α-subunit like RNA. Several experiments were conducted to verify the sequence of the α-subunit. Results obtained by direct dideoxy sequencing of bovine retinal RNA (Figure 9) were in complete agreement with the composite sequence determined from clones 7, 8, and 15 (Figure 6A), and the reported sequence of the α-subunit (29). Oligonucleotide probes representing the C-terminus and the 3' untranslated region of the α-subunit (probes C, D, Figure 7) hybridize to RNAs that appear to be of identical size and abundance (Figure 8, lanes 5, 6). The same RNAs are detected using long cDNA probes or other

oligonucleotides near the N-terminus (Figure 8, lanes 1-3). Further verification of the α-subunit sequence is provided from comparison to an isolated full length human cDNA clone (HPA-1, Figure 12). There is a marked degree of homology that extends into both the 3' and 5' untranslated regions. These results firmly establish the authenticity of the α-subunit sequence presented here (Figure 6A), and that reported (29).

Identification of α-Subunit Variants. Four closely related α-subunit like variants are also predicted to exist based on sequence analysis (Figure 5). Only one cDNA was isolated representing each of the variants suggesting they are present in low copy number in the bovine retina. However, since the cDNA analysis concentrated on only those clones that hybridized to two oligonucleotide probes, it was possible that many shorter cDNAs representing the variants went undetected. Oligonucleotide probes representing clones Pα-1, Pα-2, Pα-3 detect RNAs that are present at much lower abundance. As shown in Figure 8, lanes 1-6 represent a three hour exposure without any signal intensification, and lanes 7-9 show RNAs detected with Pα-1, -2, -3 specific probes, respectively, after a 54 hour exposure with two intensifying screens. A 5-10 fold increase in signal per time results from the use of intensifying screens. Therefore, the predicted variants do appear to represent lower abundance RNAs.

The hybridization patterns observed with oligonucleotide probes (Figure 8, lanes 2-11) are thought to represent specific hybridization. Spurious hybridization to non RNA material is extremely unlikely. Even in lane 11, in which the hybridization appears less convincing, there is a streak through the hybridizing band where no probe has hybridized.

Each lane shown in Figure 8 represents a nitrocellulose strip cut after blotting. The membrane containing blotted human RNAs was mismeasured such that each strip contains a portion of two adjacent lanes of RNA. The lack of hybridization represents the space between lanes where no RNA is present. On a shorter exposure of the results shown in lane 10 this streak is also apparent (not shown).

The absence of spurious hybridization does not rule out nonspecific hybridization to related sequences. This is unlikely, since even with a 27 base oligomer with a melting temperature significantly lower than that of the 50 base oligomers, the hybridization pattern is the same (compare lane 4 with lanes 2, 3, 5, and 6 or with a cDNA probe, lane 1). Therefore it is concluded that as many as seven RNAs are identified using clone specific probes for bovine retinal RNA, and that analagous RNAs exist in the human retina, representing at least Pα-1 and Pα-2.

The ability to detect transcripts in the bovine retinal RNA population with clone specific oligonucleotides does not provide sufficient support for the existence of the predicted RNAs. A number of steps were involved in the construction of the bovine cDNA library (144, 145), leading to the possibility of isolating artifactually contrived cDNAs. The most common types of cloning artifacts found are 5' end rearrangements (146, 147), or the joining of unrelated cDNAs during the ligation of cDNA to vector DNA (148). We cannot rule out the possibility that the 1.5 kb EcoRI fragment in clone Pα-4 (Figure 5) does result from the joining of unrelated cDNAs. These types of artifacts, however, cannot account for the organization of Pα-1, Pα-2, and Pα-4 since the 3' most EcoRI fragments, where the divergence is found, also contains sequence

common to the α-subunit. Likewise for the 5' EcoRI fragment containing the start point of divergence in Pα-3, α-subunit sequence is present. Nonetheless, it is still important to demonstrate that the clones do represent intact retinal RNAs, and not novel exceedingly rare types of cloning artifacts.

Several lines of evidence support the authenticity of the cDNAs. The cDNA library was made from polysomal RNA (14). The isolation method separates very dense polyribosomes from other less dense cellular material. Only mRNAs that are actively being translated should be isolated by this procedure. This does not rule out the possibility of obtaining artifactual cDNAs, but does essentially eliminate the chance of cloning nuclear unprocessed or partially processed RNAs.

Pα-1 and Pα-2 diverge at the same nucleotide (compare in Figure 6A, B, C,) and 29 amino acids are encoded past this point in both cDNAs before a stop codon is encountered. It is extremely unlikely that two clones would be truncated at their 3' ends at the same nucleotide, and that both of these clones would pick up unrelated cDNAs presumably by blunt end ligation. The chances become astronomical that ligation of the two unrelated cDNAs would create organizations that fortuitously lead to coding potential for 29 C-terminal amino acids past the point of divergence. Moreover, oligomers 50 nucleotides in length, that are specific for Pα-1 and Pα-2 detect transcripts in bovine and human retinal RNA (Figure 8, lanes 7, 10, and 8, 11, respectively). The Pα-1 transcripts appear to be of very similar size and abundance. Thus, it is expected that Pα-1 and Pα-2 cDNAs do represent true bovine retinal RNAs, that can be incorporated into a stable polyribosome complex.

Pα-3 specific oligonucleotide probes also detect transcripts in bovine retinal RNA, however no transcript was detected in human retinal RNA (not shown). This result may be due to the very low abundance of the RNAs, which is compounded with the technical problem of high background unexpectedly occurring during the analysis (Figure 8, lanes 10, 11). The best evidence in support of the predicted organization of Pα-3 is from DNA blotting analysis. A cDNA probe representative of the 5' end EcoRI fragment (probe 8, Figure 7) hybridizes to fragments of ~2.8 kb and 6.0 kb in HindIII and BamHI digested genomic DNA, respectively (BamHI digest is shown, Figure 11, lane 4). Identical size fragments are observed (autoradiographs are superimposable) using the adjacent EcoRI fragment in Pα-3 as a probe (not shown). Fragments of identical size are identified in BamHI digested DNA with probes 4 and 5 (Figure 11, lanes 2 and 3) consistent with the overlap of the probes (Figure 7). The isolation of two random cDNAs that hybridize to bands of identical size in genomic DNA from two separate digests is extremely unlikely. This results then suggests that the two fragments are linked and therefore supports the organization of Pα-3.

The sequence of Pα-4 predicts the possibility of alternative splicing in the 3' untranslated region. While alternative splicing is well documented (reviewed in 142,143), there are a paucity of reports that describe its occurrence in this region (149,150). This may be because most studies of cDNA analysis have concentrated on the determination of the open reading frame and such events have therefore gone unnoticed. Or, it may be that these events occur in relatively few transcripts of specific genes, and may represent an important mode of regulation. It is recognized that the 3' untranslated region contains a signal that specifies

polyadenylation (156), and it has been suggested that alternative splicing permits differential use of multiple poly (A) sites (143). Furthermore, splicing of upstream exons may in part be regulated by downstream splicing events in genes that exhibit tissue specific expression (143). The recent finding of a gene that produces a transcript apparently devoid of 3' noncoding sequence (151) increases the necessity to better understand the importance of this region. Further analysis of Pα-4 may yield important information in this regard.

Possible Functions of Predicted Isozymes. There are several potential functions for the predicted isozymes. It is possible that all of the predicted polypeptides are expressed only in the rod cell as has been suggested for the α-subunit (30). If this is so, it is possible that some of the isozymes subserve more housekeeping type functions and may be localized in other rod cell compartments. The relative abundance of the predicted isoforms is more consistent with that observed for other CN PDEs (120). The identification of a cGMP hydrolyzing PDE in the IPM consisting of smaller subunits (102) is intriguing. Perhaps the Pα-3 protein product represents this PDE. Another possibility is that one of the cDNAs represents the β-subunit. While a portion of a putative β-subunit sequence has been reported (29), confirmation of the sequnce has not been obtained.

The possibility that the isozymes are expressed in other retinal cell types or even in tissues outside the retina cannot be ruled out. Other retinal PDEs that hydrolyze cGMP have been identified. A high affinity form in the inner plexiform layer (24) and a cone outer segment PDE have shown to exist (31,100). Both of these forms are present in greatly reduced amounts in comparison to the α-subunit. It is interesting to

speculate that the α-subunit gene may be responsible for producing both highly specialized and housekeeping proteins, a phenomenon that has not, to my knowledge, been observed.

Analysis of the Bovine α-Subunit Gene. In order to understand the origin of the multiple transcripts, experiments were conducted to identify the number of genes that encode them. Both genome reconstruction (Figure 10) and DNA blot analysis (Figure 11) are consistent with a single bovine gene. The ability to detect segregation of only one chromosome in somatic cell hybrids (see Table 4) is consistent with a single human gene as well. Alternative splicing and at least two promoters are likely to be operative at the loci to explain the observed transcript heterogeneity.

The determination of a single locus allowed an estimation of the size of the gene by simply adding up the sizes of hybridizing restriction fragments identified with cDNA probes (Figure 11). During the analysis there was one unexpected observation. Probe 1 (Figure 7) does not hybridize to any fragments identified with adjacent probes 2, 4, or 6. This is only possible if intron junctions are present near each end of the region represented by probe 1. Such an arrangement is supported by the failure to detect any common hybridization pattern in HindIII or PstI digested DNA also (not shown). Further characterization of the gene is required to unambiguously verify this hypothesis.

Analysis of the Human cGMP PDE α-subunit. The implications that a defective cGMP PDE could be the primary cause of certain forms of hereditary retinal degenerations in mammals (see Literature Review), makes the gene a good target for the *candidate gene approach*. In this approach one studies a gene in which a defect is implicated as a causative factor of a disease. The first step then would be to isolate and

characterize the gene or a representative cDNA. Once isolated the cDNA or gene fragment can be used to identify the chromosomal location for comparison to disease loci already known (139). The second step is to analyze large kindreds with well established pedigrees for linkage to the disease of interest. A very high lod score (140) is expected (lod >> 3) with a recombination fraction close to zero if a defect in the gene is responsible for the disease. This method of study is particularly amenable to the study of diseases that occur in well studied, highly specialized tissue such as the retina. This approach complements the more conventional linkage studies in which random probes are used in an attempt to narrow down the location of the disease gene. The *candidate gene approach* has the advantage of allowing relatively fast assessment of the involvement of a defect in a specific gene to hereditary disease. Additionally, it guarantees that important information will be obtained at the very least from analysis of the cDNA or gene, which is not true for conventional linkage analysis.

The completion of the first step for our candidate, the cGMP PDE α-subunit is presented here. We have isolated a full length cDNA (Figure 12), and we have localized the gene to the long arm of chromosome 5 (5q; Table 4). Completion of the second step is presently underway in collaboration with investigators at the Berman Gund and Howe Laboratories of Ophthalmology at Harvard Medical School. While we were unable to find any retina specific disorders that have been mapped to 5q, two disease genes have recently been localized in the region. Familial polyposis coli (FPC) is a disease that predisposes affected individuals to the formation of multiple adenomatous colonic polyps (152). Interestingly, Gardner Syndrome which is clinically very difficult

to distinguish from FPC, has been associated with congenital hypertrophy of the RPE (153). Additionally, a susceptibility locus for schizophrenia has been mapped to 5q (154). It is difficult at present to envision a causal relationship between these disorders and defects in the cGMP PDE α-subunit gene. It is more prudent to consider the possibility that the α-subunit gene may be more closely linked than the presently available markers and thus be of importance in the study of these disorders.

Comparison of CN PDEs. The high degree of homology observed in comparison of the bovine and human α-subunits is consistent with homologies that have been shown for other phototransduction proteins (15,141). It was somewhat unexpected, however, that the α-subunits share a region of homology with a number of nonretinal CN PDEs (155). This homology found in PDEs representing many different classes (120) suggests that the majority of CN PDEs and perhaps all mammalian PDEs may make up a conserved PDE gene family.

The molecular genetics of PDE genes has only recently begun to be unravelled. The best studied gene is the Drosophila *dunce* gene which encodes a cAMP PDE. Over the past ten years a wealth of information has been disseminated about this extraordinarily complex locus (132-134). The complexity has made studies of the gene structure a very arduous task and therefore incomplete. The partial characterization of the bovine cGMP PDE α-subunit gene presented here exhibits a strikingly similar complexity. That is, a single large gene appears to encode multiple RNAs, that are likely to arise via the use of alternative splicing and multiple promoters. Further studies of PDE genes may

indeed show that such complexity is a feature common to all PDEs within this gene family.

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# APPENDIX

Figure A1: Complete Sequence of cDNA clone Pα-1. The sequence of cDNA clone Pα-1 is shown. Postion 1 in this sequence corresponds to position 914 of the α-subunit sequence (Figure 6A). Internal EcoRI sites are underlined so that relative positions can be oriented according to Figure 7.

**Clone Pa-1:**

```
   1 AATTTTTTCA TGTGTGGCCA GTCCTGATGG GGGAGGCTCC ACCCTACGCT GGTCCCAGGA CTCCGGATGG AAGGGAAATC AACTTTTACA AGGTCATTGA CTACATCCTG CATGCCAAAG
 121 AAGACATCAA AGTCATCCCG AATCCTCCTC CAGACCACTG GGCTTTAGTG AGCGGTCTCC CCACTTATGT TGCCCAAAAT GGCCTGATTT GCAACATCAT GAATGCGCCT TCAGAGGACT
 241 TTTTTGCATT CCAGAAAGAG CCTCTGGATG AGTCTGGATG GATGATTAAA AATGTCCTTT CTATGCCAAT TGTGAACAAG AAGGAGGAAA TTGTTGGGGT GGCCACGTTT TACAATCGCA
 361 AAGATGGGAA ACCCTTTGAT GAAATGGATG AGACCCTCAT GGAGTCTTTG ACCCAGTTCC TGGGCTGGTC TGTCTTAAAT CCTGACACTT ATGAGTTGAT GAACAAACTT GAAAACAGGA
 481 AGGATATTTT CCAAGACATG GTGAAATACC ACGTGAAGTG TGACAATGAA GAAATCCAGA CAATCTTGAA AACCAGAGAG GTGTATGGGA AGGAGCCGTG GGAATGCCAG GAAGAAGAAC
 601 TGGCTGAGAT CCTGCAAGGA GAGCTGCCAG ATGCAGACAA ATATGAAATT AATAAATTCC ACTTCAGCGA CCTGCCCCTG ACGGAACTGG AGCTGGTGAA ATGTCGAATT CAGATGTACT
 721 ATGAGCTCAA AGTGGTGGAT AAATTTCACA TTCCTCAGGA GGCCCTGGTG CGGTTCATGT ATTCCCTGAG TAAGGGCTAC CGCAGGATCA CCTACCACAA CTGGCGGCAC GGCTTCAACG
 841 TGGGGCAGAC CATGTTCTCC TTGCTGGTGA CCGGAAAGCT GAAGCGATAC TTCACAGACC TGGAGGCCTT GGCCATGGTC ACCGCCGCCT TCTGCCACGA CATTGACCAC AGAGGCACTA
 961 ACAATCTCTA CCAGATGAAA TCCCAGAACC CACTGCGCAA GCTCCATGGG TCCTCCATCT TGGAAAGACA CCACTTGGAG TTTGGTAAAA CACTCTTGAG AGATGAGAGC CTAAATATCT
1081 TTCAGAACCT CAATCGCAGG CAGCATGAGC ATGCAATCCA CATGATGGAC ATTGCGATCA TTGCTACAGA CCTTGCCTTG TATTTCAAGA AAAGCACGAT GTTCCAAAAG ATCGTGGATC
1201 AGTCTAAGAC ATACGAGACT CAGCAGGAGT GGACGCAGTA CATGATGCTG GATCAGACAC GGAAGGAAAT TGTCATGGCC ATGATGATGA CCGCCTGTGA TCTCTCAGCC ATCACCAAGC
1321 CTTGGGAGGT GCAGAGCAAG GTGGCTCTGC TGGTTGCTGC TGAATTCTGG GAACAAGGTG ACCTGGAGCG CACGGTGCTG CAACAGAATC CCATTCCCAT GATGGACAGA AACAAGGCGG
1441 ATGAACTCCC TAAGCTTCAG GTCGGCTTCA TCGACTTTGT TTGCACCTTT GTCTACAAGG AATTCTCCCG TTTCCACGAG GAGATCACTC CCATGCTGGA TGGGATTACC AACAACGGCA
1561 AGGAGTGGAA ACCGCTTGCT GATGAGTATG AGACCAAGAT GAAGCGGCTG GAGGAGGAGA AGCAGAAACA GCAGGCAGCC AACCAAGCAG CCGCAGGAAG TCACTGTATA TTACCAGTCA
1681 GGTGCCAGGA GTCTGAAAGA ATACACTCTT ATGGAGCTGC TTGTCATGCA TATGGAAGAG CCTTGTTTTG ACTTCCTTCG AACCAAGCAG ACCCTTGCGT ACCATGTCTA CCCTACCTGT
1801 AGCAACACAT CTGGGATTCT AGGCTTCTCT GTCACCGTGG GGACTCAGGC AACCAAATAC AACTCTGAAG TTGTTGATAA GAAGATAGAA GAGTTCCTTT AAGCTTTGAG GAGAAGATTG
1921 AGAACCTCAC TGAGGATGCA TTCAACACCC AGGTCACAGC TCTGATCAAG CTGAAGGAGT GCGAGCACAC CCACCTTGCG GAGGAAGTGG ACAGGAACTG GAATGAAGTG GTGACTCGGC
2041 AGTATCTCTT TGACCGCCTT GCCCATGAGA TTGAAGCACT GAAGTCATTC TCAAAATCAG ACCTAGTCAA CTGGTTCAAG GCCCACAGAG GACCAGGAAG TAAAATGCTC AGTGTTCATG
2161 TTGTTGGATT TGGGAAGTAC GAGCTGGAAG AGGATGGTAC CCCTTCCGGT GAGGATTCAA ACTCTTCTTG TGACGTGATG CAGCTGACTT ACCTGCCAGC TTCTCCTCTG CTGGTAGACT
2281 GTACCGTCCC CATTGTTGAT ATCAGGGGTT TCACATCAAG ACTTAACCTT CTCCCCTACC ATAAAATAGT CAAATAAACT GCAGTCTCCT TGGCCTGAAG CACTGTGCAT TGAAGTGTTT
2401 GAATTC
```

Figure A1

Figure A2: Partial Sequence Determined for cDNA Clone Pα-2. The partial sequence of cDNA clone Pα-2 is shown. The ~1.5 kb EcoRI fragment at the 3' end (Figure 5) was not completely sequenced in the determined 3' untranslated region. Postion 1 in this sequence corresponds to position 914 of the α-subunit sequence (Figure 6A). Internal EcoRI sites are underlined so that relative positions can be oriented according to Figure 7.

Clone Pa-2:

```
   1 AATTTTTTGA TGTGTGGCCA GTCCTCATGG GGCAGGCTCC ACCCTACGCT GGTCCCAGGA CTCCGGATGG AACGCAAATC AACTTTTACA AGGTCATTGA CTACATCCTG CATGCCAAAG
 121 AAGACATCAA AGTCATCCCG AATCCTCCTC CAGACCACTG GGCTTTAGTC AGCGGTCTCC CCACTTATGT TGCCCAAAAT GGCCTGATTT GCAACATCAT GAATGCGCCT TCAGAGGACT
 241 TTTTTGCATT CCAGAAAGAG CCTCTGGATG AGTCTGGATG GATGATTAAA AATGTCCTTT CTATGCCAAT TGTGAACAAG AAGGACGAAA TTGTTGCGGT GGCCACGTTT TACAATCGCA
 361 AAGATGCGAA ACCCTTTGAT GAAATGGATG AGACCCTCAT GGAGTCTTTG ACCCAGTTCC TGGGCTGGTC TGTCTTAAAT CCTGACACTT ATGAGTTGAT GAACAAACTT GAAAACAGGA
 481 ACGATATTTT CCAAGACATG GTGAAATACC ACGTGAAGTG TGACAATGAA GAAATCCAGA CAATCTTGAA AACCAGAGAG GTGTATGCGA ACGAGCCGTG GGAATGCCAG GAAGAAGAAC
 601 TGGCTGAGAT CCTGCAACGA GAGCTGCCAG ATGCAGACAA ATATGAAATT AATAAATTCC ACTTCAGCGA CCTGCCCCTG ACGGAACTGC AGCTGGTGAA ATGTGGAATT CAGATGTACT
 721 ATGAGCTCAA AGTGGTGGAT AAATTTCACA TTCCTCAGGA GGCCCTGGTG CGGTTCATGT ATTCCCTGAG TAAGGGCTAC CGCAGGATCA CCTACCACAA CTGCCGGCAC GGCTTCAACG
 841 TGCGGCAGAC CATGTTCTCC TTGCTGGTGA CCGGAAAGCT GAAGCGATAC TTCACAGACC TGGAGGCCTT GGCCATGGTC ACCGCCGCCT TCTGCCACGA CATTGACCAC AGAGCCACTA
 961 ACAATCTCTA CCAGATCAAA TCCCAGAACC CACTGGCCAA GCTCCATGGG TCCTCCATCT TGGAAAGACA CCACTTGGAG TTTGGTAAAA CACTCTTGAG AGATGAGAGC CTAAATATCT
1081 TTCAGAACCT CAATCGCAGG CAGCATGAGC ATGCAATCCA CATGATGGAC ATTGCGATCA TTGCTACAGA CCTTGCCTTG TATTTCAAGA AAAGGACGAT GTTCCAAAAG ATCGTGGATC
1201 AGTCTAAGAC ATACGAGACT CAGCAGGAGT GGACGCAGTA CATGATGCTG CATCAGACAC GGAAGGAAAT TGTCATGCCC ATGATGATCA CCGCCTGTGA TCTCTCAGCC ATCACCAAGC
1321 CTTGCGAGGT GCAGAGCAAG GTGGCTCTGC TGGTTGCTGC TGAATTCTGG GAACAAGGTG ACCTGGAGCG CACGGTGCTG CAACAGAATC CCATTCCCAT GATGGACAGA AACAAGCGCG
1441 ATGAACTCCC TAAGCTTCAG GTCGGCTTCA TCGACTTTGT TTCCACCTTT GTCTACAAGG AATTCTCCCG TTTCCACGAG GAGATCACTC CCATGCTGGA TGGGATTACC AACAACCGCA
1561 ACGACTCGAA ACCGCTTGCT CATGAGTATG AGACCAAGAT GAAGGGGCTG CAGGAGGAGA AGCAGAAACA GCAGGCAGCC AACCAAGCAG CCGCAGGAAG TCCACCTTTC AGCAGTTGCA
1681 AAAATGCAGC TCACGGACCA AGCCCAAAGC CTGAGGCCCA AGATATAGGA GGTGACACCA CCTCACCTTG ATACATAATA TTTTTCCACT TTTGTAACAG CTTGAGCACA CTTTGGTAAA
1801 AATTTGAGCA TCTCTTTTAA TCAATGACTT TTATCATTTC AAGGATCACA CTTGAAATTT TATAATCTCA ACGGTTTAAG TAAAA...
```

Figure A2

Figure A3: Complete Sequence of cDNA Clone Pα-3. The sequence of cDNA clone Pα-3 is shown. Postion 932 in this sequence corresponds to position 941 of the α-subunit sequence (Figure 6A). Internal EcoRI sites are underlined so that relative positions can be oriented according to Figure 7.

Clone Pa-3:

```
   1 AATTTGCTGT GGTCCCTGGA GCCATAGATA AAATGTATAT AACTTTTTCT TCAGATCCCC AGTGACCTCC CATTCAGACT GATGTTTCAT TTTAGAGAGC ATGTATTTTC ATGAAAGGTG
 121 ATTCCCCAAG AGTGCAAAAA CTGATTTAGG TGGCGACGAA ATCTTAGATA TTACACTGAG TCATGGCCCT CCCAAGGAGC TACAGTATGT GCACAGATAT AAAGTATGTA TTTAGTATTA
 241 AAATTTCATG GGGATGGGGA ATGATTAGGA ATAGAATGTC TAAAAGGGCT CTTTAGGGAG ATGATAGTGA AAAAAATAGA TTGAGAAATA CTCATCTGGG GTCAGTTTCT GCTTCTGAAA
 361 CTGTTCATTT TCTCTTCCTG GGGTGTTTGC TCATTTCTGA GGGCCCGGAT AAGTGGAAAT GACTTTGAGA GGTTTTGTTT TGTTTTGTTT TAGCTATTCC TTCTACAACC TTCCAAACCA
 481 TATCAGTTTG GAAGGTTTTT GGTAGGTTTT TCCAGGTGAA AAACCCATTC TCCTCTCAAC AGTCTTTGGG CACAGAAATC CTGCAGCATC CTCTGGTGTA ACCCCCAGAT CATATTCCAT
 601 GTGAAACCTT TCCATTCAAC TGGCCTTAAC TATTATATTG TCTCTGAGTT TCAGCTCACC TTTTCTAGTG TCCCACATTA AGAAAGATCC AAAAACTCTA AAAAGTAAAA CATAGGTAAA
 721 AACATACTAA ATGAGAGCTT TGTTTGCACG TCCACTAAGT ACTGGAAGCC TCTCACCAGT TGTCCTTCAG CCTTCCTTGT TTATCATCTC CCATTGGCTG AATTCTCTTG GAAGTCGTGT
 841 TGTGCCATTG CTAACATTTC TATACTTCAG TCTGAAGCCT TCCCAAAGCT GTCTATGCCA CTGTGCGTTA TAGATATAAC CAGCAGTGAA CATGTAAGAA GTGGGGGAGG CTCCACCCTA
 961 CGCTGGTCCC AGGACTCCGG ATGGAAGGGA AATCAACTTT TACAAGGTCA TTGACTACAT CCTGCATGGC AAAGAAGACA TCAAAGTCAT CCCGAATCCT CCTCCAGACC ACTGGGCTTT
1081 AGTGACCGGT CTCCCCACTT ATGTTCCCCA AAATGGCCTG ATTTGCAACA TCATGAATGC GCCTTCAGAG GACTTTTTTG CATTCCAGAA AGAGCCTCTG GATGAGTCTG GATGGATGAT
1201 TAAAAATGTC CTTTCTATGC CAATTGTGAA CAACAAGCAG GAAATTGTTG GGGTGGCCAC GTTTTACAAT CGCAAAGATG GGAAACCCTT TGATGAAATG GATGACACCC TCATGGAGTC
1321 TTTGACCCAG TTCCTGGGCT GGTCTGTCTT AAATCCTGAC ACTTATGAGT TGATGAACAA ACTTGAAAAC AGGAAGGATA TTTTCCAAGA CATGGTGAAA TACCACGTGA AGTGTGACAA
1441 TGAAGAAATC CAGACAATCT TGAAAACCAG AGAGGTGTAT GGGAAGGAGC CGTGGGAATG CGAGGAAGAA GAACTGGCTG AGATCCTGCA AGGAGAGCTG CCAGATGCAG ACAAATATGA
1561 AATTAATAAA TTCCACTTCA GCGACCTGCC CCTGACGGAA CTGGAGCTGG TGAAATGTGG AATTCAGATG TACTATGAGC TCAAAGTGGT CGATAAATTT CACATTCCTC ACGACGCCCT
1681 GGTGCGGTTC ATGTATTCCC TGAGTAAGGG CTACCGCAGG ATCACCTACC ACAACTGGCG GCACGGCTTC AACGTGGGGC AGACCATGTT CTCCTTGCTG GTGACCCGAA AGCTCAAGCG
1801 ATACTTCACA GACCTGGAGG CCTTCGCCAT CGTCACCGCC GCCTTCTGCC ACGACATTGA CCACAGAGGC ACTAACAATC TCTACCAGAT GAAATCCCAG AACCCACTGC CCAAGCTCCA
1921 TGGCTCCTCC ATCTTCGAAA GACACCACTT GGAGTTTGGT AAAACACTCT TGAGAGATGA GAGCCTAAAT ATCTTTCAGA ACCTCAATCG CAGGCAGCAT GAGCATGCAA TCCACATGAT
2041 GGACATTGCG ATCATTGCTA CAGACCTTGC CTTGTATTTC AAGAAAAGGA CGATGTTCCA AAAGATCGTG GATCAGTCTA AGACATACGA GACTCAGCAG GAGTGCACGC AGTACATGAT
2161 GCTCGATCAG ACACCGAAGG AAATTCTCAT CGCCATGATG ATGACCGCCT GTGATCTCTC AGCCATCACC AAGCCTTCGG AGGTCCAGAG CAAGGTGCCT CTGCTGGTTG CTGCTGAATT
2281 CTGCGAACAA CGTGACCTGC AGCGCACCGT GCTGCAACAG AATCCCATTC CCATGATGGA CAGAAACAAG GCGGATGAAC TCCCTAAGCT TCAGGTCGGC TTCATCGACT TTGTTTGCAC
2401 CTTTGTCTAC AAGGAATTCT CCCGTTTCCA CGACGAGATC ACTCCCATGC TGGATGGGAT TACCAACAAC CGCAAGGAGT GGAAAGCGCT TGCTGATGAG TATGAGACCA AGATGAACGG
2521 GCTGGAGGAG GAGAACCAGA AACAGCAGGC AGCCAACCAA GCAGCCGCAG GAAGTCAACA CGCCGGAAAG CAACCTGCGG GCGCGCCTGC ATCCAAGTCT TGCTGCGTCC AATAGCAGAG
2641 CGAGGACCCA GGGCCCAGGC CGGCCCCACC CTTCACCGAC GAGAGCACCC AGGCCAGTGG GAAACCATAC GCCACCTGCT CGAGAAGTCA GAGTTGTGGG GTTTCAAAAC TGAAAGAGAA
2761 TTTAGTTTAC ATCTTTCCTA GTGGCTTTCA AGCCTCTTTT TCAGCTGTGA GAACTTTTTA TTGAATGACA ACCAACATTT TAGCTTTCAC AGACTCAAAC ATTAGTGCCA GACTCACCTG
2881 CTTACCTAGA ATCTTTTGTG TTCTTATACT TTCATTTTAT AGTACACAAA TATGCAATCA TTCACAAAAG TAGAGAATTC
```

Figure A3

Figure A4: Partial Sequence Determined for cDNA Clone Pα-4. The partial sequence of cDNA clone Pα-4 is shown. The ~1.5 kb EcoRI fragment at the 3' end (Figure 5) was not completely sequenced. Postion 1 in this sequence corresponds to position 914 of the α-subunit sequence (Figure 6A). The sequences are identical up to position 1816 where the divergence begins. Internal EcoRI sites are underlined so that relative positions can be oriented according to Figure 7.

Clone Pa-4:

```
   1 AATTTTTTGA TGTGTGGCCA GTCCTGATGG GGCAGGCTCC ACCCTACGCT GGTCCCAGCA CTCCGGATGG AAGGGAAATC AACTTTTACA AGGTCATTGA CTACATCCTG CATGGCAAAG
 121 AAGACATCAA AGTCATCCCG AATCCTCCTC CAGACCACTG GGCTTTAGTG AGCGGTCTCC CCACTTATGT TGCCCAAAAT GGCCTGATTT GCAACATCAT GAATGCGCCT TCAGAGGACT
 241 TTTTTGCATT CCAGAAAGAG CCTCTCGATG AGTCTGGATG GATGATTAAA AATGTCCTTT CTATGCCAAT TGTGAACAAG AAGGAGCAAA TTGTTCGGGT GCCCACGTTT TACAATCGCA
 361 AAGATCGCAA ACCCTTTGAT GAAATGCATG AGACCCTCAT GGAGTCTTTG ACCCAGTTCC TGGGCTGGTC TGTCTTAAAT CCTGACACTT ATGAGTTCAT GAACAAACTT GAAAACAGGA
 481 AGGATATTTT CCAAGACATG GTGAAATACC ACCTGAAGTG TGACAATGAA GAAATCCAGA CAATCTTGAA AACCAGAGAG GTGTATGGGA AGGAGCCGTG GGAATCCGAG GAAGAAGAAC
 601 TGGCTCAGAT CCTGCAACGA GAGCTGCCAG ATGCAGACAA ATATGAAATT AATAAATTCC ACTTCAGCGA CCTGCCCCTG ACGGAACTGG AGCTGGTGAA ATCTCGAATT CAGATGTACT
 721 ATGACCTCAA AGTGGTGGAT AAATTTCACA TTCCTCAGGA GGCCCTGGTG CGGTTCATGT ATTCCCTGAG TAAGGGCTAC CGCAGGATCA CCTACCACAA CTGGCGGCAC GGCTTCAACG
 841 TGGCCCAGAC CATGTTCTCC TTGCTCGTGA CCGGAAAGCT GAAGCGATAC TTCACAGACC TGGAGGCCTT GGCCATGGTC ACCCCCGCCT TCTGCCACGA CATTGACCAC AGAGCCACTA
 961 ACAATCTCTA CCAGATGAAA TCCCAGAACC CACTGGCCAA GCTCCATGGG TCCTCCATCT TGGAAAGACA CCACTTGGAG TTTGGTAAAA CACTCTTGAG AGATGAGAGC CTAAATATCT
1081 TTCAGAACCT CAATCGCAGG CAGCATGAGC ATGCAATCCA CATGATGGAC ATTGCGATCA TTGCTACAGA CCTTGCCTTG TATTTCAAGA AAAGGACGAT GTTCCAAAAG ATCGTCGATC
1201 AGTCTAAGAC ATACGAGACT CAGCACGAGT GGACGCAGTA CATGATGCTG GATCAGACAC GGAAGGAAAT TGTCATGGCC ATGATGATGA CCGCCTGTGA TCTCTCAGCC ATCACCAAGC
1321 CTTGCGAGGT CCAGAGCAAG GTGGCTCTGC TCGTTGCTGC TCAATTCTGG GAACAAGGTG ACCTGGAGCG CACGGTGCTG CAACAGAATC CCATTCCCAT GATGGACAGA AACAAGCGGG
1441 ATGAACTCCC TAAGCTTCAG GTCGGCTTCA TCGACTTTGT TTGCACCTTT GTCTACAAGG AATTCTCCCG TTTCCACGAG GAGATCACTC CCATGCTGGA TGGGATTACC AACAACCGCA
1561 AGGAGTGGAA AGCGCTTGCT GATGAGTATG AGACCAAGAT GAAGGGGCTG GAGGAGGAGA AGCAGAAACA GCAGGCAGCC AACCAAGCAG CCGCAGGAAG TCAACACGGC GGAAAGCAAC
1681 CTGGGCGCCG GCCTGCATCC AAGTCTTGCT GCGTCCAATA GCAGAGCGAG GACCCAGGGC CCAGGCGGCC CCACCCTTCA CCGACGAGAG CACCCAGGCC AGTGGGAAAC CATACGCCAC
1801 CTGCTCCAGA AGTCATTCTG CCCACCGTTT CTGGCCCATT CATTAATCCT GAGAAGAGAG GCTGGTAGTG ACTCTCACGA GTAGCAACAG AAATAGTGCA GCACAACAAC TTATCTTCAA
1921 TCTACCACAT CTATGCAATC TTAAATCTCT GATGAGCAAG TTAGAAACGG ATACAAGCTC TCTACTGATT TCATAATCAG TTGAGACATC AGAGTAATCC AGGGCTCAGG TCCAGTTGCT
2041 GGCTGTGGTT AGTGTCTGTG TGGTGGCTGT GGTCTGGAGA GTCTTCAAGA ATGTGATCTC TCTGTTGAGT CATGCTTTGC AAAGCTGAGA TGAAGAGAGG CTGTAGTCAT CCTTTGATTT
2161 TTTCCTTTTC TTCTCCTTCA TGAAGTCTTC TACCAGCTTC TCCTGAGGAA TAAATATACA GCAGCCCCCA CAGGAGGCGA TAGGCTGTCT GAAGGCCTGG TCACTCTAAC ACACTGGCTC
2281 TAAACCTTCA TTGGAATATT CACTCGTTCA AGACTATAAC AAAAGAAAAA GCACTGCATG CGGTGTCAGA GGGCTCACAG AGACTCACCC TAAACTTTAC CTTCCAAGTT CTAAGGGCTA
2401 TTTCTTTGCA TCAATTCCCA GCCCTCCTTC CCAACAACAC ACGGAACATC TACAACAGGG CCACTAAACT GGATGTATCC TCACCCTAAA TGGCTTTTAC AAAGTGGTTT TGTTATTCAT
2521 CCACTGATGT GGGCTTTCTT TTGTCTACTC TTTATCTTAA AAGCTCAATT AGATAATAGG ACTGTGTCTC GTTCTGAATT AATATGTGTT TTTAAGGAAC TGTGCCTGTC TTTTAAAGCA
2641 CAGTTCTAGT TCACTCCTCA ACTCAGAGGT TCAGAGGCCA AAGCATATTA TGGAACACAG CCAACAAATC ATGTACCATG TAATCAAGCT ACATCAAGGT CAAGGGAAAT TTTGATGCTA
2761 AATCTTTTTC TGACACTTGC AGATGACCAG CCTTCTGCTC TTTCAAAGAG TTAGGAGATG GCAACAGGAT TGCACCAGTG CTAAGGGCCA GCAGTCTAAC CCTAACCCTC ACCTGCGATT
2881 GCTGTTTATT TTCACACAGG ATATGTGACA GACCAAAAGG GAGAAACATA TGGCACACAG CAAAATGTCA GCCATAGAAT AAGCACTTGA TAGAGAAGGG AATCATTTTA CATGTCGATT
3001 CTGCCACCCA AAACCTCAGT CCAATGCAAA ACAACAGTTT TTGCATCTTC TAGAATTTCC ACCCACTGCA TCCTTCTTCT CTTGCACCTG TACAGTTGTC ATGTCCCACC CCCAGTCATT
3121 CTTCAGTCAC TGAAACCTAT TTTATCTAGC CCTCAGTGTT CATTCCTAAT CCAGCTTTTG CTATAATCCC GGGTCATTTC AATACCCAGT AAACAACCAT CTGACATCCT AGCATCATGC
3241 GTATCTTGAT CACTCCAGTT TTTAAATTTT ACCATTCCAA AGTTCAGTCT CTGATATAGA TCCTATCATA CCATTTTCCA AAGACTCAGC ACCCATCCTG ATCATACTAA GGCCACTTTT
3361 TCTATGTAGA CTCTCACCTT CCTTCCATAT ACAATCTCGA TACTCAAATC CATTGGTGTT TAACTCTTCT CTTGCTAGCT GTCCATCGTT CAATCATGCT CATTCAGTCA TGTCTGACTC
3481 TTTGTGACCC TCTGGACTGT AGCCCATGCT CCTCCGTCTA TCGAATTCTC CAGGCAAGAA TACTGGAATG GGTTGCCATG CCCTCCTCCA GCGGATCTTC TCGACCCAGA GATCGAACCT
3601 GCTCATCTCC TGTGGCTCCT GCACTGCCAG CGGATTCTTT ACCACTGAGC CACCTGAGAA GCCCCTATCC CTCAATCAAG CAAAATCAAT TCTAGCGCAC CAAATGCTAC TTGTGACAAT
3721 CGCCCAAGAT TACCTTTTCT TTTCCTGAGT CTCTAATCAA GTTTCAAACT AGTTGCTCAC TTTGATATGG CCTGGATATT TCTTAAGACT CTTGTTTCTG TAATCCTGTC CCTCGTCAGC
3841 TTCCTCTCAC CCACCTCTAC AATTAAAAAT CTCCAACACC CTTATCAGTA CTGTTTCTAA TTCCTATTCT TATCACTATG CCCAGTAAAG AGGTAACTCC TTTCACTAAG GCTAGCCTCC
3961 GCCTATGTGT AACAGCAA...
```

Figure A4

Table A1: Nucleotide Differences Observed in cDNA Clones. The top half of the table shows changes in comparison of the composite sequence of the α-subunit (Figure 6A) to a published sequence (29). Only one deletion was found, which is in the 3' untranslated region. Shown at the bottom are the differences observed during sequence analysis of the cDNAs. All of the differences are noted relative to that shown in Figure 6A. The amino acid changes found in the published α-subunit sequence were at the following positions: 194, valine to alanine, 424, threonine to alanine, and 675, phenylalanine to cysteine.

Table A1: Nucleotide Differences Observed in cDNA Clones.

| α-subunit (this report) compared to published sequence (29). | | | | | | | |
|---|---|---|---|---|---|---|---|
| Position | Change | Position | Change | Position | Change | Position | Change |
| 635 | (C) | 1137 | (C) | 1281 | (A) | 1324 | (G) |
| 1704 | (C) | 1968 | (C) | 2019 | (C) | 2041 | (C) |
| 2073 | (G) | 2158 | (A) | 2451 | (A) | 2666 | (C deletion) |
| 2701 | (G) | 2906 | (G) | | | | |

| Differences in cDNA sequences in common region. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Position | Change | Clone | Position | Change | Clone | Position | Change | Clone |
| 310 | (C) | 8 | 1137 | (C) | 8 | 1195 | (G) | 8 |
| 1281 | (A) | 8 | 1968 | (G) | 8 | 2019 | (C) | 8 |
| 2046 | (C) | 8 | 2158 | (A) | 8 | 2451 | (A) | 8 |
| 635 | (C) | 7 | 1710 | (A) | 15 | 1851 | (C) | Pα-1,2,3 |