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**Total synthesis and expression of a gene for the  $\alpha$ -subunit of bovine rod outer segment guanine nucleotide-binding protein (transducin)**

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**ABSTRACT**

To facilitate structure-function studies by site-specific mutagenesis, we have synthesized a gene for the  $\alpha$ -subunit of the bovine rod outer segment (ROS) guanine nucleotide-binding protein (transducin). The gene codes for the native amino acid sequence and contains, by design, 38 unique restriction sites which are uniformly spaced. This enables mutagenesis in any part of the gene by restriction fragment replacement. The gene is 1076 base pairs in length. It was constructed from 44 synthetic oligonucleotides which were joined enzymatically *in vitro* into four fragments which were cloned. The synthetic transducin gene and cDNA encoding transducin were expressed to similar levels in monkey kidney cells (COS-1) using a vector in which transcription was under the control of the adenovirus major late promoter.

**INTRODUCTION**

Guanine nucleotide-binding proteins (G proteins) play a central role in transmembrane signal transduction by linking a membrane receptor to a specific effector. G proteins appear to have remarkable structural and functional similarities (1). G proteins are heterotrimeric ( $G\alpha\beta\gamma$ ). The  $\alpha$ -subunits of the various classes of G proteins characterized to date share a relatively high degree of amino acid sequence homology. The  $\beta$ -subunits for many of the different classes of G proteins are identical. The receptors that activate G proteins also appear to have remarkable structural similarities including seven putative transmembrane helices and sites for glycosylation and phosphorylation (2). Each distinct class of receptors interacts with a specific class of G proteins. Upon activation of a receptor by an agonist, the  $\alpha$ -subunit ( $G\alpha$ ) interacts with receptor and exchanges bound GDP for GTP. The GTP-bound form of  $G\alpha$  then stimulates the appropriate effector system.  $G\alpha$  has intrinsic GTPase activity, and hydrolysis of bound GTP to GDP causes  $G\alpha$  to cycle back to the receptor.

We have been studying transmembrane signaling in the visual system. In visual transduction, the photopigment rhodopsin absorbs light and undergoes a series of structural changes which result in the activation of the rod outer segment (ROS) G protein, transducin. The  $\alpha$ -subunit of transducin ( $T\alpha$ ) then dissociates from the  $\beta\gamma$ -subunits ( $T\beta\gamma$ ) and rhodopsin and activates the ROS cyclic GMP phosphodiesterase. A decrease in cyclic GMP levels is thought to decrease plasma membrane cation conductance and lead to hyperpolarization of the

rod cell. Our aim has been to answer questions concerning the nature of the structural changes in proteins which lead to altered protein-protein interactions in the visual cascade.

To facilitate structure-function studies of bovine rhodopsin, a synthetic gene for this protein was synthesized (3) and expressed in monkey kidney (COS-1) cells (4). Rhodopsin mutants were prepared by *in vitro* site-specific mutagenesis to probe the sites of interaction between photolyzed rhodopsin and transducin (5). A complementary approach to the study of transducin-rhodopsin interactions in which site-specific mutagenesis is applied to T $\alpha$  would be clearly desirable. Therefore, we have carried out the synthesis of a gene for the  $\alpha$ -subunit of bovine transducin which was designed to facilitate site-specific mutagenesis by restriction fragment replacement (6). In this paper, we report on the T $\alpha$  gene synthesis and its expression in monkey kidney (COS-1) cells. Further, we review our extensive experience with the strategies and techniques involved in gene synthesis by the chemical-enzymatic approach.

#### DESIGN OF THE SYNTHETIC GENE

While a number of methods are available for site-specific mutagenesis, the use of a properly designed synthetic gene offers many advantages in cases where extensive mutagenesis is planned. Mutagenesis using a synthetic gene involves replacement of a restriction fragment by a synthetic counterpart that contains the desired codon alteration(s). In addition to single amino acid substitutions, extensive changes such as deletions, insertions, and multiple substitutions can be introduced as desired. Because all of the restriction sites within the gene are unique, cloning is "directional" and no screening is necessary for identification of the desired recombinant transformant. Our experiences in mutagenesis studies of bovine rhodopsin (5) and bacteriorhodopsin (7) support this approach and justify the initial investment of effort in gene synthesis.

Therefore, in this context, the aim of gene synthesis is to prepare a DNA duplex which encodes the amino acid sequence of a particular protein while containing an optimal number of unique restriction sites. The choice of restriction endonuclease sites to be considered in the design of the transducin gene was made according to the following criteria: 1). reliable availability, 2). freedom from any exonuclease activity, 3). a recognition sequence of five or more nucleotides, and 4). the generation of staggered rather than blunt ends. Because of the degeneracy of the genetic code, a very large number of potential nucleotide sequences could encode the 350 residue amino acid sequence of the  $\alpha$ -subunit of transducin (8,9). This potential variability in nucleotide sequence generates a large number of potential restriction sites. All of the potential restriction sites were identified based on the amino acid sequence of the protein. From this pool, 38 unique sites were chosen according to the above criteria to be as uniformly spaced throughout the gene as possible. For comparison, the native cDNA sequence contained only 7 unique restriction sites generating staggered ends. Sites for enzymes generating blunt ends were used only if long gaps were present after all enzymes generating

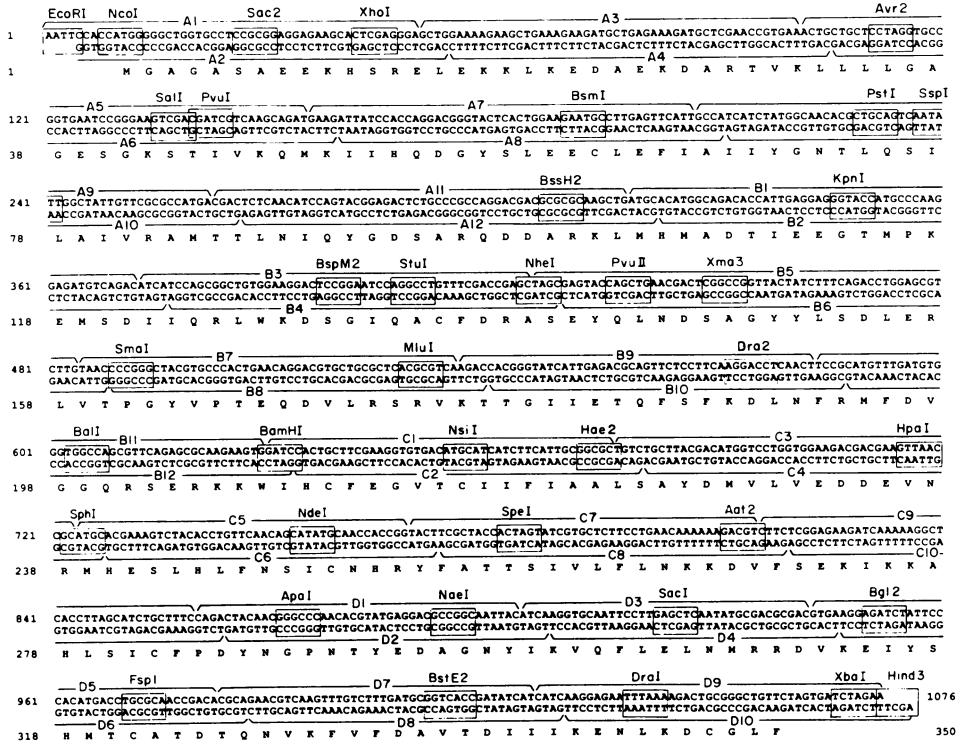


Figure 1. Plan for the synthetic gene. The nucleotide sequence, restriction map, and amino acid translation are shown. The gene was assembled from 4 duplexes (A through D). The oligonucleotides synthesized for each fragment are numbered (A1, A2...; B1, B2...; etc.) and enzymatic joining points are noted.

staggered ends were considered. A total of 9 sites for blunt end enzymes were introduced, no two blunt end enzymes were chosen consecutively so that "directional" cloning of all potential restriction fragments in the gene would be possible. Convenient cloning sites were chosen for each end of the gene. An *Eco* RI site was employed at the 5'-end and a *Hind* 3 site was employed at the 3'-end.

The nucleotide sequence of the gene was determined to yield the native amino acid sequence and the desired restriction map determined as above. Stretches of four or more guanosines or cytosines were avoided where possible to minimize potential difficulties in oligonucleotide synthesis and DNA sequencing. Two stop codons were placed at the 3'-end of the coding portion of the gene. No attempt was made to optimize codon usage for a particular expression system. However, a CCACC consensus sequence (10) was introduced immediately preceding the initiation codon.

The total 1076 base pair gene was synthesized in four fragments (A through D) of

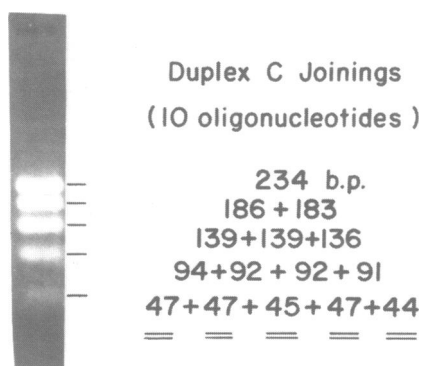


Figure 2. Agarose gel electrophoresis of the duplex C joining reaction. As described in Experimental Procedures, the 10 oligonucleotides for duplex C were mixed, annealed, and joined with T4 DNA ligase. The mixture was run on a 4% agarose gel and DNA was visualized by staining with ethidium bromide. The yield of the full length 234 base pair duplex was 24.2%.

roughly equal length as shown in figure 1. Fragments A and B were assembled from 12 oligonucleotides each, and fragments C and D were assembled from 10 oligonucleotides each. The 44 total oligonucleotides ranged in length from 39 to 59 nucleotides. To allow for characterization of the synthetic oligonucleotides by 5'-end analysis as previously described (3), the 5'-terminal nucleotide of each oligonucleotide was different from the penultimate nucleotide. The strategy of joining the single-stranded oligonucleotides to form the duplexes was based on principles developed earlier (3,11). The length of the cohesive overhangs in the complementary oligonucleotides was four bases in all cases. No cohesive end in any of the oligonucleotides was self-complementary, and each of the four fragments was assembled from oligonucleotides with unique cohesive ends to allow for unambiguous joinings within each fragment. Thus, the joining the oligonucleotides to form the four fragments was independent of restriction sites. The joining of the 10 oligonucleotides to form duplex C is shown in figure 2.

The ends of the four duplexes (A through D) were designed to facilitate cloning. The 5'-end of duplex A was compatible with an *Eco* RI cohesive end, and the 3'-end was compatible with the 5'-cohesive end of duplex B. The four base cohesive overhang used to join duplexes A and B was nonpalindromic to avoid the formation of concatamers during cloning. The 3'-end of duplex B was compatible with a *Bam* HI cohesive end. Likewise, the 5'-end of duplex C was compatible with a *Bam* HI cohesive end, and the 3'-end was compatible with the 5'-cohesive end of duplex D. The cohesive overhang joining duplexes C and D was nonpalindromic. The 3'-end of duplex D was compatible with a *Hind* 3 cohesive end. This strategy allowed the cloning of fragments A and B together and the cloning of fragments C and D together (figure 4) before subcloning to assemble the entire gene (figure 5).

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## EXPERIMENTAL PROCEDURES

### Materials

O-Methyl-protected N,N-diisopropylphosphoramidites and solid supports were obtained from Applied Biosystems, Inc.  $\beta$ -Cyanoethyl-protected phosphoramidites were obtained from American Bionetics, Inc. Reagents and solvents used for oligonucleotide synthesis were purified according to standard procedures (12). T4 polynucleotide kinase was from Pharmacia P-L Biochemicals. Radionuclide compounds were from Amersham, Corp. The expression vector, pMT-2 (13) was generously provided by Dr. R.J. Kaufman, Genetics Institute, Inc. Cloning vectors (pTZ) were obtained from United States Biochemical Co. Rabbit polyclonal anti-transducin antiserum was prepared by Dr. K. Yatsunami as previously described (8).

### Oligonucleotide Synthesis

Oligonucleotides were synthesized on an Applied Biosystems Model 380A automated DNA synthesizer using approximately 0.2  $\mu$ mol of solid support-bound nucleoside per synthesis. Average coupling times were 30 seconds for O-methyl-protected phosphoramidites and 150 seconds for  $\beta$ -cyanoethyl-phosphoramidites. Dimethoxytrityl-protecting groups were removed by treatment with 3% (wt/vol) dichloroacetic acid in dichloromethane for 60 seconds for adenosine, thymidine, and cytidine, and 50 seconds for guanosine. Deprotection of oligonucleotides was performed according to standard procedures (14).

### Purification and Characterization of Oligonucleotides

Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis as previously described (3). After 5'-end phosphorylation (15), characterization by gel electrophoresis and 5'-end analysis were performed (3).

### Joining of Oligonucleotides by T4 DNA Ligase

Enzymatic joining of oligonucleotides to form duplexes A through D was performed as previously described (3). The oligonucleotide which formed the 5'-overhang of each duplex was not phosphorylated in the reaction mixture as to prevent concatamer formation. Each assembled full length duplex was purified by agarose gel electrophoresis on Nu-Sieve agarose (FMC Corp.) followed by electroelution onto DEAE-cellulose NA45 membrane (Schleicher and Schuell). The joining of duplex C is shown in figure 2 as an example. Agarose gel electrophoresis of purified duplexes A through D are shown in figure 3.

### Cloning of Synthetic DNA Duplexes

The cloning of duplexes A through D is presented schematically in figure 4. Purified duplexes A and B were ligated into a pTZ plasmid linearized by successive digestions with *Eco* RI and *Bam* HI. Purified duplexes C and D were ligated into a pTZ plasmid linearized by successive digestions with *Bam* HI and *Hind* 3. Ligation mixtures were used to transform  $\text{CaCl}_2$ -treated *Escherichia coli* strain DH1. CsCl-purified plasmid DNA was prepared from ampicillin-resistant colonies.

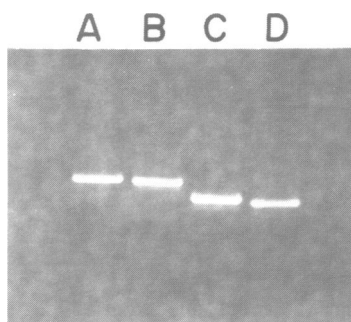


Figure 3. Agarose gel electrophoresis of the purified gene fragments A through D. DNA was visualized by ethidium bromide staining.

The synthetic gene fragments were subcloned into the mammalian expression vector pMT2', which had been modified to contain an *Eco* RI-*Not* I cloning site as described (5). The strategy employed is presented in figure 5-A. A four-component ligation mixture contained: 1). the 5.5 kilobase *Eco* RI-*Not* I linearized expression vector, 2). the 633 base pair synthetic gene *Eco* RI-*Bam* HI restriction fragment, 3), the 447 base pair synthetic gene *Bam* HI-*Hind* 3 restriction fragment, and 4). a 21 base pair *Hind* 3-*Not* I synthetic duplex adapter.

#### Cloning of cDNA into Expression Vector

The cloning of bovine transducin  $\alpha$ -subunit cDNA was previously reported (8). This cDNA was subcloned from M13 phage into the mammalian expression vector to permit comparisons of synthetic gene expression and cDNA expression as presented in figure 5-B. The 5'-noncoding end of the cDNA was reconstructed to include the same CCACC consensus sequence (10) found in the synthetic gene. A five-component ligation mixture contained: 1). the 5.5 kilobase *Eco* RI-*Not* I linearized expression vector, 2). a 20 base pair *Eco* RI-*Nco* I synthetic duplex adapter containing a CCACC consensus sequence immediately preceding the initiation codon as in the synthetic gene, 3). the 713 base pair cDNA *Nco* I-*Sph* I restriction fragment, 4). the 402 base pair cDNA *Sph* I-*Hind* 3 restriction fragment which included 43 base pairs of 3'-untranslated sequence, 5). a 21 base pair *Hind* 3-*Not* I synthetic duplex adapter. The reconstructed 5'- and 3'-ends were sequenced.

#### DNA Sequence Analysis

The nucleotide sequence of the cloned synthetic gene was confirmed by the chain terminator method for DNA sequencing using [ $^{35}$ S]dATP $\alpha$ S on CsCl-purified plasmid DNA (16,17). Eighteen 21-mer sequencing primers were synthesized and purified as above. Using these primers, both strands of the gene were sequenced independently twice. The sequence was correct except for a single unexpected C-G base pair insertion at nucleotide position 582 in the gene.

### Correction of the Unexpected C-G Base Pair in the Synthetic Gene

To correct the single unexpected C-G base pair insertion at nucleotide position 582, the synthetic gene was excised from the expression vector by successive digestions by *Not* I and *Eco* RI and both resulting fragments were isolated. The gene fragment was further digested with *Dra* 2 and *Bam* HI to yield a 5'-end fragment of 573 base pairs and a 3'-end fragment of 447 base pairs. A 56 base pair duplex corresponding to the restriction fragment *Dra* 2-*Bam* HI was synthesized to correct the unexpected sequence and characterized. A four-component ligation mixture contained: 1). the 5.5 kilobase *Eco* RI-*Not* I linearized expression vector, 2). the 573 base pair *Eco* RI-*Dra* 2 gene restriction fragment, 3). the 447 base pair *Bam* HI-*Not* I gene restriction fragment, and 4). the 56 base pair *Dra* 2-*Bam* HI synthetic duplex. Plasmid DNA cloned from this mixture was resequenced to confirm that the correction had been introduced.

### Expression of the Synthetic Gene in COS-1 Cells

The synthetic gene and cDNA were expressed in COS-1 cells by transfection with CsCl-purified plasmid DNA by treatment with DEAE-dextran (18) as previously reported (4). After incubation for 72 hours, the cells were solubilized and subjected to polyacrylamide gel electrophoresis and immunoblotting as below.

### SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (19), and immunoblotting (20) was performed using rabbit anti-transducin antiserum and Protein A which was iodinated with Na<sup>125</sup>I using chloramine T (21).

## RESULTS

### Preparation of Oligonucleotides

Synthetic oligonucleotides of high purity were an essential prerequisite to accomplish *in vitro* enzymatic joining of large numbers of oligonucleotides to form synthetic duplexes A through D. Preparative polyacrylamide gel electrophoresis was successful in purifying oligonucleotides of up to about 60 nucleotides. Purity was assessed by denaturing gel electrophoresis and 5'-end analysis after quantitative phosphorylation of oligonucleotides and was estimated to be greater than 95%. Oligonucleotides used to assemble duplexes A and B were synthesized from  $\beta$ -cyanoethyl-phosphoramidites while those used to assemble duplexes C and D were synthesized from O-methyl-phosphoramidites. The final yields of the oligonucleotides ranged between about 15% and 35% and were not dependent on the type of phosphoramidite used.

### Enzymatic Joining of Oligonucleotides to Form Duplexes A through D

Phosphorylated oligonucleotides were combined, annealed, and treated with T4 DNA ligase in a single step for each duplex (A through D). The joining of duplex C is shown in figure 2 as an example. Each expected full length duplex was successfully purified from all of

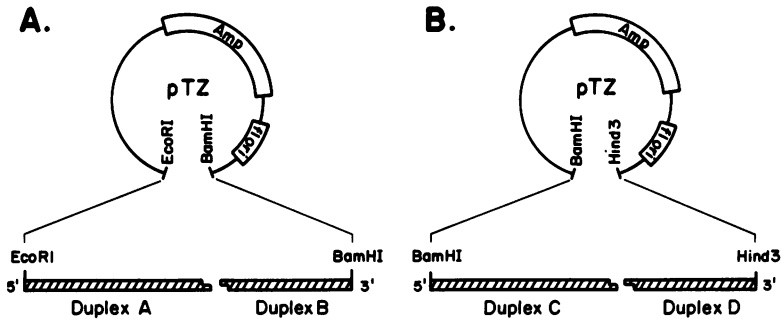


Figure 4. Cloning of the purified gene fragments A through D. A. - Duplexes A and B were cloned into a pTZ plasmid linearized with *Eco* RI and *Bam* HI. B. - Duplexes C and D were cloned into a pTZ plasmid linearized with *Bam* HI and *Hind* 3.

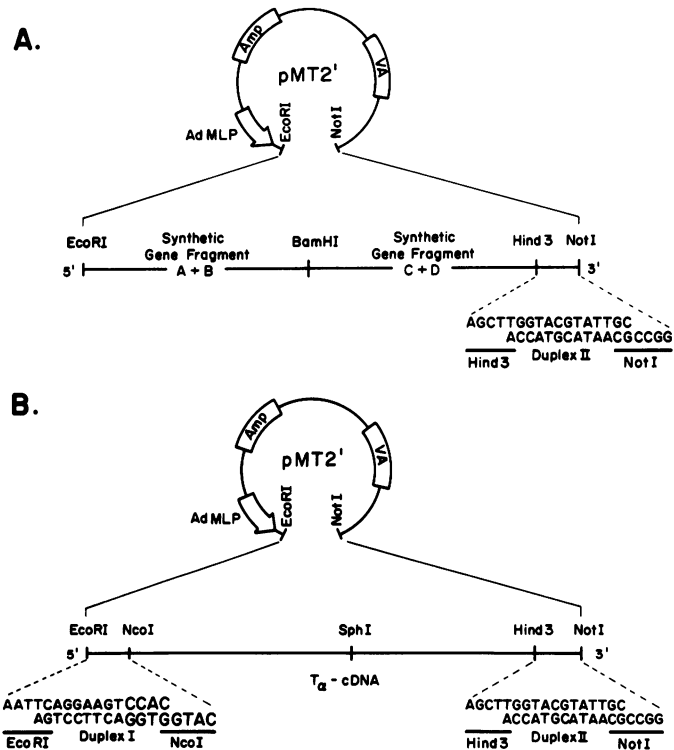


Figure 5. Cloning of the synthetic gene and cDNA for the  $\alpha$ -subunit of transducin into the mammalian expression vector. A. - synthetic gene fragment A + B and fragment C + D were cloned into pMT2' linearized with *Eco* RI and *Not* I. A synthetic duplex adapter (Duplex II) was used at the 3'-end. B. - T $\alpha$ -cDNA was cloned into the same linearized vector. The 5'-end of the cDNA was reconstructed to include a CCACC consensus sequence using Duplex I.



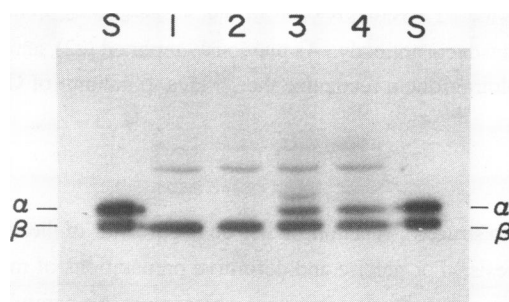


Figure 6. Immunoblot analysis of the  $\alpha$ -subunit of transducin expressed in COS-1 cells. Lane S, transducin purified for bovine ROS (100 ng). Lane 1, mock transfected COS-1 cells. Lane 2, COS-1 cells transfected with expression vector alone, without insert. Lane 3, COS-1 cells transfected with vector + synthetic gene. Lane 4, COS-1 cells transfected with vector + cDNA. The endogenous COS-1 G protein 36 kDa  $\beta$ -subunits cross-react with anti-holotransducin antibody.

the incompletely joined fragments by agarose gel electrophoresis as described above. The final yields of full length duplex were estimated by monitoring radioactivity to be as follows: duplex A (12 oligonucleotides, 322 base pairs), 38.1%; duplex B (12 oligonucleotides, 315 base pairs), 24.2%; duplex C (10 oligonucleotides, 234 base pairs), 24.2%; duplex D (10 oligonucleotides, 217 base pairs), 7.2%. The purified duplexes are shown in figure 3.

#### Cloning of Synthetic Duplexes A through D

The synthetic gene was designed so that the duplexes prepared *in vitro* could be cloned in pairs. Duplexes A and B were cloned into a cloning plasmid independently from duplexes C and D, as shown in figure 4, for amplification of the DNA before subcloning. The two resulting gene fragments were transferred into the expression vector as described above and presented schematically in figure 5. Sequencing of the gene was accomplished directly in the expression vector. A single unexpected C-G base pair was found at position 582. This error was definitively corrected by restriction fragment replacement of a *Dra* 2-*Bam* HI synthetic duplex.

#### Expression of the Synthetic Transducin Gene and cDNA

The synthetic gene and cDNA encoding the  $\alpha$ -subunit of transducin were expressed in monkey kidney cells (COS-1) under the control of the adenovirus major late promoter. The consensus sequence CCACC (10) was introduced in both synthetic gene and cDNA constructions. The levels of expression were identical as judged by immunoblotting as shown in figure 6. The levels of transducin produced were about 0.3% of total cellular protein. These results indicate that, when compared to the cDNA sequence, the nucleotide changes introduced to tailor a specific restriction map in the synthetic gene were not detrimental in terms of production of protein in mammalian cells. Furthermore, the level of endogenous G protein  $\beta$ -

subunit was not affected by overexpression of T $\alpha$  as judged by immunoblotting using a polyclonal anti-holotransducin antibody. As previously reported (22), antibodies raised against rod outer segment holotransducin recognize the 36 kDa  $\beta$ -subunit of G proteins from other tissues.

## DISCUSSION

Our approach to structure-function studies on the proteins of the visual system involves site-specific mutagenesis. For precise and definitive preparations of mutant DNA, we have relied upon the technique of restriction fragment replacement in a carefully designed synthetic gene. For example, our laboratory reported the synthesis of a gene for bovine rhodopsin (3) and used this gene to prepare and characterize mutations involving domains of rhodopsin which may be involved in the activation of transducin (5). We propose to use the synthetic transducin gene described in this report to prepare mutant proteins to answer specific questions about the structural elements of transducin which interact with rhodopsin. Other mutagenesis studies, including the introduction or exchange of entire putative functional domains from other related G proteins, will also be facilitated by use of the synthetic transducin gene described in this report.

The design of the synthetic gene allowed 38 unique restriction sites to be introduced into the 1076 base pair gene. This compares with the 7 unique sites with overhanging cohesive ends in the native cDNA sequence. The design and construction of the gene in fragments which were assembled *in vitro* and then purified by agarose gel electrophoresis was a departure from methods reported previously (3, 23). The yields of DNA duplexes in enzymatic joinings were between 7.2% and 38.1% when 10 to 12 oligonucleotides were joined enzymatically in one reaction mixture was significantly higher than yields previously reported of 1% to 4% (3) and 4% to 9% (23) from the joining of up to 8 oligonucleotides. Oligonucleotides of 39 to 59 nucleotides in length were carefully purified and 5'-end phosphorylated to maximize fragment joining yields. No errors were detected in any of the joinings. Based on these findings, it should be possible to join as many as 20 oligonucleotides in a single reaction and isolate and clone the resulting duplex.

A single unexpected C-G base pair was identified upon sequencing a single clone of the synthetic gene. This error was easily corrected by restriction fragment replacement. The etiology of this error is not known. It may have resulted from an n+1 impurity in one of the oligonucleotides due to a condensation of two cytosines onto the growing oligonucleotide at the same cycle. This error rate of about one per 1000 cloned base pairs is similar to that encountered in this laboratory over the last several years with the exception of the higher rate reported for the synthesis of the bacterio-opsin gene (23). Even at these low frequencies of unexpected "mutations", it is vital to carefully sequence cloned synthetic DNA.

The synthetic gene was expressed in monkey kidney cells by transient transfection with

a vector in which transcription was under the control of the adenovirus major late promoter. The level of expression of the synthetic gene was identical to that of the corresponding cDNA, indicating that the nucleotide changes introduced in the design of the gene did not adversely affect transcription. Furthermore, given the level of expression obtained, we hope to devise a satisfactory method to purify the transducin from the COS-1 cells, and to assay for nucleotide-binding and GTPase activities. When this goal is achieved, mutagenesis of T $\alpha$  will be carried out to probe the structural domains responsible for receptor binding specificity and other relevant functions.

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