the operator sequence is located ~530 bp downstream of the initiation site with the SV40 P_e and ~550 bp from the MLP transcriptional start. Construct C3 (Fig. 1) has been previously described (*24*) and the operator is centered 752 bp downstream of the RNA start site.
10. In each experiment each of 150 individual cells [rabbit kidney cell lines RKT3 and RKT3i (*11*)] was injected with 0.2 pl of DNA at 1 μg/ml (between 5 and 20 molecules per sample) with a Zeiss Automated Injection System. For induction, 50 m*M* IPTG was present in the DNA solution, and the medium contained 10 m*M* IPTG. The cells were fixed 12 hours after injection and stained with mouse antibody to T-antigen and rabbit antiserum to mouse immunoglobulin G conjugated with fluorescein isothiocyanate (FITC).
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14. In vitro transcription was assayed in 25-μl reactions containing 1 μg of linearized plasmid DNA, 40% HeLa nuclear extract (*13*), 2% polyvinyl alcohol, 4 m*M* creatine phosphate, 4 m*M* $MgCl_2$, 300 μ*M* ATP, CTP and GTP, 30 μ*M* UTP, and 0.25 μ*M* [α-^{32}P]UTP. Where indicated, 1 μg of purified *lac* repressor, 500 μ*M* IPTG, and α-amanitin were added to the reactions. After incubation for 20 min at 20°C, nucleotides were added and the reaction continued for 30 min at 30°C. The transcripts were purified by organic extraction and ethanol precipitation, and analyzed on 4% sequencing gels.
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A 49-Kilodalton Phosphoprotein in the *Drosophila* Photoreceptor Is an Arrestin Homolog

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The gene encoding the 49-kilodalton protein that undergoes light-induced phosphorylation in the *Drosophila* photoreceptor has been isolated and characterized. The encoded protein has 401 amino acid residues and a molecular mass of 44,972 daltons, and it shares approximately 42 percent amino acid sequence identity with arrestin (S-antigen), which has been proposed to quench the light-induced cascade of guanosine 3′,5′-monophosphate hydrolysis in vertebrate photoreceptors. Unlike the 49-kilodalton protein, however, arrestin, which appears to bind to phosphorylated rhodopsin, has not itself been reported to undergo phosphorylation. In vitro, Ca^{2+} was the only agent found that would stimulate the phosphorylation of the 49-kilodalton protein. The phosphorylation of this arrestin-like protein in vivo may therefore be triggered by a Ca^{2+} signal that is likely to be regulated by light-activated phosphoinositide-specific phospholipase C.

ALTHOUGH THE ROLE OF G PROTEIN–mediated guanosine 3′,5′-monophosphate (cGMP) hydrolysis in the visual transduction pathway of vertebrates has been elaborated (*1*), the corresponding pathway in invertebrate photoreceptors has not been clearly delineated. A 49-kD protein of *Drosophila melanogaster* undergoes light-induced, reversible phosphorylation in vivo (*2*). In *norpA* (no receptor potential A) mutants, which are likely to be defective in an intermediate process of visual transduction (*3*), the light-induced phosphorylation of this protein is blocked (*2*). The 49-kD protein is abundant (*2*) and has an epitope that has been observed in all of the photoreceptors of the compound eye (*4, 5*) as well as in the ocelli and larval photoreceptors (*4*). The epitope has been immunolocalized to the rhabdomeres and to the cell bodies and axons (*4*), and the 49-kD protein has been isolated from a *Drosophila* rhabdomere preparation (*5*). These results suggest that the reversible phosphorylation of the 49-kD protein may be important in visual transduction. We now report the molecular cloning of the gene encoding the 49-kD protein and show that its amino acid sequence resembles that of vertebrate arrestin. Arrestin has been proposed to interact with

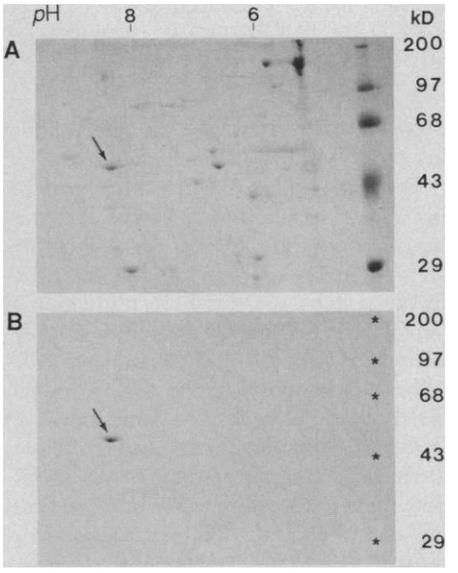

Fig. 1. In vitro translation products of poly(A)$^+$ mRNA hybrid-selected by the putative 49-kD recombinant DNA clone cTYp1-2. (**A**) Coomassie blue–stained gel. (**B**) ^{35}S autoradiogram. Approximate *p*H of the first dimension isoelectric focusing (IEF) and the molecular masses of internal markers in the second dimension [SDS–polyacrylamide gel electrophoresis (SDS-PAGE)] are indicated. The location of the 49-kD protein is indicated by arrows. A *Drosophila* cDNA library constructed in λgt11 from Canton S adult head poly(A)$^+$ mRNAs was screened with the monoclonal antibody P27 to the 49-kD protein (*4*) as a probe. One of two positive clones, cTYp1-2, was localized to the left arm of the third chromosome (66D) by in situ hybridization to the DNA of salivary gland polytene chromosomes (*28*). This cTYp1-2 clone was positively identified as representing the gene for the 49-kD protein by hybrid selection followed by in vitro translation in a rabbit reticulocyte lysate (Amersham) in the presence of [^{35}S]methionine (*29*). The translated protein sample was dissolved, together with 200 dissected eyes (dark-adapted), in an IEF lysis buffer and subjected to two-dimensional gel analysis (*2*). The identity of this spot as the 49-kD protein was confirmed by staining the immunoblot with polyclonal antibody to the 49-kD protein (*30*) and also by the fact that this spot shifts in the acidic direction upon light adaptation (*31*). The presence of a faint spot to the right of the 49-kD protein in the autoradiogram may be due to 49-kD protein phosphorylated by an endogenous protein kinase. Asterisks indicate location of markers.

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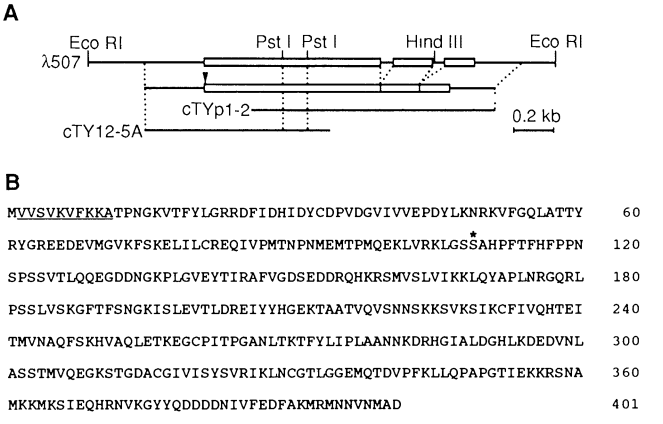

Fig. 2. (**A**) Map of the 49-kD protein gene DNA of *D. melanogaster*; λ507 represents the 2.3-kb Eco RI fragment of the genomic DNA clone λ507 reported by Levy *et al.* (*8*). The cDNA map drawn below λ507 was constructed from the two cDNA clones, cTYp1-2 and cTY12-5A. (**B**) Putative amino acid sequence (*9*) of the 49-kD protein. The translational initiation site, shown in (A) by an arrowhead, was unequivocally determined based on the NH_2-terminal amino acid sequence (underlined) (*7*) and by the existence of a TAA termination signal (nucleotides −7 to −9) upstream from the assigned methionine codon (*31*). The NH_2-terminal methionine of the 49-kD protein appears to be posttranslationally cleaved. Northern (RNA) blot analysis indicated that the cDNA clone cTYp1-2 hybridizes to only one mRNA band of about 1.8 kb (*31*). Therefore, the composite cDNA map covers almost the full length of the 49-kD protein mRNA. We observed a slight nucleotide polymorphism between the cDNAs cTYp1-2 and cTY12-5A. Seven cases of third nucleotide substitution leading to no differences at the amino acid level and one case of second and third nucleotide substitution changing the amino acid from S (asterisk) to N were found. These variations may originate from differences in genetic background. The sequence analysis indicates that the nucleotide sequence of the protein coding region of the gene for the 49-kD protein is ≈55% identical to that of bovine arrestin [analyzed by the FastA program of University of Wisconsin Genetic Computer Group (UWGCG)]. The sequence of the 49-kD protein gene has been deposited in GenBank (accession number, M32141).

phosphorylated rhodopsin, thereby inhibiting the cGMP hydrolytic cascade (*6*).

The recombinant cDNA clone representing the gene for the 49-kD protein was selected from a cDNA expression library by antibody screening (Fig. 1). The authenticity of the clone was verified by translation in vitro of mRNA hybrid-selected from polyadenylated [poly(A)$^+$] mRNAs of *Drosophila* heads with the putative 49-kD protein cDNA clone cTYp1-2. Two-dimensional gel analysis of the translation products shows the incorporation of ^{35}S radioactivity specifically into the 49-kD protein spot, indicating that the clone cTYp1-2 encodes this protein (Fig. 1). Furthermore, we sequenced the NH_2-terminal ten amino acids of the 49-kD protein (*7*); the amino acid sequence obtained matches that derived from the nucleotide sequence of the cDNA clone (Fig. 2B).

The head-specific genomic clone λ507 reported by Levy *et al.* (*8*) overlaps with cTYp1-2. We further screened an additional library by using cTYp1-2 as a probe and isolated the cDNA clone cTY12-5A (*8*). The restriction enzyme maps and the deduced amino acid sequence (*9*) of the gene for the 49-kD protein constructed from these clones are shown in Fig. 2. The gene encodes a polypeptide consisting of 401 amino acids [with a calculated molecular mass of 44,972 daltons and an isoelectric point (*p*I) of 8.9]. The amino acid sequence of the 49-kD protein is similar to that of vertebrate arrestin (*10–12*); the two proteins share ≈42% amino acid identity, and the conserved amino acids are concentrated in several regions of the proteins (Fig. 3A) (*13*). Some of the homologous domains include the three putative phosphoryl binding sites of vertebrate arrestin (*10–12*): amino acids 73 to 76 (DVMG), 135 to 141 (APQDVGK), and 292 to 298 (GIALDGK) of bovine arrestin versus amino acids 68 to 71 (EVMG), 130 to 136 (EGDDNGK), and 286 to 292 (GIALDGH) of the 49-kD protein, respectively (identical residues are underlined). However, the major consensus features of a phosphoryl binding site (*14*), D-X-X-G and (A/G)-X-X-X-X-G-K (where X represents any amino acid), are imperfect in the 49-kD protein.

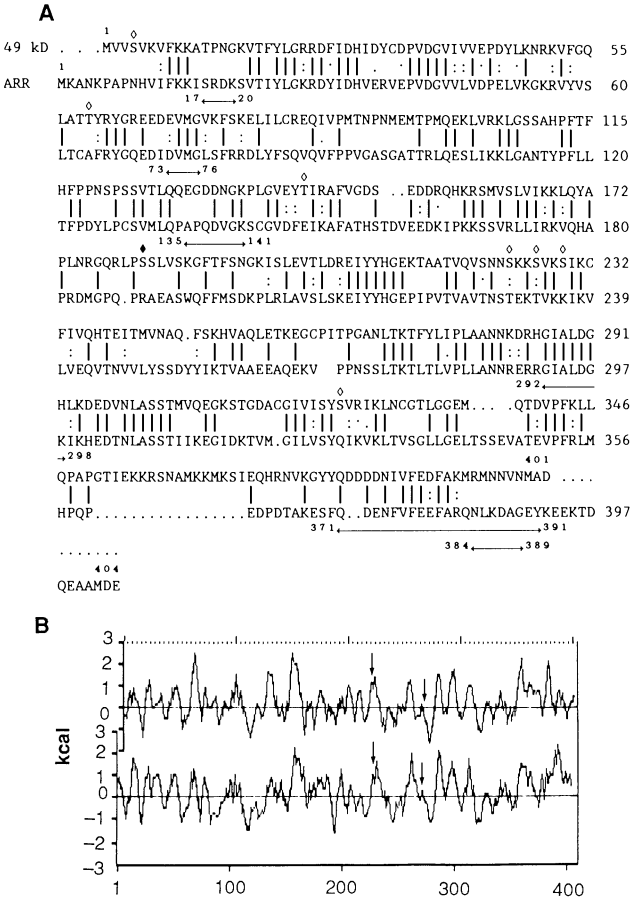

Fig. 3. (**A**) Homology comparison between the *Drosophila* 49-kD protein and bovine arrestin (ARR). Identical pairs of amino acids are indicated by a bar. Homologous pairs of amino acids are indicated by a colon. The amino acid sequence of the 49-kD protein is 41.5% identical to that of bovine arrestin (analyzed by the Gap program of UWGCG). The arrestin sequences discussed in the text are underlined. The potential phosphorylation sites for protein kinase C [(S/T)-X-(R/K)] (◇) (*32*) or Ca^{2+}/calmodulin–dependent protein kinase II [R-X-X-(S/T)] (◆) (*33*) are shown. (**B**) Hydropathy profiles of the *Drosophila* 49-kD protein (upper curve) and bovine arrestin (lower curve). The positive value on the ordinate indicates hydrophilicity, and the negative value indicates hydrophobicity (*34*). The abscissa represents amino acid residue number. Potential glycosylation sites [N-X-(S/T)] of both arrestin and the 49-kD protein are shown by arrows. The residue window size used was six. The 49-kD protein curve is shifted six amino acids toward the COOH-terminus. Both curves were generated by DNASIS (Hitachi).

A small stretch of arrestin sequence (amino acids 371 to 391) at the COOH-terminus resembles the α subunit of transducin. This region has been proposed to be a putative rhodopsin binding site (*10–12*). The corresponding sequence in the 49-kD protein (amino acids 380 to 400) is similar to that of arrestin only in the first half of the sequence. The arrestin sequences that resemble the adenosine diphosphate–ribosylation sites of transducin—amino acids 17 to 20 (SRDK) and 384 to 389 (NLKDAG)—of bovine arrestin (*10, 11*) are not found in the 49-kD protein.

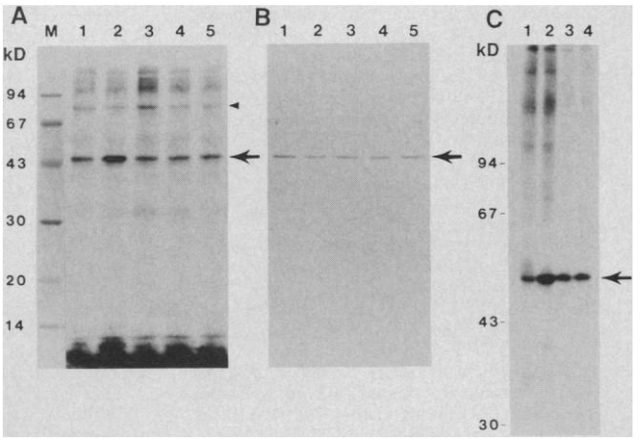

Fig. 4. Ca^{2+} activates the phosphorylation of the *Drosophila* and *Musca* 49-kD proteins in vitro. Wild-type *Drosophila* flies were dehydrated by acetone at −20°C (*2*). The retinas of *Musca* compound eyes were freshly dissected. For the cell-free suspension of the compound eye sample, the retinas from 70 *Drosophila* or 5 *Musca* were dissected and homogenized in 210 μl of 10 m*M* tris-HCl (*p*H 7.5) containing 0.5 m*M* EGTA. Proteins (30 μl) of these homogenates and 10 μl of each effector were mixed and incubated at 25°C for 5 min. The reaction was initiated by adding 30 μl of [^{32}P]ATP (adenosine triphosphate) mixture [0.1 m*M* [γ-^{32}P]ATP (10 μCi), 10 m*M* $MgCl_2$, and 25 m*M* tris-HCl (*p*H 7.5)]. The final concentrations of each ingredient were 15 m*M* tris-HCl (*p*H 7.5), 0.21 m*M* EGTA, 43 μ*M* [γ-^{32}P]ATP (10 μCi), and 4.3 m*M* Mg^{2+}. The reaction mixtures were incubated for 5 min at 25°C. The reaction was stopped by the addition of trichloroacetic acid at a final concentration of 15%. The resulting precipitates were separated by centrifugation, washed by a mixture of ether and ethanol (1:1), dissolved in 0.125*M* tris-HCl (*p*H 6.8) containing 5% β-mercaptoethanol and 2% SDS, and subjected to SDS-PAGE. The gel was transferred electrophoretically to nitrocellulose. The nitrocellulose was stained with polyclonal antibody to the 49-kD protein (*30*) and a horseradish peroxidase–conjugated goat antibody to rabbit immunoglobulin G. An autoradiogram was taken from each nitrocellulose filter. The final concentration of each effector was Ca^{2+} [354 μ*M* (final total concentration); 140 μ*M* (estimated effective concentration after compensation by EGTA], cAMP (10 μ*M*), cGMP (10 μ*M*), IP_3 (10 μ*M*), and TPA (1 μ*M*). (**A**) Ca^{2+} activation of the *Drosophila* 49-kD protein phosphorylation: autoradiogram taken from the immunoblot shown in (B). (**B**) Immunoblot showing the *Drosophila* 49-kD protein in each lane. Lane 1, control (no effector); lane 2, Ca^{2+}; lane 3, cAMP; lane 4, cGMP; and lane 5, IP_3. M, molecular size markers. In (A) and (B) the positions of the 80-kD protein and the 49-kD protein (*2*) are indicated by an arrowhead and an arrow, respectively. (**C**) Autoradiogram showing the Ca^{2+} activation of the *Musca* 49-kD protein phosphorylation. The 49-kD protein is indicated by an arrow. The experimental conditions used for the *Drosophila* retinas shown in (A) and (B) were repeated for the experiment shown in (C), except that freshly dissected *Musca* eye homogenates were used. Lane 1, control (no effector); lane 2, Ca^{2+}; lane 3, Ca^{2+} and phosphatidylserine (1 mg/ml); and lane 4, Ca^{2+}, phosphatidylserine (1 mg/ml), and TPA.

Arrestin and the 49-kD protein share almost identical hydropathic structural domains (Fig. 3B). Also, the positions of two possible glycosylation sites predictable in both arrestin and the 49-kD protein are well conserved (Fig. 3B). Such a resemblance in hydropathy profiles is surprising because the two proteins have different *p*I's. The *p*I of the 49-kD protein is basic (*p*I = 8.9), whereas that of arrestin is acidic (*p*I = 6.0).

To characterize the phosphorylation of this arrestin-like protein, we examined its phosphorylation in vitro. We evaluated possible activators of protein kinases: Ca^{2+}, adenosine 3′,5′-monophosphate (cAMP), cGMP, inositol 1,4,5-trisphosphate (IP_3), and 12-*O*-tetradecanoyl phorbol-13-acetate [TPA, an analog of diacylglycerol (DAG)]. We found that only Ca^{2+} activated 49-kD protein phosphorylation in vitro (Fig. 4) (*15*), whereas cAMP activated the phosphorylation of an 80-kD protein (Fig. 4A, lane 3) that is also a photoreceptor-specific phosphoprotein and which undergoes light-induced, reversible phosphorylation (*2*). TPA did not activate 49-kD protein phosphorylation in homogenates of *Musca* retina (Fig. 4C) (*16*); however, calcium activated 49-kD protein phosphorylation.

In invertebrate photoreceptors, the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) is likely to follow the excitation of rhodopsin which leads to plasma membrane depolarization (*17*). The hydrolytic products of PIP_2, DAG and IP_3, are known in both mammalian and invertebrate systems to activate protein kinase C and to increase the concentration of cytosolic Ca^{2+}, respectively (*18*). Phosphoinositide-specific phospholipase C (PI-PLC), which is responsible for the hydrolysis of PIP_2, is defective in the compound eyes of *Drosophila norpA* mutants (*19*). In fact, the *norpA* gene has been shown to encode a putative PI-PLC (*20*). Thus, it appears that the block of 49-kD protein phosphorylation in the *norpA* mutants is due to a defect in the activation mechanism of the protein kinase rather than to a lack of kinase or its substrate (*21*).

The 49-kD protein of the *Drosophila* photoreceptor resembles vertebrate arrestin in its amino acid sequence and hydropathy profile; however, there are distinctions between these proteins. For example, the 49-kD protein is a phosphoprotein (*22*), whereas no phosphorylation of arrestin has been reported (*23*). In addition, their *p*I's differ (*24*). Although the sequences similar to the presumed phosphoryl binding and rhodopsin binding sites of arrestin are present in the 49-kD protein, there is always a deviation of the 49-kD sequence from the strict consensus (*25*). Such differences in biochemical properties may reflect the differences in their physiological functions. As the light-induced phosphorylation of the 49-kD protein is blocked in the *norpA* mutants, which carry a defective PI-PLC gene, the phosphorylation of the 49-kD protein may be regulated in vivo by cytosolic Ca^{2+} through the activation of PI-PLC (*26*). Because arrestin homologs have been implicated in transduction systems other than photoreceptors, such as β-adrenergic receptors and muscarinic acetylcholine receptors (*27*), our finding of this arrestin-like protein and its phosphorylation by Ca^{2+} may provide an insight into regulation of transduction systems of both vertebrates and invertebrates.

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7. The 49-kD protein extracted from 600 eyes that had been dissected from dark-adapted wild-type *Drosophila* was separated on two-dimensional gels. The gels were blotted onto polyvinylidene difluoride membrane, and the 49-kD protein spots were subjected to gas-phase sequencing [P. Matsudaira, *J. Biol. Chem.* **262**, 10035 (1987)]. We obtained signals through the tenth cycle from the NH_2-terminus: [V]VSVKVFKKA (brackets indicate uncertainty).
8. By Southern (DNA) [E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975)] hybridization and direct sequencing, we found that a head-specific genomic DNA clone λ507 [L. S. Levy, R. Ganguly, N. Ganguly, J. E. Manning, *Dev. Biol.* **94**, 451 (1982)] overlaps with cTYp1-2. We subcloned the cTYp1-2 Eco RI and λ507 Eco RI inserts into pBluescript II (Stratagene) and sequenced DNAs by the dideoxynucleotide termination method with the use of synthetic primers and double-stranded DNA templates [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977); M. Hattori and Y. Sasaki, *Anal. Biochem.* **152**, 232 (1986)]. In all cases both strands were sequenced. The DNA sequencing of these clones suggested that cTYp1-2 lacks the 5′ noncoding and about 240 bp of the 5′ coding sequence. Therefore, we further screened a commercially available *D. melanogaster* adult (y^2w^{bf}; strain unidentified) [C. B. Bridges and K. S. Brehme, *The Mutants of* Drosophila melanogaster (Publ. No. 552, Carnegie Institution of Washington, Washington, DC, 1944), pp. 222–236] cDNA library constructed in λZAP II (Stratagene). The inserts from several

positive clones were excised in vivo from the λZAP II vector into pBluescript II by coinfection with helper phage R408 [J. M. Short, J. M. Fernandez, J. A. Sorge, W. D. Huse, *Nucleic Acids Res.* **16**, 7583 (1988)]. From this screening we obtained cTY12-5A, which overlaps with cTYp1-2 and extends toward the 5′ untranslated end of the 49-kD protein mRNA.
9. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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15. We quantified the phosphorylation by scanning the autoradiograms with a Molecular Dynamics Computing Densitometer Model 300A and found that Ca^{2+} increased the 49-kD protein phosphorylation by 95 ± 53% (n = 5, with 99% confidence limit). We suspected that the *Drosophila* 49-kD protein may be homologous to phosphoproteins found in photoreceptors of other invertebrate species. There are at least two phosphoproteins that are phosphorylated in vitro in response to Ca^{2+}: a 46-kD protein of *Limulus* ventral and lateral eyes [E. M. Wiebe, A. C. Wishart, S. C. Edwards, B.-A. Battelle, *Vis. Neurosci.* **3**, 107 (1989)] and a 47- to 49-kD protein of the crab *Leptograpsus variegatus*, which exhibits 4-nitrophenylphosphatase activity [S. C. Trowell and M. Carter, in *Molecular Physiology of Retinal Proteins*, T. Hara, Ed. (Yamada Science Foundation, Osaka, Japan, 1988), pp. 415–416]. However, the polyclonal antibody to the 49-kD protein (*30*) failed to stain any protein bands in these molecular mass regions on immunoblots of *Limulus* ventral or lateral photoreceptors or of *Leptograpsus* photoreceptors (N. Komori and H. Matsumoto, unpublished data).
16. The decrease in phosphorylation of the 49-kD protein in lanes 3 and 4 of Fig. 4C by the addition of phosphatidylserine may be due to the decrease in the effective Ca^{2+} concentration by chelation. The reason for the lower background phosphorylation in lanes 3 and 4 is unknown.
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21. When we homogenized the retina of the *norpA* mutant flies, the phosphorylation of the 49-kD protein occurred in vitro (H. Matsumoto, unpublished data). Therefore, the *norpA* mutant flies carry both the functional protein kinase for the 49-kD protein and the substrate.
22. Potential phosphorylation sites of the 49-kD protein by protein kinase C or Ca^{2+}/calmodulin–dependent protein kinase II are shown in Fig. 3A.
23. Because we suspected that vertebrate arrestin might be a phosphoprotein, we attempted to phosphorylate it. Under illuminated or nonilluminated conditions under which several unidentified proteins became phosphorylated in bovine whole retinal homogenates, we failed to observe any phosphorylation of arrestin (N. Komori and H. Matsumoto, unpublished data).
24. In general, *p*I is one of the important parameters that determines the way in which a protein interacts with other macromolecular structures.
25. The rhodopsin binding site may differ between arrestin and the 49-kD protein because the COOH-terminal structures of the corresponding rhodopsins differ substantially [W. L. Pak, *Photobiochem. Photobiophys.* **13**, 229 (1986)].
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A Bacterial Enhancer Functions to Tether a Transcriptional Activator Near a Promoter

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The nitrogen regulatory protein NtrC of enteric bacteria activates transcription of the *glnA* gene by catalyzing isomerization of closed complexes between RNA polymerase and the *glnA* promoter to open complexes. NtrC binds to sites upstream of *glnA* that have properties of eukaryotic transcriptional enhancers. NtrC-binding sites were found to facilitate open complex formation when these sites and the *glnA* promoter were located on different rings of a singly linked catenane, but not when the two rings were decatenated. The results provide evidence that NtrC contacts RNA polymerase–promoter complexes in a process mediated by formation of a DNA loop. NtrC-binding sites serve to tether NtrC near the *glnA* promoter, thereby increasing the frequency of collisions between NtrC and polymerase-promoter complexes.

EUKARYOTIC TRANSCRIPTIONAL ENhancers are DNA sequences that serve as binding sites for proteins that increase (or in some cases decrease) the rate of transcription of nearby genes (*1*). Defining characteristics of enhancers include their ability to function efficiently over large distances, at least in vivo (*2*), and to function downstream as well as upstream of transcriptional start sites.

Sequences analogous to transcriptional enhancers have been identified in prokaryotes (*3*). One of the best studied of these is the enhancer upstream of the *glnA* gene of enteric bacteria (*4*), which encodes glutamine synthetase. This enhancer, which is composed of multiple binding sites for the nitrogen regulatory protein NtrC (*5*), is amenable to detailed analysis because *glnA* transcription can be studied in a purified in vitro system with well-defined DNA templates. Moreover, the functions of the enhancer-binding protein NtrC and its target protein σ^{54}-holoenzyme, an alternative holoenzyme form of RNA polymerase, are comparatively well understood. NtrC catalyzes isomerization of closed recognition complexes between σ^{54}-holoenzyme and the *glnA* promoter to open complexes in which DNA around the transcription start site is

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