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Diurnal Expression of Transducin mRNA and Translocation of Transducin in Rods of Rat Retina

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The messenger RNA (mRNA) that encodes α subunit of the guanosine triphosphate–binding protein transducin (T_α) and T_α immunoreactivity were localized and measured in the rat retina during the light-dark cycle with in situ hybridization and immunohistochemistry. Both T_α mRNA and T_α immunoreactivity were observed only in photoreceptors. Within the photoreceptor T_α mRNA was present primarily in the inner segments and to a lesser extent in the outer nuclear layer at all times during the day and night. However, the distribution of T_α immunoreactivity varied profoundly with the light-dark cycle; during the day, T_α immunoreactivity was highest in the inner segments, and at night the outer segments were more immunoreactive. The amounts of T_α mRNA and T_α immunoreactivity also depended on the light-dark cycle. Levels of T_α mRNA were high immediately before and after lights on; levels were low for the rest of the light-dark cycle. During the day, T_α immunoreactivity increased in the inner segments following the increase in T_α mRNA. After the lights were turned off, T_α immunoreactivity decreased in the inner segments and increased in the outer segments. Thus, it appears that T_α is synthesized in the inner segments after a morning increase in T_α mRNA. Newly synthesized T_α remains in the inner segments until it is transported to the outer segments at night, where it may be involved in the increase in the sensitivity of photoreceptor rods at night.

TRANSDUCIN IS A GUANOSINE TRIphosphate (GTP)–binding (G) protein that mediates the stimulation of guanosine 3′,5′-monophosphate phosphodiesterase by light-activated rhodopsin in photoreceptor rods. Transducin is a membrane-associated protein that consists of three subunits—α, β, and γ. The α subunit (T_α) is structurally and functionally homologous to the α subunits of the G proteins that mediate inhibition of adenylate cyclase by neurotransmitter receptors. The β subunit of transducin may be identical to the β subunit of the G proteins that are associated with adenylate cyclase (*1*). Although the structure of transducin and the molecular characteristics of its coupling with rhodopsin have been extensively investigated, little information is available concerning either the regulation of the synthesis or the subcellular distribution of transducin by changes in physiological states. We localized and measured the expression of T_α messenger RNA (mRNA) and the subcellular distribution of T_α in photoreceptors during the light-dark cycle. T_α immunoreactivity was measured with immunohistochemistry and T_α mRNA was measured with in situ hybridization histochemistry.

Male Sprague-Dawley rats were maintained under a 12-hour light-dark cycle for 4 weeks before the localization studies were performed. Four animals were sacrificed every 4 hours during the light-dark cycle (under dim red light during the night); eyes were immediately removed, frozen on powdered dry ice, and stored at −80°C until 12-μm frozen sections could be prepared and mounted on gelatin-coated slides. Before the histochemical procedures, the slide-mounted tissue sections were thawed and kept at room temperature for 10 minutes, then fixed in 4% formaldehyde for 20 minutes. Immunohistochemical localization of T_α was performed with the peroxidase antiperoxidase (PAP) method (*2*) with a highly selective polyclonal antibody against T_α (*3*). The PAP reaction product was measured in photoreceptor inner and outer segments (*4*) and T_α mRNA was localized and measured (*5*). The synthetic oligodeoxynucleotide probe, complementary to nucleotides 1070 to 1117 of bovine T_α (*6*), was made by solid-phase synthesis on an Applied Biosystems DNA synthesizer and labeled with terminal deoxynucleotidyl transferase (BRL) and [^{35}S]deoxyadenosine triphosphate (NEN). Retinal sections were incubated with the complementary DNA (cDNA) probe at 37°C for 24 hours and washed under conditions of high stringency (18°C below the theoretical melting temperature). The distribution of labeled mRNA was evaluated by autoradiography (*5*, *7*).

The specificity of our cDNA-T_α mRNA hybridization procedures was verified (*7*) by meeting four criteria: (i) Only cells within the photoreceptor cell layer were positive for T_α immunoreactivity and T_α mRNA; in the rat retina this cell layer almost exclusively contains photoreceptor rods. (ii) Hybridizations were performed under conditions of high stringency that precluded cross-hybridization even with mRNA's for related G proteins. (iii) A negative control for sequence-independent hybridization was established with a synthetic probe for tyrosine hydroxylase mRNA, which is not expressed within photoreceptors. (iv) A single band on a Northern blot of total RNA was observed with our cDNA probe at a hybridization stringency identical to that used for in situ hybridization.

T_α mRNA was only present in photoreceptors, was more abundant in the inner segments than the outer nuclear layer, and was absent from the outer segments (Fig. 1A). Qualitatively this distribution of T_α mRNA was the same at all times of day and night. On the other hand, the distribution of T_α immunoreactivity was distinctly different at night than during the day. During the day, T_α immunoreactivity was most pronounced in the inner segments, was less evident in the outer nuclear and plexiform layers, and was low in the outer segments (Fig. 1B). At night, T_α immunoreactivity was most dense in the outer segments, relatively little was present in the inner segments, and none was observed in the outer nuclear and plexiform layers (Fig. 1C). The only cells other than photoreceptors observed to have T_α immunoreactivity were in the pigment epithelium where immunoreactivity was most dense at 3:00 a.m. and 7:00 a.m., the time of peak disk shedding. Because these cells phagocytose the disks shed from the distal end of photoreceptors, this localization of T_α immunoreactivity in the pigment epithelium indicates that some T_α is shed with the photoreceptor disks.

T_α mRNA levels varied with the light-dark cycle (Fig. 2B). At night, levels of T_α mRNA were low, but they increased just prior to the onset of light. In the morning, T_α mRNA was at its highest level. By mid-

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day the amount of T_α mRNA was at its lowest. The relation of the light-dark cycle to T_α immunoreactivity in the inner and outer segments is also shown in Fig. 2A. In the morning, T_α immunoreactivity increased in the inner segments following the increase in T_α mRNA. T_α immunoreactivity reached its highest level by midday and remained high in the inner segments until the lights were turned off. After the lights were turned off, T_α immunoreactivity decreased in the inner segments and increased in the outer segments. It is unlikely that these changes in immunoreactivity represent changes in the availability of antigenic sites on T_α molecules (*8*).

Thus, T_α is synthesized in the inner segments after a morning increase in T_α mRNA, and newly synthesized T_α remains in the inner segments until it is transported to the outer segments at night. This regulation of the synthesis and distribution of T_α is unlike that of opsin (the protein component of rhodopsin). Although opsin mRNA is also located in the inner segments (*7*), the synthesis and distribution of opsin appears to be either not influenced or only weakly regulated by the light-dark cycle (*9*). After it is synthesized, opsin is inserted into disk membranes at the proximal end of the outer segments where it remains until the disks are shed from the distal end several days later (*9*).

In rats, the shedding of disks is under diurnal control with a peak in shedding occurring in the morning (*10*), a time course similar to that for T_α mRNA. It is thus possible that the control of photoreceptor shedding and T_α synthesis may be related. Although the mechanism of the control of photoreceptor shedding is not completely understood, dopamine (*11*) and melatonin (*12*) may be involved. The demonstration of dopamine receptors on photoreceptors (*13*) and the ability of dopamine to control light-adaptive movements of photoreceptors in lower vertebrates (*14*) support a role for dopamine in light- and dark-adaptive responses of photoreceptors.

At night and after short periods of darkness photoreceptor rods are much more sensitive to light than they are during the day (*15*). Although our data do not distinguish direct effects of illumination from possible circadian events (both are involved in the control of photoreceptor sensitivity), photoreceptors may alter the distribution of T_α as a mechanism for controlling sensitivity. That is, during the day T_α levels may be limiting in the outer segments, leading to low photoreceptor sensitivity. At night, T_α is transported to the outer segments from the inner segments, possibly leading to an increase in sensitivity. By analogy, other systems that utilize G proteins (for example, hormone and neurotransmitter receptors) may alter the expression and subcellular distribution of these proteins as a means of regulating receptor sensitivity.

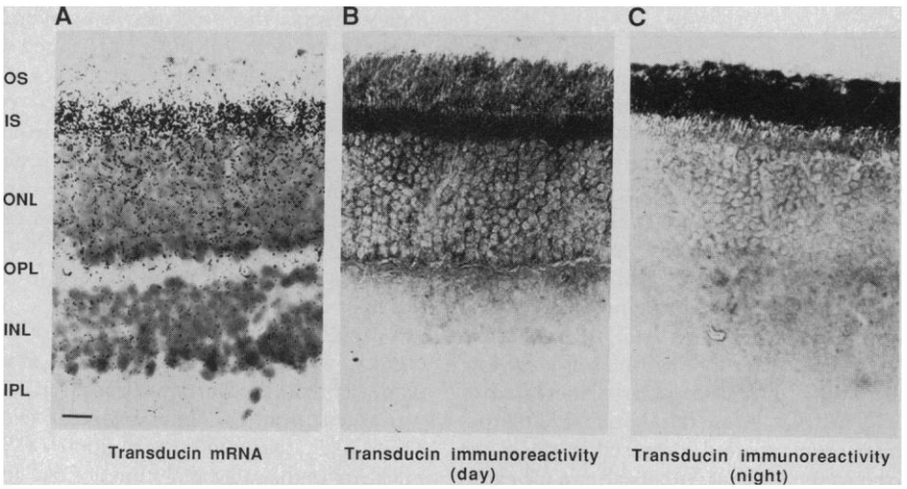

Fig. 1. Distribution of transducin mRNA and transducin immunoreactivity in rods. (**A**) Transducin mRNA was localized by in situ hybridization histochemistry with a synthetic [^{35}S]oligodeoxynucleotide probe. In order to localize bound probe, sections were dipped in emulsion and exposed for 4 weeks. Transducin immunoreactivity was localized by the PAP method with a polyclonal antibody, raised to purified T_α in sections of rat retinas removed during the day (**B**) and night (**C**). Scale bar, 35 μm. Abbreviations: OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; and IPL, inner plexiform layer.

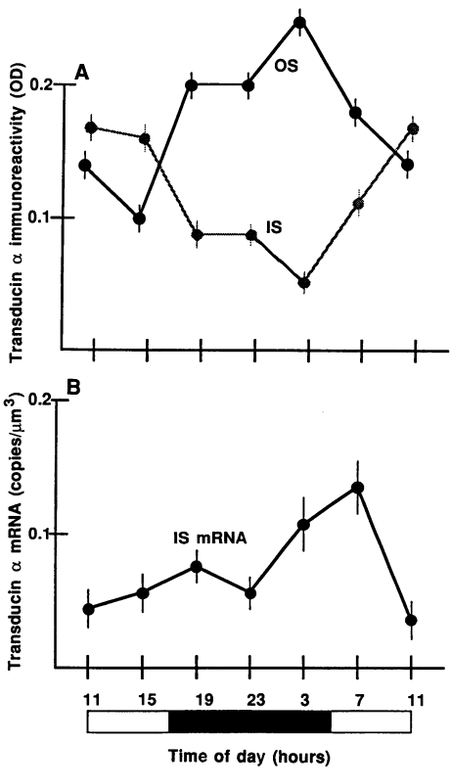

Fig. 2. Diurnal rhythm of transducin immunoreactivity and transducin mRNA. Twenty-eight rats were entrained to a 12-hour light-dark cycle; lights were turned on at 5:00 a.m. (5 hours) and turned off at 5:00 p.m. (17 hours). Four rats were killed every 4 hours. Two sections separated by 200 μm were taken from each retina and processed for measurement of (**A**) transducin immunoreactivity in the outer segment (OS) and the inner segment (IS) and (**B**) transducin mRNA (IS mRNA). Transducin immunoreactivity was measured by PAP immunocytochemistry, with the reaction product quantitated densitometrically. Data points represent the means ± SEM (n = 16; data from each section were treated as independent observations since variation was independent of the animal and retina). Transducin mRNA was measured by in situ hybridization histochemistry (quantitated autoradiographically). Data points represent the means ± SEM (n = 8; data from each slide, two sections per slide, were treated as independent observations since variation was independent of the animal and retina; covariance was observed for sections mounted on the same slide).

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2. After fixation in 4% formaldehyde, slide-mounted sections were processed for immunohistochemistry with the PAP method [L. A. Sternberger, *Immunocytochemistry* (Wiley, New York, ed. 2, 1979)]. The sections were incubated for 24 hours at 4°C in T_α antiserum (supplied by A. Spiegel) at a dilution of 1:1000. The sections were incubated (30 minutes) sequentially in a goat antibody to rabbit immunoglobin G (1:100) and rabbit PAP (1:1000) (Accurate Chemical) at room temperature. Before the incubation in each reagent, the sections were rinsed in phosphate-buffered saline and incubated for 30 minutes in 3% normal goat serum. Because the rat retina contains very few cones, we were not able to determine if cones were labeled with this antibody.

In the monkey retina we have observed an exclusive labeling of rods [L. V. Cohen, W. S. Young, III, M. R. Brann, *Soc. Neurosci. Abstr.* **12**, 630 (1986)] consistent with the work of others (*3*).

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4. The immunohistochemical reaction product was developed by incubation of all sections simultaneously in 0.05% diaminobenzidine hydrochloride (Sigma) and 0.01% hydrogen peroxide for 7 minutes. Optical densities of regions of retinal sections were evaluated with bright-field illumination through a microscope (Ortholux, Leitz) at ×320 with a video camera–based image analysis system (Loats Associates, Westminister, MD). All optical density readings were corrected for background (which was determined in an unlabeled region of the inner retina). Within each microscopic field, three regions over the inner segments and three regions over the outer segments were measured. To reduce sampling error, two microscopic fields taken from separate sections (separated by at least 200 μm) were quantitated from each retina.
5. In situ hybridization histochemistry was performed with procedures previously employed for localization and measurement of neuropeptide mRNA's [W. S. Young III, E. Mezey, R. E. Siegel, *Neurosci. Lett.* **70**, 198 (1986); W. S. Young III, T. I. Bonner, M. R. Brann, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9827 (1986)]. We modified the published procedures by increasing the hybridization temperature from 22°C to 37°C and by quantitating autoradiograms from x-ray film under bright-field illumination with the same image analysis system as that used to quantitate immunoreactivity. Background values were determined by comparison of inner layers of the retina to sections incubated with an equivalent concentration of tyrosine hydroxylase cDNA probe. This probe was prepared in the same way as the T_α cDNA probe and was complementary to bases 1441 to 1488 of rat tyrosine hydroxylase [B. Grima *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 617 (1985)]. These values were equivalent and always less than 10% of those obtained with specific labeling. On each section, the optical density over three randomly selected domains, selected without knowledge of section grouping, were averaged to reduce sampling error. Standards were prepared by mixing known amounts of [^{35}S]deoxyadenosine in brain paste. These standards were subsequently sectioned and exposed to film with the retinal sections. Optical densities were converted to copies per cubic micrometer with nonlinear regression of the data from the standards (*7*). Due to the moderate resolution of our autoradiographic technique and the rarity of cones in the rat retina, we were not able to determine if cones were labeled in addition to rods. Recent work [C. L. Lerea *et al.*, *Science* **234**, 77 (1986)] indicates the sequence of our cDNA probe is restricted to rods in the bovine retina, and that cones have a different transducin-like mRNA.
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Site-Specific Nick in the T-DNA Border Sequence as a Result of *Agrobacterium vir* Gene Expression

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The T-DNA transfer process of *Agrobacterium tumefaciens* is activated by the induction of the expression of the Ti plasmid virulence *(vir)* loci by plant signal molecules such as acetosyringone. The *vir* gene products act in trans to mobilize the T-DNA element from the bacterial Ti plasmid. The T-DNA is bounded by 25–base pair direct repeat sequences, which are the only sequences on the element essential for transfer. Thus, specific reactions must occur at the border sites to generate a transferable T-DNA copy. The T-DNA border sequences were shown in this study to be specifically nicked after *vir* gene activation. Border nicks were detected on the bottom strand just after the third or fourth base (± one or two nucleotides) of the 25–base pair transfer-promoting sequence. Naturally occurring and base-substituted derivatives of the 25–base pair sequences are effective substrates for acetosyringone-induced border cleavage, whereas derivatives carrying only the first 15 or last 19 base pairs of the 25–base pair sequence are not. Site-specific border cleavages occur within 12 hours after acetosyringone induction and probably represent an early step in the T-DNA transfer process.

Agrobacterium tumefaciens HARBORing a Ti (tumor-inducing) plasmid induces tumors in plants by transferring and integrating a specific DNA segment, T-DNA, into the plant nuclear genomes [reviewed in (*1*)]. The T-DNA transfer process is mediated by products of the Ti plasmid virulence *(vir)* (*2*) and chromosome virulence *(chv)* (*3*) loci. While *chv* expression is constitutive, *vir* expression is tightly regulated (*2, 4*). Activation of the *vir* genes is the direct result of the recognition by *Agrobacterium* of signal molecules produced by wounded plant cells (*5*), and this activation initiates the transfer process.

The T-DNA is bounded by imperfect 25–base pair (bp) direct repeats (*1*). While deletion of the left border repeat has no significant effect on oncogenicity (*6*), deletion of the right repeat totally abolishes it (*7–9*). When the orientation of the right border is reversed with regard to its natural orientation, the efficiency of transfer or integration (or both) of the T-DNA is greatly attenuated (*7, 9*). Since only the T-DNA element is found integrated in the plant nuclear genome, specific reactions are expected to occur at the T-DNA borders (especially at the right border) to generate a transferable T-DNA copy. Recent experiments have shown that *vir* induction by acetosyringone (AS), a purified plant signal molecule, results in the production of S1 nuclease–sensitive structures at the T-DNA borders (*10*). The data described below indicate that this S1 sensitivity is due to AS-induced specific single-stranded cleavage within the 25-bp repeat sequence.

Various constructs (Fig. 1A), including a 3.3-kbp Hind III fragment overlapping the right T-DNA border of the nopaline-type Ti plasmid as well as several derivatives of the transfer-promoting 25-bp border sequence (*7*), were tested for their sensitivity to S1 nuclease after AS induction. Border nicks began to occur within 4 hours and reached a maximal level by 12 hours, after the addition of AS to bacterial cultures grown in Murashige and Skoog medium, with sucrose and phosphate (MSSP) (*10*). Thus, the different *Agrobacterium* strains were induced with AS for 12 hours, and total DNA was prepared

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