Localization and quantitation of opsin and transducin mRNAs in bovine retina by in situ hybridization histochemistry

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Oligodeoxynucleotide probes complementary to a portion of bovine opsin mRNA and transducin mRNA were used for in situ hybridization histochemistry. Within the retina, only photoreceptors expressed mRNAs detectable with these probes, and the majority of both mRNAs were in photoreceptor inner segments. More opsin mRNA was detected than transducin mRNA. In the inner segments 0.54 ± 0.05 copies/μm^3 of opsin mRNA and 0.34 ± 0.05 copies/μm^3 of transducin mRNA were detected. In the outer nuclear layer, 0.39 ± 0.06 copies/μm^3 of opsin mRNA and 0.27 ± 0.04 copies/μm^3 of transducin mRNA were detected.

Rhodopsin Transducin Hybridization (Retina) Photoreceptor Guanine nucleotide-binding protein

1. INTRODUCTION

Visual excitation is initiated by the bleaching of the visual pigment rhodopsin by light. Bleached rhodopsin couples to the GTP-binding protein transducin which in turn mediates light-induced activation of the enzyme cGMP-dependent phosphodiesterase. The proteins involved in this cascade of events have been purified and extensively characterized. Rhodopsin consists of an apoprotein, opsin, covalently linked to 11-*cis*-retinal and transducin is a protein consisting of 3 subunits: α, β and γ [1,2]. The α-subunit of transducin is structurally similar to, but not identical to, the α-subunits of N_i and N_o, the GTP-binding proteins which mediate signal transduction by neurotransmitter receptors [1,2]. The β-subunit of transducin may be identical to the β-subunits of N_i and N_o [1–3]. The sequences of the genes encoding human [4] and bovine [5] opsin and the α- [6–8], β- [3] and γ- [9] subunits of bovine transducin have been sequenced. Based on the sequences of opsin, and the α-subunit of transducin, we prepared two ^{35}S-labeled synthetic oligodeoxynucleotide probes for measurement of these mRNAs by quantitative in situ hybridization histochemistry. We report the first anatomical localization and quantitation of opsin and transducin mRNAs in bovine retina.

2. MATERIALS AND METHODS

2.1. *Tissue preparation*

Retinas were dissected from bovine eyes (Trueth & Sons, Baltimore, MD), mounted, frozen on powdered dry ice and stored at −80°C. 12 μm sections were cut and thaw-mounted onto gelatin-coated slides. The sections were then warmed for 2 min at 40°C and stored at −80°C until use.

2.2. *Preparation of probes*

Synthetic oligodeoxynucleotide probes complementary to nucleotides 4139–4185 of opsin [4,5], 1070–1117 of bovine transducin [7,8], and 1441–1488 of rat tyrosine hydroxylase [10] were made by solid-phase synthesis on an Applied Biosystems DNA synthesizer (courtesy of Dr M.J. Brownstein, NIMH). The region chosen for the opsin probe exhibits 100% base sequence homology between the human and bovine genes. The tyrosine hydroxylase probe was used as a con-

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trol for nonspecific binding. The probes were subsequently purified by preparative gel electrophoresis using 8% polyacrylamide and 8 M urea. The purified probes were labeled by tailing the 3′-end using terminal deoxynucleotidyl transferase (BRL) and (α-^{35}S)-labeled deoxyadenosine triphosphate (NEN, >1000 Ci/mmol). The specific activity of the resultant probes was 2050 and 2260 Ci/mmol for rhodopsin and transducin, respectively.

2.3. *In situ hybridization histochemistry*

Hybridizations were performed essentially as described [11]. Briefly, sections were warmed to room temperature for 10 min and then fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 5 min. Sections were rinsed in PBS and incubated in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl (pH 8) for 10 min at room temperature. Sections were then washed with 2 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7) and transferred through 70 (1 min), 80 (1 min), 95 (2 min), and 100% ethanol (1 min); 100% chloroform (5 min); and 100 (1 min) and 95% ethanol (1 min); and air-dried. Hybridizations were performed in 4 × SSC, 50% formamide, 1 × Denhardt's (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 250 μg/ml yeast tRNA, 500 μg/ml sheared single-stranded salmon sperm DNA), and 10% dextran sulfate. 5 × 10^5 dpm of probes in 45 μl buffer were applied to each section. Sections were covered with a parafilm coverslip and incubated for 20 h at room temperature. The coverslips were removed and the sections rinsed in four 15-min changes of 2 × SSC/50% formamide, the first at room temperature and remaining three at 40°C. These 40°C washes were performed at 18°C below the theoretical T_m values of the opsin and transducin probes [12,13]. Finally, sections were washed twice for 1 h at room temperature in 2 × SSC and air dried.

2.4. *Autoradiography*

Autoradiography was performed by one of three methods. First, sections were apposed to X-ray film for 20 h to test hybridizations and check background. This method gave the most rapid results but the lowest resolution. For higher resolution, sections were dipped in NTB3 nuclear emulsion (Kodak) and exposed for 3 days. For quantitative experiments, sections were apposed to emulsion-coated coverslips and exposed for 1 week as described [11,14]. Standards for quantitation were prepared by mixing known amounts of (γ-^{35}S)-labeled deoxyadenosine triphosphate in brain paste. These standards were sectioned and exposed concurrently with the retinal sections [11]. Grain reflectances were measured under dark field illumination using a Loats Associates (Westminster, MD) image analysis system.

2.5. *Data analysis*

Probe concentrations were calculated essentially as in [11]. Briefly, the relationship of grain reflectance to concentration of probe was determined by nonlinear regression of the data from the standards as illustrated in fig.1. The measured radioactivity concentrations (dpm corrected for half-life decay of the ^{35}S) in the standards were converted to probe copy number with the following formula:

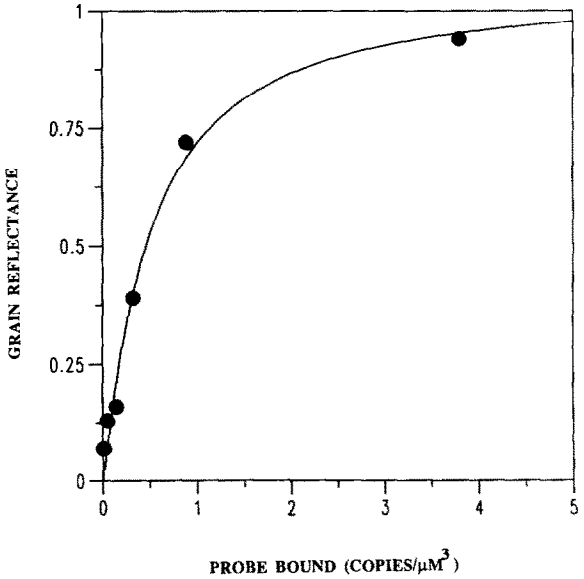

Fig.1. Relationship of grain reflectance to probe concentration. The line is a computer-generated least-squares fit of the data (●) from standards to the function grain reflectance = $ax^b/c/(1 + x^b/c)$: where a = grain reflectance of emulsion at saturation (1.0 ± 0.1), x = probe bound (copies/μm^3), b = a cooperativity constant (1.1 ± 0.1), and c = concentration where the emulsion is half-saturated (0.5 ± 0.2). Experimental values were calculated from measured reflectances using this function (see data analysis).

probe copies/μm^3 = dpm/mg × 1 × 10^{-9} mg/μm^3 × 1/spec. act. (dpm/mol) × 6.02 × 10^{-23} copies/mol (Avogadro's number). Molar equivalents of probes to standards were calculated based on their relative specific activities.

3. RESULTS

Fig.2 shows autoradiograms of sections of bovine retina after hybridization with the probes for opsin and transducin mRNAs. Both probes labeled the outer nuclear layer and the inner segments of the photoreceptors, the latter most intensely. Neither probe labeled the outer plexiform layer or the inner layers of the retina.

In the outer nuclear layer we detected 0.39 ± 0.06 copies/μm^3 of opsin mRNA and 0.27 ± 0.04 copies/μm^3 of transducin mRNA. In the inner segments we detected 0.54 ± 0.05 copies/μm^3 of opsin mRNA and 0.34 ± 0.05 copies/μm^3 of transducin mRNA. Data are means ± SD of determinations from 3 retinae. These data must be considered minimum estimations, as we do not know the amount of mRNA lost during the tissue processing or the efficiency of hybridization.

To test for nonspecific labeling by our opsin and transducin probes, we hybridized retinal tissue with a probe complementary to rat tyrosine hydroxylase mRNA, a mRNA which is not expressed by photoreceptors. This probe had the same length and was synthesized and labeled by the same method as our transducin probe. Only background labeling was observed with this probe, i.e. it labeled the entire retina with the same low density as our opsin and transducin probes labeled the inner layers of the retina. Thus, the labeling observed by our opsin and transducin probes is sequence-dependent. It is unlikely that our transducin probe cross-hybridized with other GTP-binding protein mRNA. First, because no other GTP-binding proteins have been localized to photoreceptors, and second the known structural

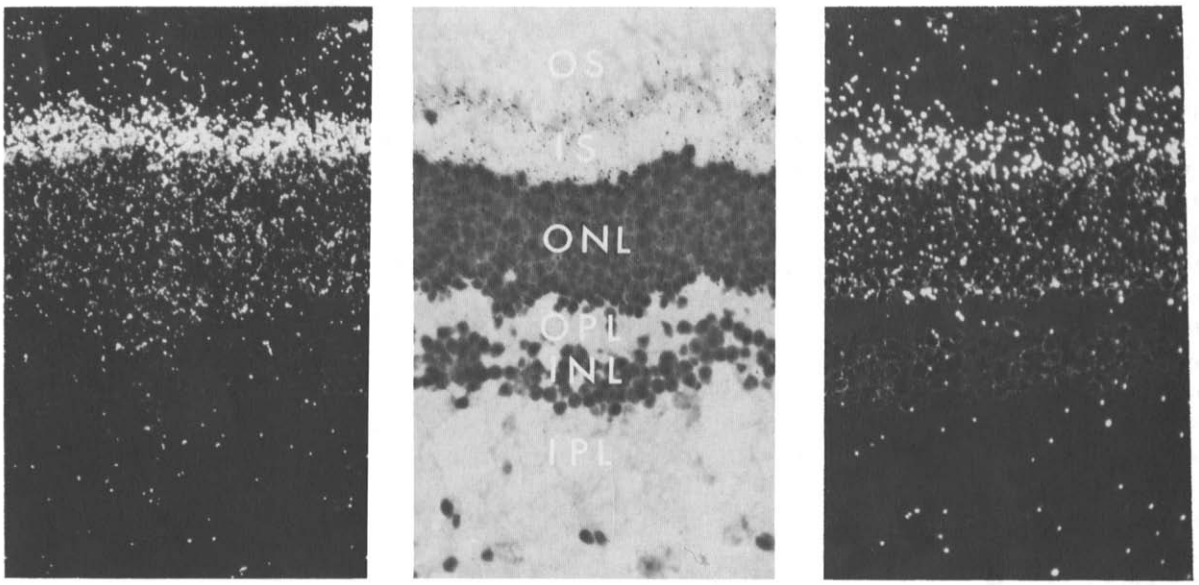

Fig.2. In situ hybridization of opsin and transducin oligodeoxynucleotide probes in bovine retina. Cells were stained with cresyl violet, and autoradiographic localization of mRNAs in dipped sections were performed as described in section 2. Outer segment (OS), inner segment (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL).

homologies between transducin and these proteins is limited [1,2], making cross-hybridization unlikely under conditions of high stringency as used here.

4. DISCUSSION

Immunocytochemical methods have shown that both opsin and transducin are located on photoreceptors, neither protein is present in the inner layers of the retina, and both proteins are highly concentrated in the disk membranes of the outer segments [15,16]. Our observations that mRNAs encoding opsin and transducin are restricted to photoreceptors confirms and extends to the gene level the conclusions of the immunocytochemical studies. Furthermore, these mRNAs are concentrated in the inner segments suggesting that translational activity occurs in this region of the photoreceptors [17]. The higher levels of opsin mRNA than transducin mRNA are consistent with the higher levels of rhodopsin than transducin in disk membranes [16]. Interestingly, while opsin and transducin mRNA are efficiently transported to the inner segments from their site of synthesis in the nuclei of photoreceptors, no mRNA is transported in the other direction toward the synaptic processes in the outer plexiform layer. These data suggest that the polarity in the distribution of proteins within photoreceptors may at least in part be defined by unidirectional transport of mRNAs.

Methods identical to those reported here have been applied to the measurement of mRNAs encoding several neuropeptides, and illustrate the abundance of opsin and transducin mRNAs relative to other mRNAs of known function. Opsin and transducin mRNAs in rods are approx. 10-times as abundant as corticotropin-releasing hormone mRNA in the parvocellular region of the hypothalamic paraventricular nucleus [11], and one-tenth as abundant as vasopressin and oxytocin mRNAs in magnocellular paraventricular and supraoptic nuclei [18].

Our localization and quantitation of opsin and transducin mRNAs in bovine retina illustrates the utility of in situ hybridization histochemistry in the study of retinal function. Currently, we are using this technique to investigate the effects of light and dark on retinal mRNAs.

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