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G protein mRNA mapped in rat brain by *in situ* hybridization

(signal transduction/adenylyl cyclase/inositol phospholipid cycle)

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ABSTRACT **Guanine nucleotide-binding regulatory proteins (G proteins) mediate many receptor-coupled signal transduction events. We have localized in rat brain by *in situ* hybridization the mRNA for the G protein subunits—$G_{\alpha s}$, $G_{\alpha o}$, and G_{β}. Oligonucleotide probes were radiolabeled by a technique that resulted in a probe of defined specific activity and uniform length. mRNA species encoding $G_{\alpha s}$ and G_{β} occur in high densities heterogeneously throughout the brain, especially in large neuronal cell bodies—e.g., hippocampal pyramidal cells, granule cells of the dentate gyrus, hypothalamic nuclei, and neurons of brainstem nuclei and the reticular formation. $G_{\alpha o}$ mRNA has a more limited distribution and abundance, being detectable in the claustrum, endopiriform nucleus, habenula, hippocampal pyramidal cells, granule cells of the dentate gyrus, and cerebellar Purkinje cells.**

Guanine nucleotide-binding regulatory proteins, also designated G proteins, couple receptors for neurotransmitters, hormones, growth factors, light, and other stimuli to biochemical second messenger systems and to ion channels (1, 2). Receptor modulation of intracellular cAMP levels involves the G proteins, G_s and G_i, which, respectively, stimulate and inhibit adenylyl cyclase activity. The inositol phospholipid second messenger system also requires a G protein to activate the cleavage of phosphatidylinositolbisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol. The molecular identity of the G protein associated with the inositol phospholipid cycle has not been definitively established, although localization studies suggest a role for G_o (3). G proteins have also been implicated in the linkage of neurotransmitter receptors directly to potassium and calcium channels (4–8).

Structurally, G proteins exist as cytoplasmic-facing, membrane-associated heterotrimers of α, β, and γ subunits. The β and γ subunits form a stable complex ($\beta\gamma$), which is closely similar if not identical for all G proteins. In contrast, the α subunit differs among the various G proteins and, by convention, functionally defines individual types of G protein (e.g., α_s subunit for G_s, α_i subunit for G_i, and α_o subunit for G_o, etc.). In most systems, the α subunit appears to directly regulate second messenger systems or ion channels (1, 8–12), although some recent evidence suggests that $\beta\gamma$ subunits may independently modulate adenylyl cyclase activity and ion-channel function (5, 13).

The requirement for G proteins in many types of neurotransmission is widely appreciated, yet the integrative role of the various G protein species in brain function remains unclear. Discrete localizations of biochemical systems within the brain often provide insight into neural function. For example, similarities in the immunohistochemical mapping of G_o and the neuroanatomical distribution of protein kinase C, a component of the inositol phospholipid cycle, suggest a role for G_o protein in this second-messenger pathway (3). Recently, cDNA clones encoding the α and β subunits for several G proteins have been isolated and characterized (see refs. 1 and 14). In the present study, we have used oligonucleotide probes specific for the α subunits of G_s ($G_{\alpha s}$) and G_o ($G_{\alpha o}$) as well as the G_{β} subunit to map by *in situ* hybridization the spatial expression of G protein mRNA throughout the rat brain.

MATERIALS AND METHODS

Rats (175–250 g, Sprague–Dawley) anesthetized with sodium pentobarbital were perfused via the left cardiac ventricle with 200 ml of 50 mM sodium phosphate (pH 7.5) containing 100 mM NaCl, followed by 200 ml of 50 mM sodium phosphate (pH 7.5) containing 0.3 M sucrose. Brains were removed and mounted in a 1:1 mixture of bovine brain paste/Tissue-tek (Miles), frozen rapidly at −40°C and then stored at −20°C. Sections (10 μm) were cut at −18°C and thaw mounted onto gelatin/chrome alum-coated slides. Before use, sections were warmed to room temperature and "pre-treated." Sections were fixed in 4% formaldehyde for 5 min at room temperature and, after rinsing in 2× SSC (0.3 M NaCl/0.05 M sodium citrate), were acetylated by incubation in 0.25% acetic anhydride in 0.1 M triethanolamine/0.15 M NaCl (pH 8.0) for 10 min at room temperature. The sections were briefly rinsed in 2× SSC, dehydrated and delipidated by an ethanol/chloroform series: 70% (1 min), 80% (1 min), 95% (2 min), and 100% (1 min) ethanol; 100% chloroform (5 min); 100% (1 min) and 95% (1 min) ethanol; and dried on a slide warmer (45°C).

Oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) Model 380B synthesizer. The oligonucleotide probe for $G_{\alpha o}$ was directed to a unique 3′ untranslated region (nucleotides 1881–1926) while the $G_{\alpha s}$ probe was targeted to a divergent portion of the coding region (nucleotides 476–521) (14). The G_{β} probe was directed to a coding sequence unique to the rat $G_{\beta 1}$ species and corresponds to the N-terminal 16 amino acids (nucleotides +3 to +48) (R.R.R. and Paul Feinstein, unpublished data). This probe is not expected to recognize the less abundant $G_{\beta 2}$ species (15). The oligonucleotide probe for olfactory marker protein (OMP) was centered around the ATG start codon (nucleotides −29 to +17) (B.L.L., R.R.R., and Ernest Barbosa, unpublished data). The oligonucleotide probes were constructed by a technique involving the synthesis of a 58-mer oligonucleotide containing 46 nucleotides of "target-specific" sequence followed by an additional 12 nucleotides—the

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Abbreviations: G proteins, guanine nucleotide-binding regulatory proteins; CAR, common annealing region; OMP, olfactory marker protein.

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"common annealing region" (CAR) (Fig. 1). A second oligonucleotide (22-mer), consisting of $(dT)_{10}$ followed by the sequence complementary to the CAR region, was annealed to the 58-mer oligonucleotide and the 3′ OH adjacent to the $(dT)_{10}$ extended with dATP[α-^{35}S] ($\approx$1300 Ci/mmol; 1 Ci = 37 GBq) by the Klenow fragment of DNA polymerase I. Reactions were performed in the presence of 100 μM dCTP to prevent 3′–5′ exonucleolytic digestion of the annealed CAR region. After the extension reaction, radiolabeled oligonucleotide was phenol extracted and precipitated with 2.5 M NH_4OAc/70% ethanol to remove free dATP[α-^{35}S], before resuspension in "hybridization solution" [4× SSC/50% deionized formamide/1× Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/500 μg of salmon sperm DNA per ml/250 μg of tRNA per ml/10 mM dithiothreitol/10% dextran sulfate]. Additional oligonucleotide probes for $G_{\alpha s}$ and G_o (labeled by 3′ tailing) were obtained from New England Nuclear–Dupont.

Oligonucleotide probe (1×10^6 cpm; $\approx$13,000 Ci/mmol) was applied to each section in 50 μl of "hybridization solution," covered with a parafilm coverslip and allowed to hybridize for 18–24 hr at 27°C. After hybridization, the parafilm coverslips were removed under 2× SSC and the sections were dipped twice in 2× SSC to remove excess probe. The sections were then washed at room temperature in 2× SSC for 30 min, at 55°C in 1× SSC for 4 × 15 min, and finally at room temperature in 2× SSC for an additional 30 min. To dry, sections were dipped three times in 300 mM NH_4OAc to remove NaCl and dried under a blow dryer followed by 30 min on a slide warmer set at 55°C. Dried sections were apposed to film (βmax, Amersham; 1–10 days at 4°C) or dipped in nuclear emulsion (NTB-3, Kodak; 2–6 weeks at 4°C) to generate autoradiograms.

RESULTS

In situ hybridization histochemistry can be performed with various types of radiolabeled probes—e.g., cDNA clones, ribonucleotide probes enzymatically transcribed from cDNA clones, or chemically synthesized oligonucleotide probes. Because of the high degree of conservation among the G proteins at the amino acid and nucleotide levels, we have generated specific oligonucleotides directed to regions most divergent in sequence to distinguish among the various members of this family. The ^{35}S-labeled oligonucleotide probes used in this study, which we refer to as CAR probes, are constructed in an unusual fashion (see *Materials and Methods*, Fig. 1). These offer several advantages over oligonucleotide probes radiolabeled by 3′-tailing reactions or the "syn-probe" technique (16). The CAR probes are of a defined, uniform length and constant specific activity and, compared to syn-probe oligonucleotides, are simpler to prepare. Oligonucleotide probes radiolabeled by 3′ tailing with terminal deoxynucleotidyl transferase have a single-stranded homopolymeric tail of variable length (and variable specific activity), which significantly increases the opportunity for nontarget hybridization. By contrast, the radiolabeled portion of CAR probes is maintained as uniform length double-stranded DNA and, thus, is less susceptible to nonspecific interactions.

To verify the specificity of these probes, we conducted RNA blot analyses with rat brain mRNA. Each of the probes recognizes specific bands corresponding to those previously demonstrated to represent the mRNA encoding their respective subunit proteins (14). As a prelude to *in situ* hybridization analyses, we examined the variation in $G_{\alpha s}$, $G_{\alpha o}$, and G_β message expression in dissected brain regions by quantitative slot blot hybridization. Significant variations in levels of $G_{\alpha s}$ message are apparent, with the highest levels in the pituitary gland; successively lower levels in the medulla, hypothalamus, midbrain, hippocampus, pons, cerebral cortex, and thalamus; and the lowest levels in the caudate putamen (data not shown).

For *in situ* hybridization studies, several control experiments were performed to confirm specificity. As a measure of nonspecific hybridization, we used an oligonucleotide probe directed to mRNA encoding OMP, a protein whose abundant expression is restricted to primary olfactory neurons in the olfactory epithelium and absent from the brain, with the exception of the olfactory bulb (17). By using the OMP probe, only very faint densities of autoradiographic grains are detected in several brain areas (e.g., pyramidal cells of the piriform cortex, hippocampal formation, and cerebellar granule cells), presumably reflecting nonspecific signal. For all probes used, cohybridization with 100-fold excess nonradioactive oligonucleotide reduces the autoradiographic signal to the levels observed with OMP probe. Double-stranded versions of each of the G protein oligonucleotide probes were constructed by the addition of all four dNTPs after the initial radiolabeling reaction. Likewise, these probes, which lack a single-stranded target-specific sequence, demonstrate grain densities similar to those for the OMP control. In brain sections treated with RNase A (100 μg/ml) to degrade RNA present in the tissue, essentially no autoradiographic signal is observed for any of the probes. Interestingly, observed grain density after RNase A treatment is lower than in control experiments with double-stranded probes, excess unlabeled probe, or OMP probe. This suggests that the low nonspecific signal observed in the latter control experiments involves interaction with tissue RNA. These results support the application of the OMP oligonucleotide probe as a general measure of background nonspecific signal in rat brain sections for the probes used in this study. Oligonucleotide probes were also constructed for three separate G_i-like subunits (G_{i1}, G_{i2}, and G_{i3}) recently cloned from rat tissues (14). *In situ* hybridization studies with these probes reveal discrete distributions upon long periods of film exposure. However, these grain densities are similar to those in sections hybridized with OMP probe, making it difficult to ensure the authenticity of hybridization signals.

The abundance and distribution of mRNA for the α subunits of G_s and G_o as well as the G_β subunit have been evaluated for numerous levels of rat brain coronal sections (Fig. 2). The discrete localizations of the various probes differ markedly. At anterior forebrain levels, $G_{\alpha s}$ message is most highly concentrated in the septal nuclei, diagonal band of Broca, and in pyramidal cells of the piriform cortex, with

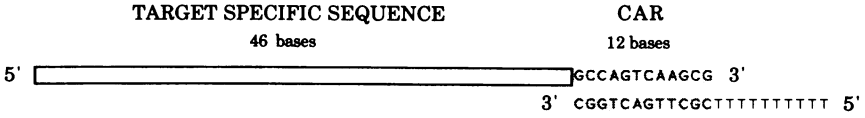

FIG. 1. Scheme of CAR oligonucleotide probe construction. CAR probes consist of two annealed oligonucleotides sharing a complementary sequence (called the common annealing region; CAR). The first oligonucleotide contains a 46-nucleotide target-specific sequence, while the second provides a tail of $(dT)_{10}$, which serves as a template for quantitative extension by the Klenow fragment of DNA polymerase with dATP[α-^{35}S] resulting in a probe of defined length and specific activity.

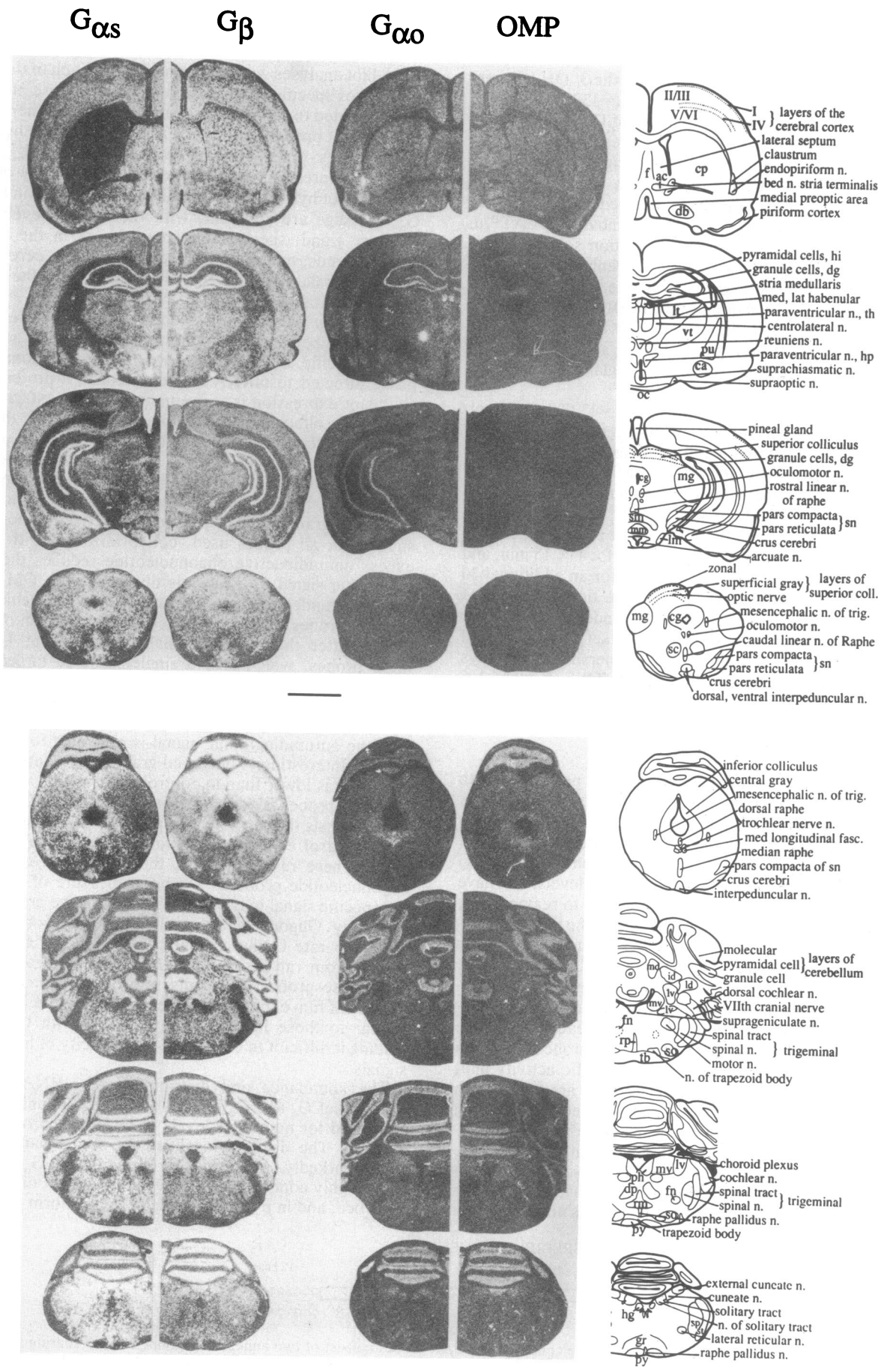

FIG. 2. *(Legend appears at the bottom of the opposite page.)*

substantial labeling in other cellular layers of the cerebral cortex. In marked contrast, the caudate putamen exhibits extremely low densities of $G_{\alpha s}$ hybridization, comparable to those of the OMP control, although moderate levels of $G_{\alpha s}$ message are detected in the globus pallidus. The $G_{\alpha o}$ probe labels only the pyramidal cell layer of the piriform cortex, the claustrum and the endopiriform nucleus, areas also labeled by the $G_{\alpha s}$ probe. The G_{β} probe demonstrates more extensive hybridization with a pattern somewhat similar to that of the $G_{\alpha s}$ probe except for marked labeling of the caudate putamen.

In the diencephalon, the bed nucleus of the stria terminalis and the central nucleus of the amygdala show prominent $G_{\alpha s}$ hybridization but low levels of $G_{\alpha o}$ and G_{β} messages. Within the hypothalamus, intense grain density associated with $G_{\alpha s}$ message is apparent in the paraventricular, supraoptic, and suprachiasmatic nuclei, while moderate levels of the G_{β} message are apparent in these areas and no $G_{\alpha o}$ message is detectable. In the hippocampal formation, substantial grain density is evident for all three probes over the pyramidal cell layer of the hippocampus and the granule cell layer of the dentate gyrus. Quinolinic acid injections abolish the autoradiographic grains, establishing that these occur in intrinsic neurons (data not shown).

More caudally in the diencephalon, the corticomedial and basolateral nuclei of the amygdala contain $G_{\alpha s}$ message in lower levels than the central nucleus of the amygdala. Lesser amounts of G_{β} message are apparent in these nuclei, with no signal evident for $G_{\alpha o}$. Similarly, the dorsal hypothalamic area possesses substantial amounts of $G_{\alpha s}$ but not G_{β} or $G_{\alpha o}$ message. The arcuate nucleus of the hypothalamus has extremely high densities of $G_{\alpha s}$ message but negligible levels of $G_{\alpha o}$ and modest amounts of G_{β} message. The pineal gland exhibits the most intense density of $G_{\alpha s}$ message in this study, yet has negligible levels of G_{β} and $G_{\alpha o}$ message. The habenula, which contains substantial amounts of G_{β} message, is one of the few areas of the brain with similar densities for $G_{\alpha o}$ and $G_{\alpha s}$ message.

In the midbrain, the interpeduncular nucleus, a prominent target nucleus for projections from the habenula, possesses substantial levels of $G_{\alpha s}$ and G_{β} message. The periaqueductal gray and superficial aspect of the superior colliculus exhibit substantial amounts of $G_{\alpha s}$ message, a moderate amount of G_{β} message and negligible $G_{\alpha o}$ message. Like the superior colliculus, the superficial aspect of the inferior colliculus possesses substantial message levels for $G_{\alpha s}$ and G_{β}, but not $G_{\alpha o}$. Similarly the zona compacta of the substantia nigra displays moderate amounts of $G_{\alpha s}$ and G_{β} message. The dorsal raphe and median raphe nuclei, which are the major sources of serotonin projections in the brain, possess very high levels of $G_{\alpha s}$ and G_{β} message and modest amounts of $G_{\alpha o}$ message.

Throughout the midbrain and brainstem, reticular formation structures possess substantial levels of message for $G_{\alpha s}$, more modest levels for G_{β}, and essentially no significant levels for $G_{\alpha o}$. Brainstem nuclei with intense grain densities for $G_{\alpha s}$ and G_{β} message include the pontine nuclei, locus coeruleus, the vestibular nuclei, and cochlear nuclei. $G_{\alpha s}$ and G_{β} message are abundant in several vagal nuclei, in the nucleus ambiguus, solitary nucleus, hypoglossal nucleus, and inferior olivary complex.

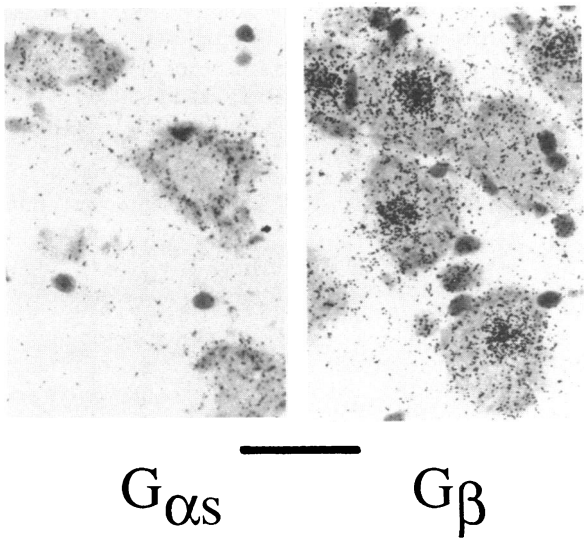

FIG. 3. Cellular distribution of hybridization signal for $G_{\alpha s}$ and G_{β}. Photomicrographs are from nuclear emulsion dipped slides of brain sections hybridized with the appropriate oligonucleotide probe. Sections were stained with toluidine blue after emulsion development (NTB-3, Kodak; 30-day exposure at 4°C). The cell bodies are from the pontine central gray neuronal field. (Bar = 20 μm.)

The cerebellum is the one area with noticeable grain densities for the OMP probe. The autoradiographic signal is most concentrated over the granule cell layer and presumably reflects nonspecific interactions of the oligonucleotide probe with the RNA content of this dense population of cells. The modest grain density observed for the $G_{\alpha o}$ probe in the granule cell layer of the cerebellum does not consistently exceed that apparent with the OMP probe and thus probably represents nonspecific signal. By contrast, the substantially higher density of G_{β} and $G_{\alpha s}$ probe-associated grains over the granule cell layer of the cerebellum presumably represents authentic message hybridization. A discrete layer of $G_{\alpha o}$ message-associated grains overlies the cerebellar Purkinje cells, and examination of emulsion-coated slides at higher magnification confirms labeling of these neurons. A similar analysis for $G_{\alpha s}$ and G_{β} probes reveals intense labeling of cerebellar Purkinje cells for G_{β}, while $G_{\alpha s}$-associated grains are nearly absent from these neurons.

Analysis of the cellular distribution of exposed grains over large neuronal cell bodies reveals distinct differences between $G_{\alpha s}$ and G_{β} (Fig. 3). Message for $G_{\alpha s}$ is predominantly restricted to the cytoplasm, similar to that seen for several other neuronal messages. In contrast, grains representing G_{β}

FIG. 2. *(on opposite page)* Distribution of mRNA for $G_{\alpha s}$, G_{β}, and $G_{\alpha o}$ in rat brain. Autoradiograms of brain sections are presented in a rostral–caudal progression, with each level representing a series of serial coronal sections. Increased whiteness in the photographs, printed directly from βmax film (10-day exposure at 4°C), corresponds to higher levels of probe hybridization. Identical autoradiographic and photographic exposures were used for all photographs. As a measure of background signal for these probes, sections hybridized with OMP oligonucleotide probe are provided for comparison at each level. Camera lucida drawings of the levels presented are labeled for reference. ac, Anterior commissure; ca, central amygdaloid nucleus; cg, central gray; coll., colliculus; cp, caudate putamen; db, horizontal nucleus of diagonal band of Broca; dg, dentate gyrus; dp, dorsal paragigantocellular nucleus; f, fornix; fasc., fasciculus; fn, facial nerve nucleus; gr, gigantocellular reticular nucleus; hg, hypoglossal nucleus; hi, hippocampus; hp, hypothalamus; id, intermediate deep cerebellar nucleus; lat, lateral; ld, lateral deep cerebellar nucleus; lm, lateral mammillary nucleus; lt, laterodorsal nucleus of thalamus; lv, lateral vestibular nucleus; med, medial; md, medial deep cerebellar nucleus; mg, medial geniculate; mm, medial mammillary nucleus; mv, medial vestibular nucleus; n., nucleus; oc, optic chiasma; ph, prepositus hypoglossal nucleus; py, pyramid; pu, putamen; rm, raphe magnus; rp, raphe pallidus; sc, superior cerebellar peduncle; sm, supramammillary nucleus; sn, substantia nigra; so, superior olivary nucleus; sp, spinal nucleus of trigeminal; st, spinal tract of trigeminal; tb, trapezoid body; th, thalamus; trig., trigeminal; vt, ventroposterior nucleus of thalamus. (Bar = 3 mm.)

mRNA hybridization are largely confined to the nucleus. A similar pattern of nuclear hybridization is seen for a number of large developmentally regulated transcripts in *Drosophila*, where this pattern represents the accumulation of unprocessed mRNA precursor in the nucleus (18). Preliminary evidence suggests that G_β mRNA transcripts may undergo complex processing (B. Birren, M. Simon, R.R.R., Ron Sosnowski, and Paul Feinstein, unpublished observations).

DISCUSSION

Several lines of evidence ensure that the observed autoradiographic localizations validly reflect the distribution and relative abundance of mRNA encoding $G_{\alpha s}$, $G_{\alpha o}$, and G_β proteins. In RNA blot analyses, each oligonucleotide probe interacts only with mRNA species corresponding to the known message(s) for each G protein subunit. Slot blot analyses exhibit similar regional distributions to those observed by *in situ* hybridization. The depletion of grain density by RNase A treatment, use of a double-stranded oligonucleotide probe, and cohybridization with nonradioactive probe provide compelling evidence for the specificity of the *in situ* hybridization procedure. Moreover, the discrete and characteristic differences in localization observed between the various G protein probes also indicate specificity. Equivalent distribution patterns were observed for *in situ* hybridization studies with commercially available oligonucleotide probes for $G_{\alpha s}$ and $G_{\alpha o}$ (New England Nuclear–DuPont) labeled by 3′ tailing and targeted to different, less divergent sequence regions. A recent report, with oligonucleotide probes directed to regions of the mRNA distinct from the CAR probes of this study, described a limited mapping for $G_{\alpha s}$, $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha o}$ mRNAs in rat brain (19). Their data for $G_{\alpha s}$ and $G_{\alpha o}$ message largely correspond with our results.

For the G_α subunits, both slot blot analysis and *in situ* hybridization reveal highest densities throughout the brain for $G_{\alpha s}$, lowest levels for $G_{\alpha i}$, and intermediate values for $G_{\alpha o}$. By contrast, steady-state levels of protein for $G_{\alpha o}$ in brain are ≈10 times those for $G_{\alpha s}$ and 5 times those for $G_{\alpha i}$ (20, 21). Since mRNA levels parallel synthetic rates for most proteins, our findings suggest that $G_{\alpha s}$ protein turns over more rapidly than $G_{\alpha o}$ or $G_{\alpha i}$.

The pattern of G_β message localization is less discrete than for $G_{\alpha s}$ or for $G_{\alpha o}$. For the most part, G_β message distribution resembles that of $G_{\alpha s}$. Since the G_β subunit is thought to be used in common by all G proteins, one might expect its mRNA distribution to parallel the collective abundance of all the G_α mRNAs, a quantity largely influenced by $G_{\alpha s}$ message. The predominantly nuclear partitioning of G_β message may reflect a longer residence time in the nucleus than for other G protein messages. Conceivably, G_β message passage from nucleus to cytoplasm regulates the level of G_β mRNA and, therefore, G_β subunit protein. This regulation could modulate the function of all G protein systems by moderating the abundance of $G_{\alpha\beta\gamma}$ complexes—in essence, affecting the tonality of the system. If the processing of primary G_β transcripts is complex and regulated, this regulation may well represent the point of cellular coordinate regulation of G protein function.

Prominent $G_{\alpha o}$ message expression occurs in only a few areas, such as the claustrum, endopiriform nucleus, the pyramidal cell layer of the piriform cortex, pyramidal cells of the hippocampus and granule cell layer of the dentate gyrus, the habenula, and the Purkinje cells of the cerebellum. These mRNA localizations are consistent with the distribution of $G_{\alpha o}$ protein determined by immunohistochemistry (3).

Immunohistochemical maps for the $G_{\alpha s}$ protein have not yet been determined. $G_{\alpha s}$ protein transduces receptor-mediated stimulation of adenylyl cyclase activity. Adenylyl cyclase has been mapped in the brain by autoradiography with [^{3}H]forskolin, which binds to the activated adenylyl cyclase–$G_{\alpha s}$ complex (22). The localizations of adenylyl cyclase and message for $G_{\alpha s}$ differ markedly, with highest levels of adenylyl cyclase in the caudate putamen, which possesses the lowest levels of $G_{\alpha s}$ message in the brain. If G_s protein levels were to parallel adenylyl cyclase distribution, then posttranscriptional regulation of $G_{\alpha s}$ expression would presumably vary regionally. Alternatively, adenylyl cyclase may not couple exclusively through $G_{\alpha s}$, or $G_{\alpha s}$ in other areas of the brain may effect transduction by different mechanisms. Conceivably, in some neurons $G_{\alpha s}$ may be involved more in coupling receptors to ion channels than in stimulating adenylyl cyclase. Interestingly, the brainstem nuclei enriched in $G_{\alpha s}$ message are more closely associated with amino acid transmitters that typically act through ion channels rather than with adenylyl cyclase.

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