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Localization of glucocorticoid receptor mRNA in the male rat brain by *in situ* hybridization

(steroid hormone receptor/rat brain/regional distribution)

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ABSTRACT **The localization and distribution of mRNA encoding the glucocorticoid receptor (GR) was investigated in tissue sections of the adult male rat brain by *in situ* hybridization and RNA blot analysis. GR mRNA levels were measured by quantitative autoradiography with ^{35}S- and ^{32}P-labeled RNA probes, respectively. Strong labeling was observed within the pyramidal nerve cells of the CA1 and CA2 areas of the hippocampal formation, in the granular cells of the dentate gyrus, in the parvocellular nerve cells of the paraventricular hypothalamic nucleus, and in the cells of the arcuate nucleus, especially the parvocellular part. Moderate labeling of a large number of nerve cells was observed within layers II, III, and VI of the neocortex and in many thalamic nuclei, especially the anterior and ventral nuclear groups as well as several midline nuclei. Within the cerebellar cortex, strong labeling was observed all over the granular layer. In the lower brainstem, strong labeling was found within the entire locus coeruleus and within the mesencephalic raphe nuclei rich in noradrenaline and 5-hydroxytryptamine cell bodies, respectively. A close correlation was found between the distribution of GR mRNA and the distribution of previously described GR immunoreactivity. These studies open the possibility of obtaining additional information on *in vivo* regulation of GR synthesis and how the brain may alter its sensitivity to circulating glucocorticoids.**

Adrenal steroids have a wide range of actions in the central nervous system, where they have been shown to affect growth and differentiation of nerve cells as well as mental state, perception, learning, and sleep (for a review, see ref. 1). Glucocorticoids, as well as other steroid hormones, exert at least a part of their action via binding to specific intracellular receptor proteins, acting as modulators of the transcriptional activity of specific networks of genes (for a review, see ref. 2). Corticosterone binds to two distinct receptor systems in the rat brain (4)—the mineralocorticoid–receptor-like type I receptor and the classical glucocorticoid receptor (GR). Recently, it has been possible to map out GR immunoreactive nerve cell populations (5–9) and glucocorticoid recognition sites (10, 11) in many areas of the central nervous system by means of mouse monoclonal antibodies against the rat liver GR (12) and specific GR radioligands, respectively. High levels of glucocorticoid binding (11) and strong GR immunoreactivity (5, 8) were found within the monoaminergic neurons, in the hippocampal formation, in the corticotropin-releasing factor immunoreactive neurons, and in the mediobasal hypothalamus.

Studies performed with ligand binding and quantitative immunocytochemistry techniques indicate that cellular GR levels vary as a result of, e.g., endocrine manipulations (13), stress (14, 15), and aging (16, 17). By means of an *in situ* hybridization technique, it has been possible in the present paper to detect and map out the distribution of GR mRNA in the male rat brain. These results make it possible to study the regulation of GR synthesis in discrete nerve cell populations of the brain.

MATERIALS AND METHODS

Animals. Male specific pathogen-free Sprague–Dawley rats (body weight, 150–200 g) were used. They were kept under a regular day and night cycle (lights on at 6 a.m. and off at 6 p.m.) and were given food pellets and water ad libitum.

Probes. A 700-base-pair *Pst* I/*Eco*RI rat GR cDNA corresponding to the 3′ portion of the coding region was isolated from pRM9 (18) and subcloned into the pGEM1 vector (Promega Biotec, Madison, WI). RNA probes were produced from the sense and antisense strands of the fragment using [^{32}P]GTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) for RNA blot analysis and ^{35}S-labeled UTP (1000 Ci/mmol; NEN) for *in situ* hybridization analysis, generating probes of a specific activity of $\approx 3 \times 10^9$ cpm per μg of RNA.

Isolation of RNA and RNA Blot Analysis. The rats were decapitated and selective brain regions were immediately dissected out. Total RNA was prepared according to Chromczinski and Sacci (19). RNA (20 μg) was separated on 0.9% (wt/vol) agarose/formaldehyde gels and blotted onto nitrocellulose filters (20). Hybridization and washing conditions were according to Okret *et al.* (21). The relative mRNA levels were evaluated by measurements of the mean gray tone values on autoradiograms with an IBAS image analyzer equipped with a Bosch video camera.

***In Situ* Hybridization.** The method is a modification of the protocol developed by Brahic and Haase (22). Rats were anesthetized with pentobarbital (60 mg/kg i.p.) and perfused with ice-cold saline via the ascending aorta. The brains were dissected out and immediately frozen with powdered dry ice. Cryostat sections (10 μm) were thawed onto poly-L-lysine-coated slides and air-dried for 5 min followed by fixation for 30 min in 4% paraformaldehyde/phosphate-buffered saline (PBS), pH 7.4. After rinsing in PBS (pH 7.4) the sections were stored in 70% ethanol at 4°C or were taken directly for permeabilization with 0.1 M HCl for 10 min. The slides were then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 20 min followed by prehybridization in a humid chamber at 37°C for 1–2 hr with a solution containing 50% formamide, 300 mM NaCl, 50 mM Tris·HCl (pH 7.6), 5 mM

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Abbreviations: GR, glucocorticoid receptor; cRNA, complementary RNA.

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EDTA, 0.1% Ficoll, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, and tRNA (200 μg/ml). The sections were then blotted dry and hybridized overnight with the ^{35}S-labeled GR complementary (cRNA) probe in hybridization buffer (0.01 ng/μl), under siliconized coverslips in a humid chamber at 37°C. The hybridization buffer included 10% dextran sulfate, polyadenylic acid (100 μg/ml), and 100 mM dithiothreitol. The sections were then washed by rinsing in 2× SSC (0.3 M NaCl/0.03 M sodium citrate, pH 7.0) at room temperature followed by incubation in 2× SSC/50%

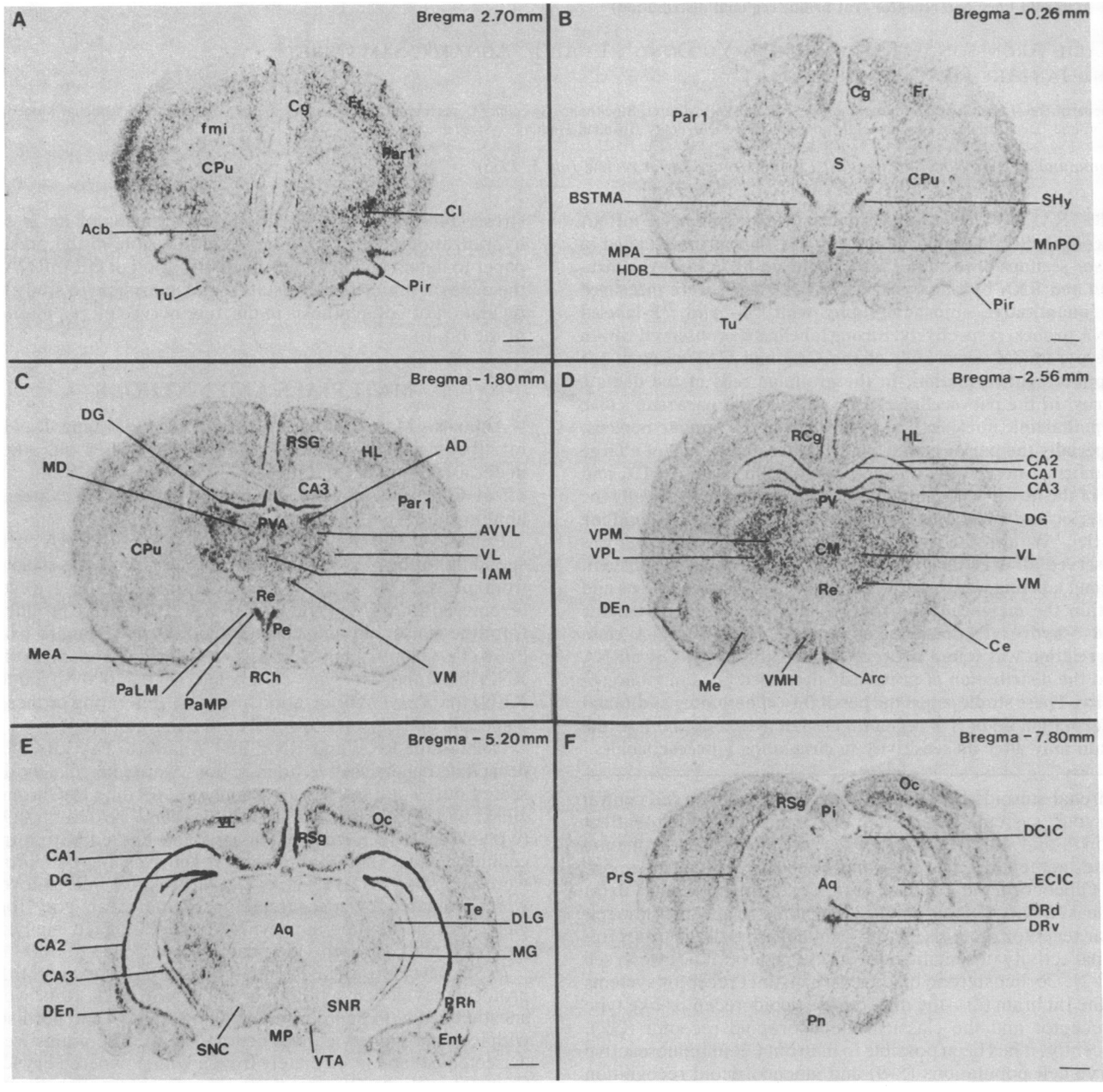

FIG. 1. *In situ* hybridization of rat brain sections with the ^{35}S-labeled GR cRNA probe. The sections were exposed to ^{3}H Ultra film (LKB) for 7 days. Acb, accumbens nucleus; Aq, aqueduct (Sylvius); Arc, arcuate hypothalamic nucleus; AVVL, anteroventral thalamic nucleus, ventrolateral part; BSTMA, bed nucleus of the stria terminalis, medial division, anterior part; CA1 to 3, fields CA1 to 3 of Ammon's horn; Ce, central amygdaloid nucleus; cg, cingulum; Cl, claustrum; CM, central medial thalamic nucleus; CPu, caudate putamen (striatum); DCIC, dorsal cortex of the inferior colliculus; DEn, dorsal endopiriform nucleus; DG, dentate gyrus; DLG, dorsal lateral geniculate nucleus; DR, dorsal raphe nucleus; ECIC, external cortex of the inferior colliculus; Ent, entorhinal cortex; fmi, forceps minor of the corpus callosum; fr, fasciculus retroflexus; HDB, nucleus of the horizontal limb of the diagonal band; HL, hindlimb area of the cortex; IAM, interanteromedial thalamic nucleus; MD, mediodorsal thalamic nucleus; Me, medial amygdaloid nucleus; MeA, medial amygdaloid nucleus, anterior part; MG, medial geniculate nucleus; MnPO, median preoptic nucleus; MP, medial mammillary nucleus, posterior part; MPA, medial preoptic area; oc, olivocerebellar tract; PaLM, paraventricular hypothalamic nucleus, lateral magnocellular part; PaMP, paraventricular hypothalamic nucleus, medial parvocellular part; Par1, parietal cortex, area 1; Pe, periventricular hypothalamic nucleus; Pi, pineal gland; Pir, piriform cortex; Pn, pontine nuclei; PRh, perirhinal cortex; PrS, presubiculum; PV, paraventricular thalamic nucleus; PVA, paraventricular thalamic nucleus, anterior part; RCh, retrochiasmatic area; Re, reuniens thalamic nucleus; RSG, retrosplenial granular cortex; S, subiculum; SHy, septohypothalamic nucleus; SNR, substantia nigra, reticular part; Te, terete hypothalamic nucleus; Tu, olfactory tubercle; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VMH, ventromedial hypothalamic nucleus; VPL, ventral posterolateral thalamic nucleus; VPM, ventral posteromedial thalamic nucleus; VTA, ventral tegmental area (Tsai).

formamide at 48°C for 5 hr, a final rinse in 2× SSC, and dehydration with ethanol.

Unspecific hybridization was determined by parallel incubation of adjacent sections with the sense GR ^{35}S-labeled RNA probe. Autoradiograms were obtained by exposure to LKB Ultrofilm sheets. A glass with a set of eight drops containing different concentrations of labeled cRNA (concentrations ranging from 5 to 200 pg/μl) was exposed together with the sections to obtain a film response curve.

Quantitation of *in Situ* Hybridization Autoradiograms. By means of an IBAS image analyzer equipped with a Bosch video camera, three measurements of mean gray tone value (MGV) were taken for each region under study: (*i*) the background value [i.e., the mean gray value obtained by labeling outside the section $(MGV)_B$], (*ii*) the unspecific value [i.e., the mean gray value of the nonspecific labeling obtained by hybridization performed with the sense GR probe $(MGV)_u$], (*iii*) the total value [i.e., the mean gray value of labeling obtained with the antisense GR probe $(MGV)_T$]. An evaluation of the specific labeling in terms of transmittance was obtained by means of the expression

$$T = \frac{(MGV)_T}{(MGV)_B} - \frac{(MGV)_u}{(MGV)_B}.$$

A film response curve was obtained by evaluating the transmittance values $[T = (MGV)_T/(MGV)_B]$ for each standard spot.

The curve was interpolated by means of a logistic curve (least-squares method) and we assessed whether the transmittance values observed in the biological samples fell into the linear part of the film response curve.

RESULTS

***In Situ* Hybridization Analysis.** Specific labeling with the ^{35}S-labeled cRNA probe was obtained in coronal brain sections at various rostrocaudal levels analyzed from the forebrain to the medulla oblongata (Figs. 1 and 2). At most rostrocaudal levels, marked differences in the degree of specific labeling were observed between various nuclei.

Telencephalon. As shown in Figs. 1 and 2 and Table 1, strong labeling was observed in the CA1 and CA2 areas of the gyrus hippocampi, in the dentate gyrus, in the medial posteroventral amygdaloid nucleus, and in the septohypothalamic nucleus. Moderate labeling was observed in layers II, III, and VI of the frontoparietal and occipital cortex, of the cingulate and retrosplenial cortex, and in several amygdaloid nuclei, especially the central amygdaloid nucleus, the dorsal endopiriform nucleus, and the presubiculum. Weak labeling was found in the frontal lobe, in the entire nucleus caudatus putamen, tuberculum olfactorium, nucleus accumbens, and within the septal area.

No specific labeling was found in the C3 and C4 areas of the gyrus hippocampi, in many lateral amygdaloid nuclei, and in most of the layers of the piriform cortex.

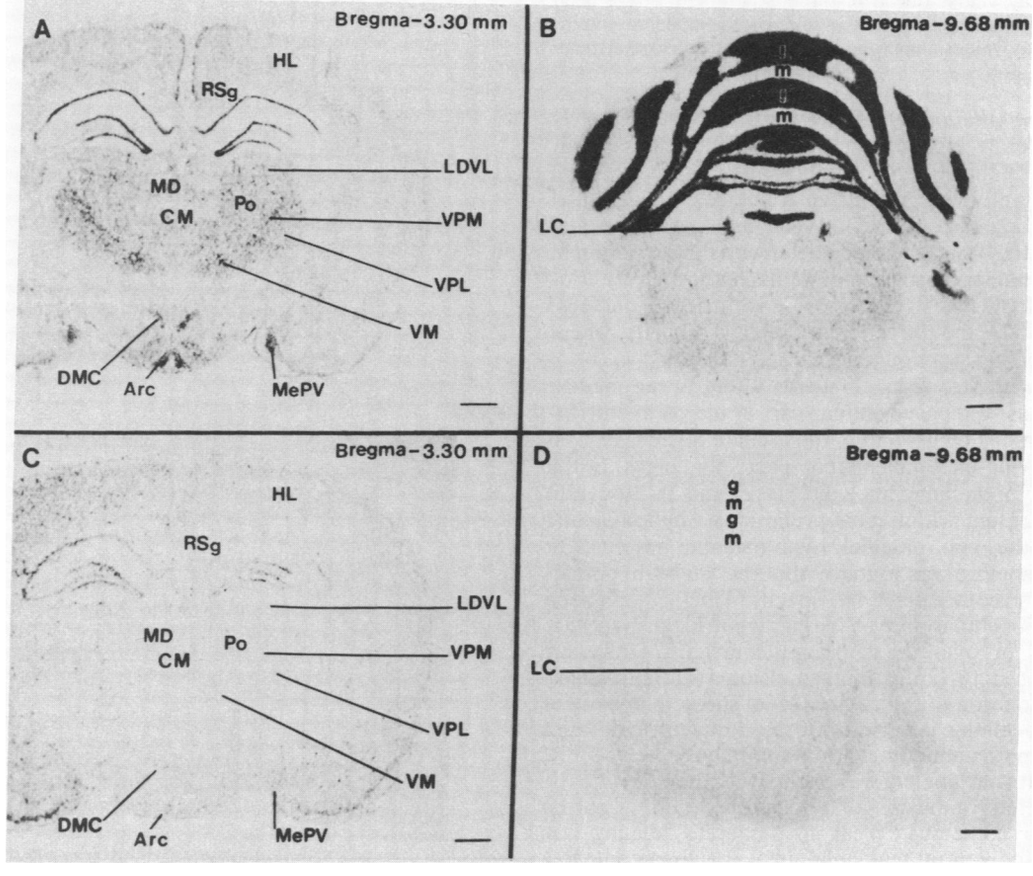

FIG. 2. (*A* and *B*) *In situ* hybridization of rat brain section with the ^{35}S-labeled GR cRNA probe. (*C* and *D*) *In situ* hybridization autoradiograms obtained by hybridization with the ^{35}S-labeled GR sense probe. The sections were exposed for 7 days. Arc, arcuate hypothalamic nucleus; CM, central medial thalamic nucleus; DMC, dorsomedial hypothalamic nucleus, compact part; HL, hindlimb area of the cortex; LC, locus coeruleus; LDVL, laterodorsal thalamic nucleus, ventrolateral part; MD, mediodorsal thalamic nucleus; MePV, medial amygdaloid nucleus, posteroventral part; Po, posterior thalamic nuclear group; RSG, retrosplenial granular cortex; VM, ventromedial thalamic nucleus; VPL, ventral posterolateral thalamic nucleus; VPM, ventral posteromedial thalamic nucleus.

Table 1. Regional GR mRNA levels as shown by *in situ* hybridization

Area	%
Telencephalon	
CA1, CA2 area of gyrus hippocampi	486
Dentate gyrus	443
Medial posteroventral amygdaloid nucleus	141
Cortex	106
Nucleus caudatus putamen	100
Diencephalon	
Ventral thalamus	113
Dorsomedial thalamus	98
Paraventricular hypothalamic nucleus, parvocellular part	196
Dorsomedial hypothalamic nucleus	127
Lower brainstem and cerebellar cortex	
Ventromedial hypothalamic nucleus	106
Arcuate hypothalamic nucleus	155
Dorsal subnucleus of dorsal raphe nucleus	139
Ventral subnucleus of dorsal raphe nucleus	224
Dorsal cortex of inferior colliculus	152
Cerebellar cortex, granular layer	462
Locus coeruleus	280

By means of the densitometric programs of the image analyzer the specific mean gray values (mean of three experiments) have been determined in various regions and nuclei of the male rat brain. Specific mean gray values are expressed as percentage striatal mean gray value.

Diencephalon (thalamus). At all the rostrocaudal levels analyzed, moderate labeling was found in the anterior thalamic and ventral thalamic nuclear complexes and in several midline nuclei (anterior periventricular nucleus, mediodorsal nucleus, centromedial nucleus). Weak labeling was noted within most of the other thalamic nuclei with the exception of the habenular nuclei, certain midline nuclei in the posterior part of the thalamus (e.g., posterior periventricular nucleus) (see Figs. 1 and 2), and the reticular thalamic nucleus, where no specific labeling was found.

Hypothalamus. Strong labeling was found in the median preoptic nucleus, the parvocellular part of the paraventricular hypothalamic nucleus, and in the arcuate nucleus as well as within the wing-like nucleus (compact subnucleus) of the dorsomedial hypothalamic nucleus (Figs. 1 and 2). Moderate labeling was present within most preoptic and hypothalamic nuclei—e.g., the ventromedial hypothalamic nucleus. In general, low labeling was found within the lateral hypothalamus and the preoptic area. No specific labeling was seen over the magnocellular part of the paraventricular hypothalamic nucleus or over the supraoptic nucleus. However, sections exposed to liquid film emulsion (NTB2, Kodak) allowing a resolution at the cellular level showed labeling of certain magnocellular cells in both the paraventricular and supraoptic hypothalamic nuclei (data not shown).

Midbrain. Strong labeling was exclusively found within the area of the nucleus raphe dorsalis and within the dorsal cortex of the inferior colliculus. Weak to moderate labeling was found within the ventral tegmental area, the zona compacta of the substantia nigra, and the pontine nuclei. In the other parts of the midbrain, weak and sometimes moderate labeling could be demonstrated.

Cerebellar cortex, pons, and medulla oblongata. Strong labeling was found over the granular layer of the entire cerebellar cortex and within the entire locus coeruleus at all rostrocaudal levels analyzed (Fig. 2). In many other nuclei of the pons and of the medulla oblongata, weak or no labeling was present.

Correlation Between GR mRNA Levels Obtained by RNA Blot Analysis and *in Situ* Hybridization. The RNA blot analysis showed a major 7-kilobase transcript similar to what has been described for the brain (23) as well as other organs (12, 23). To evaluate the validity of the *in situ* hybridization measurements, these results were compared to GR mRNA levels obtained by RNA blot analysis. For both methods, the mean gray values were expressed as percentage respective striatal mean gray value. As shown in Fig. 3 and Table 1, the mean gray values obtained with the *in situ* hybridization technique correlated well with the mean gray values obtained with RNA blot analysis.

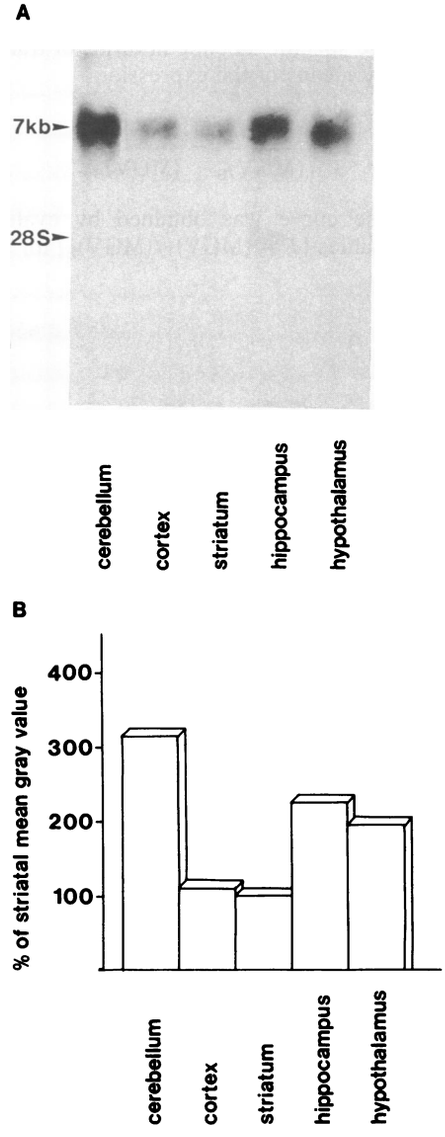

FIG. 3. RNA blot analysis of total RNA from different regions of the male rat brain. (*A*) Total RNA (20 μg per lane) was separated on 0.9% (wt/vol) agarose/formaldehyde gel, blotted onto nitrocellulose filters, and hybridized with the ^{32}P-labeled GR cRNA probe. The positions of 28S rRNA and GR mRNA transcript are indicated. kb, Kilobases. (*B*) Densitometric analysis of selected brain areas by RNA blot analysis. The specific mean gray values (mean of three experiments) are given as percentage striatal mean gray value.

DISCUSSION

The present paper gives evidence that GR mRNA can be detected and its distribution mapped out in brain sections by means of an *in situ* hybridization technique and that the levels of GR mRNA can be evaluated in a semiquantitative manner. The relative amount of GR mRNA obtained in various brain areas in the RNA blot analysis agrees well with the mean gray values obtained in the *in situ* hybridization analysis, underlining the validity of the *in situ* hybridization technique.

The results in general show a high degree of correspondence between the areas with high levels of GR mRNA and nuclei showing strong nuclear GR immunoreactivity. This is true for the CA1 and CA2 areas of the gyrus hippocampi, the granular layer of the dentate gyrus, the parvocellular part of the paraventricular hypothalamic nucleus, the arcuate nucleus, the nucleus raphe dorsalis, and the locus coeruleus. The widespread distribution of GR mRNA and GR immunoreactivity in neuronal populations in all parts of the brain indicates that information handling and metabolic events in large numbers of central neuronal populations are under direct control by glucocorticoids in the brain circulation.

In agreement with previous work (7), the dopamine cell groups of the midbrain, especially the nigral dopamine cells, appear to contain lower levels of GR than the norepinephrine and 5-hydroxytryptamine cell groups of the lower brainstem. It is of interest that by using the liquid emulsion technique, GR mRNA was found in some magnocellular neurons of the supraoptic nucleus where GR immunoreactivity is found only after a special type of fixation (3). Since Southern blot analysis (18) indicates that the GR is expressed from a single copy gene, these data taken together may indicate that specific posttranscriptional processing of the GR takes place in these magnocellular neurons.

Sometimes strong labeling with the GR cRNA probe corresponded to nerve cell groups with a weak to moderate nuclear GR immunoreactivity. This is true, e.g., for the septohypothalamic nucleus, the median preoptic nucleus, the medial amygdaloid nucleus, and especially the granular layer of the entire cerebellar cortex. In agreement, a strong signal was obtained in RNA blot analysis of the cerebellar cortex, confirming the specificity of the signal. However, sections exposed to liquid film emulsion (data not shown) showed a moderate number of silver grains per cell all over the highly densely packed granular layer, indicating that the strong hybridization signal obtained by RNA blot analysis and *in situ* hybridization film sheet autoradiograms reflects a high density of GR mRNA-containing cells rather than a cell population with an especially high GR mRNA copy number.

The present *in situ* hybridization protocol provides a rapid and sensitive tool for detection of glucocorticoid receptor mRNA in the rat brain. The use of ^{3}H films in combination with computerized image analysis makes it possible to semiquantitate the GR mRNA levels and to study regulation of GR synthesis in discrete nerve cell populations of the rat brain.

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