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Thyroid hormones regulate levels of thyrotropin-releasing-hormone mRNA in the paraventricular nucleus

(*in situ* hybridization/negative feedback/triiodothyronine/neuropeptides/rat brain)

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ABSTRACT **Cellular levels of messenger RNA encoding thyrotropin-releasing hormone (TRH) were measured in the paraventricular nucleus of the hypothalamus and the reticular nucleus of the thalamus in male rats after chemical thyroidectomy and thyroid hormone replacement. TRH mRNA levels were measured by quantitative *in situ* hybridization histochemistry using a ^{35}S-labeled synthetic 48-base oligodeoxynucleotide probe and quantitative autoradiography. Chemical thyroidectomy, produced by the administration of 6-(*n*-propyl)-2-thiouracil (PrSur), reduced plasma thyroxine below detection limits and significantly increased TRH mRNA in the paraventricular nucleus. Treatment with exogenous L-triiodothyronine (T_3) reduced TRH mRNA to the same level in both hypothyroid and euthyroid animals. Neither PrSur treatment nor T_3 replacement influenced TRH mRNA levels in the reticular nucleus of the thalamus. Blot hybridization analysis of electrophoretically fractionated total RNA from pituitaries of these animals indicated that thyrotropin-β mRNA levels were elevated after thyroidectomy and reduced by T_3 treatment, showing that the pituitary–thyroid axis was indeed stimulated by PrSur treatment. These results suggest that thyroid hormones are involved, either directly or indirectly, in regulating the biosynthesis of TRH in the thyrotropic center of the hypothalamus.**

Thyroid hormones [thyroxine (T_4) and triiodothyronine (T_3)] exert a negative feedback effect on thyrotropin (thyroid-stimulating hormone, TSH) secretion from the pituitary (1). However, while thyroliberin (thyrotropin-releasing hormone, TRH) is known to regulate pituitary–thyroid function (2), the effect that thyroid hormones have on TRH has remained unclear. Thyroid hormones do seem to inhibit TRH release, since thyroxine implants in the hypothalamus of the rat and cat inhibit thyroid function (3, 4). Furthermore, thyroxine treatment has been reported to decrease hypothalamic TRH immunoreactivity in intact rats (5) and increase hypothalamic TRH immunoreactivity in thyroidectomized rats (6). However, these findings are not universally accepted (7). Perhaps the discrepant results reported by members of different laboratories are due to the fact that tissue levels of peptides do not necessarily correlate with secretion.

Peptide biosynthesis, on the other hand, may provide a reliable index of long-term changes in secretion (8–13). TRH is synthesized from a precursor protein, which is translated from a specific mRNA (14), and we can now measure cellular levels of TRH mRNA by quantitative *in situ* hybridization histochemistry (15). This technique provides an approach with sufficient sensitivity and neuroanatomical resolution to examine the regulation of TRH biosynthesis in specific subpopulations of TRH-positive cells. Although TRH-containing neurons are widespread throughout the brain (16), the cells involved in regulating thyroid function reside almost exclusively within the paraventricular nucleus (PVN) (17, 18). In this report, we describe experiments designed to determine whether thyroid hormones play a role in the regulation of TRH biosynthesis specifically in the thyrotropic center of the hypothalamus, the PVN. The results demonstrate that thyroid hormones significantly reduce TRH mRNA levels in the PVN but have no effect on TRH mRNA in the reticular nucleus of the thalamus, a TRH-positive area (16) that is not involved in the regulation of thyrotropin secretion (17).

METHODS

Animal Treatments. Adult male Sprague–Dawley rats (200–250 g; Zivic–Miller Laboratories, Allison Park, PA) were fed a normal diet or a low-iodine diet containing 0.05% (wt/wt) 6-(*n*-propyl)-2-thiouracil (PrSur; Zeigler Brothers, Gardners, PA). They were housed in diurnal lighting conditions (lights on from 0600 to 1800) at 25°C and were given food and water ad libitum. The rats were given daily subcutaneous injections (100 μl) of either L-triiodothyronine (20 μg/kg; ref. 19) or 0.9% NaCl for 12 days. They were killed by decapitation, trunk blood was collected, and brains and pituitaries were removed and frozen on dry ice within 5 min.

Oligonucleotide-Probe Preparation and Labeling. A 48-base oligodeoxyribonucleotide complementary to the coding region of TRH (cDNA base numbers 319–366, as described by Lechan *et al.*; ref. 14) was synthesized by M. J. Brownstein on an Applied Biosystems 380A DNA synthesizer according to the manufacturer's instructions and purified by electrophoresis in a preparative denaturing acrylamide gel. The oligonucleotide probe (5 pmol) was labeled to a specific activity of 3500 Ci/mmol (1 Ci = 37 GBq), using terminal deoxynucleotidyltransferase [100 units; Bethesda Research Laboratories (BRL), Gaithersburg, MD] and [α-[^{35}S]thio]-dATP (1 μM; 1300 Ci/mmol; New England Nuclear) under the conditions recommended by BRL.

***In Situ* Hybridization Histochemistry.** Twelve-micrometer coronal sections from each brain were cut in a cryostat. Sections used for quantitation contained the rostral-most extent of the PVN of the hypothalamus as well as the reticular formation of the thalamus. Other sections were prepared to determine whether a hybridization signal could be detected in additional brain areas known to contain TRH-precursor immunoreactivity (16). These areas included the preoptic area, the bed nucleus of the stria terminalis, and the bed nucleus of the anterior commissure. Sections were thaw-mounted onto gelatin-coated microscope slides (two sections per slide) and stored at −70°C until hybridization. *In situ* hybridization histochemistry was performed as described

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Abbreviations: TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; T_3, triiodothyronine; PVN, paraventricular nucleus; PrSur, 6-(*n*-propyl)-2-thiouracil.

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(20). In brief, the sections were warmed to room temperature, fixed in 4% formaldehyde, and soaked in 0.25% acetic anhydride/0.1 M triethanolamine hydrochloride/0.9% NaCl. Tissues were then dehydrated through a series of ethanol solutions, delipidated in chloroform, rehydrated in 95% ethanol, and air-dried. Each slide was hybridized in a complex buffer containing 4× SSC (600 mM NaCl/60 mM sodium citrate, pH 7.0) and labeled probe (600,000 dpm), for 16 hr at 37°C in humid chambers. Slides were washed in 1× SSC at 55°C (approximately 20°C below the calculated melting temperature for the probe·mRNA hybrids; refs. 21 and 22). After drying, the slides were exposed to Kodak X-Omat AR film for 1 week or dipped in Kodak NTB-3 nuclear-track emulsion and allowed to expose for 2 weeks.

Quantitative Analysis of *in Situ* Hybridization. The intensity of the hybridization signal was measured as described (23, 24). The optical density and area of the hybridization signal on x-ray film over each nucleus were analyzed using an image-analysis system (Loats Associates, Westminster, MD). To estimate TRH mRNA content of the PVN and reticular nucleus, optical densities were subtracted from background and averaged over six replicates for each animal and expressed as percent control (saline-injected animals). To validate that the optical densities of the hybridization signals were below saturation of the film, ^{35}S-impregnated brain-paste standards were exposed to all films. Brain-paste standards were prepared by adding known amounts of [α-[^{35}S]thio]dATP to homogenized brain, freezing the homogenate to cryostat chucks, and sectioning at 12 μm. Nine standards were thaw-mounted onto a single microscope slide and exposed to film at the same time hybridized sections were exposed. Differences among groups were analyzed by two-way analysis of variance. Bonferroni's *t* test was used where appropriate to detect differences among individual means. The criterion for significance was $P < 0.01$.

RNA Isolation and Blot Hybridization Analysis. Total RNA was isolated from individual pituitaries by the SET pK method (25). Each pituitary was sonicated (10% of full setting; three times, 10 sec) in 250 μl of SET pK buffer [1% $NaDodSO_4$/5 mM EDTA, pH 8/10 mM Tris·HCl, pH 7.5, containing 100 μg of proteinase K (Boehringer Mannheim, RNase-free) per ml]. An additional 250 μl of buffer was added to each tube before incubation at 42°C for 2 hr. The reaction mixture was extracted twice with 500 μl of phenol/chloroform (1:1, vol/vol), and the nucleic acids in the aqueous phase were precipitated with 400 mM NaOAc and 2.5 volumes of ethanol at −20°C overnight. Total RNA from rat hypothalamus or from tissue punches of rat PVN or supraoptic nucleus was isolated by the guanidinium thiocyanate procedure (26) and ethanol-precipitated twice. The precipitated material was centrifuged at 10,000 × *g* for 10 min at 4°C. The resulting pellet was resuspended in 2.2 M formaldehyde/50% formamide/20 mM Mops/5 mM sodium acetate/1 mM EDTA, pH 7.0/0.025% bromophenol blue and denatured at 56°C for 15 min.

For pituitary RNA, one-third of the isolated material was electrophoresed in 1.4% agarose gels containing 2.2 M formaldehyde/20 mM Mops/5 mM sodium acetate/1 mM EDTA. These gels were stained in dilute ethidium bromide (4 μg/ml) for 10 min, washed in running double-distilled water for 10 min, and destained for 16 hr in electrophoresis buffer. The relative amount of RNA loaded onto each lane was measured by scanning the optical density, with a Beckman DU-8B spectrophotometer, of the 18S and 28S rRNA bands as they appeared on the negative of a Polaroid photograph taken of the destained gel illuminated with UV light. The RNA was transferred to GeneScreen (New England Nuclear) and baked at 80°C under reduced pressure for 90 min. A *Pst* I fragment (420 base pairs) encoding the cDNA for TSH-β (27) was ^{32}P-labeled, using the random-primer method (28), to a specific activity of 6.7×10^8 dpm/μg. The filters were prehybridized for 16 hr at 45°C in 5× SSPE (750 mM NaCl/50 mM sodium phosphate, pH 7.4/5 mM EDTA)/1× Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/50% formamide/0.1% $NaDodSO_4$ containing tRNA at 250 μg/ml and sheared single-stranded salmon sperm DNA at 125 μg/ml. Hybridization with denatured, labeled probe (10^6 dpm/ml) was continued for 45 hr under the same conditions. The filters were washed in 0.1× SSPE/0.1% $NaDodSO_4$ at 65°C and placed against x-ray film in a cassette equipped with two intensifying screens for 14 hr at −70°C. For hypothalamic RNA, 10 μg of each type of RNA was electrophoresed in a 1.2% agarose/2.2 M formaldehyde denaturing gel. The gel was stained and destained, and the RNA was transferred as above. The TRH-specific 48-base oligonucleotide was labeled, using terminal deoxynucleotidyltransferase and [α-^{32}P]dATP (3000 Ci/mmol, New England Nuclear), to a specific activity of 3900 Ci/mmol. The filter was prehybridized with the same buffer as above for 16 hr at 37°C, and hybridization with probe (10^6 dpm/ml) was continued an additional 16 hr. The filter was washed in 1× SSC/0.1% $NaDodSO_4$ at 55°C and exposed to x-ray film as above for 16 hr at −70°C. RNA "ladders" (Bethesda Research Laboratories) were used as size markers for all gels.

Measurement of Serum Thyroxine. Frozen sera were thawed at room temperature, and a 25-μl aliquot was used to measure circulating thyroxine levels with a thyroxine radioimmunoassay kit according to the supplier's instructions (Total T_4 Coat-a-Count; Diagnostic Products, Los Angeles, CA). The limit of sensitivity of the assay was 6.4 nM (0.5 μg/dl); the intraassay variation was less than 5%. All samples were measured in duplicate.

RESULTS

Specificity of TRH Probe Hybridization. Blot hybridization analysis of total RNA isolated from rat hypothalamus, PVN, or supraoptic nucleus was performed using the 48-base oligonucleotide labeled by 3′-tailing with [α-^{32}P]dATP as a probe (Fig. 1). Under the same hybridization and washing conditions used for *in situ* hybridization, a single RNA species of approximately 1.75 kilobases was detected by the probe in both the hypothalamus and the PVN. No hybridization was seen to RNA from supraoptic nucleus. These results are consistent with the labeling of a single mRNA species of the correct size for the complementary DNA encoding TRH (14). Hybridization specificity was also examined by *in situ* hybridization histochemistry. The distribution of labeled cells corresponded to the immunoreactive localization of the TRH prohormone (ref. 17; reticular nucleus of the thalamus, preoptic area, bed nuclei of the stria terminalis and of the anterior commissure; results not shown).

Quantitation of the Hybridization Signal. Representative optical density readings for signal and background are listed in Table 1. Optical densities over the PVN and reticular nucleus were 2- to 4-fold above background. The area (mm^2) of the hybridization signal from which optical density measurements were taken for each nucleus was not different among the experimental groups (data not shown). Five of the nine standards produced a signal above background on the film and were below saturation. All experimental observations were below saturation and fell on the linear part of the response curve of the film.

PrSur Treatment Increases Central TRH mRNA Levels. The autoradiographic signal obtained after hybridization of ^{35}S-labeled probe to brain sections containing the PVN and reticular nucleus of the thalamus is shown in Fig. 2. The intensity of labeling of the PVN in PrSur-treated animals was

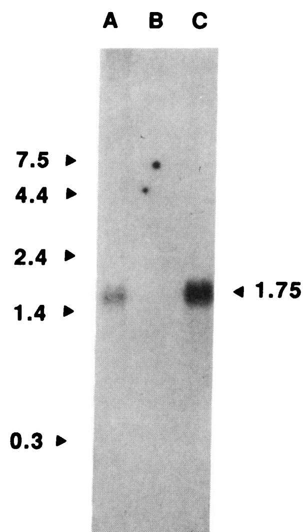

FIG. 1. Blot hybridization of the TRH 48-base probe to hypothalamic RNA. Total RNA (10 μg per lane) from hypothalamus (lane A), supraoptic nucleus (lane B), and PVN (lane C) was electrophoresed in a 1.2% agarose/2.2 M formaldehyde denaturing gel. The 48-base TRH probe used for *in situ* hybridization histochemistry was labeled with [α-^{32}P]dATP and terminal deoxynucleotidyltransferase. After transfer, the RNA blot was hybridized with the 48-mer probe and washed under the same conditions (1× SSC/0.1% $NaDodSO_4$, 55°C) as the *in situ* sections. Numbers at left give sizes of RNA markers in kilobases.

visibly stronger, in both x-ray film and nuclear-track emulsion, than in PrSur-treated animals given T_3 replacement (Fig. 2). Quantitative analysis of these autoradiograms revealed that PrSur treatment increased optical densities over the PVN by more than 2-fold ($P < 0.001$; Fig. 3*A*), whereas injection of T_3 into PrSur-treated and control animals decreased optical densities to the same level (47.5% ± 7.7% and 45.3 ± 12.0% of control, respectively; $P < 0.001$ vs. control). The hybridization signal observed using nuclear-track emulsion appeared as dense aggregations of grains specifically over cell bodies (Fig. 2, *Insets*). Neither PrSur treatment nor T_3 replacement significantly altered the hybridization signal over the reticular nucleus (Fig. 3*B*).

PrSur Treatment Increases Pituitary TSH-β mRNA and Decreases Serum Thyroxine. TSH-β mRNA levels were measured by blot analysis of total RNA isolated from the pituitaries of experimental animals (Fig. 4). TSH-β mRNA levels in PrSur-treated animals (lane B) were higher than control (lane A). T_3 replacement in both treated and untreated animals (lanes C and D, respectively) caused a decrease in TSH-β mRNA. Serum thyroxine levels (56.2 ± 1.9 nM in control rats) were reduced to below the limits of detection for the assay by PrSur treatment (<6.4 nM).

Table 1. Representative optical density measurements from x-ray film

	Optical density			
	Paraventricular nucleus		Reticular nucleus	
	Signal	Background	Signal	Background
Control	0.40	0.17	0.30	0.13
PrSur	0.58	0.14	0.32	0.14
T_3	0.28	0.15	0.34	0.15
PrSur/T_3	0.28	0.16	0.32	0.18

Optical density was measured using an image-analysis system (see *Methods*). Values above refer to optical density of an individual PVN or reticular nucleus from an animal within the specified experimental group.

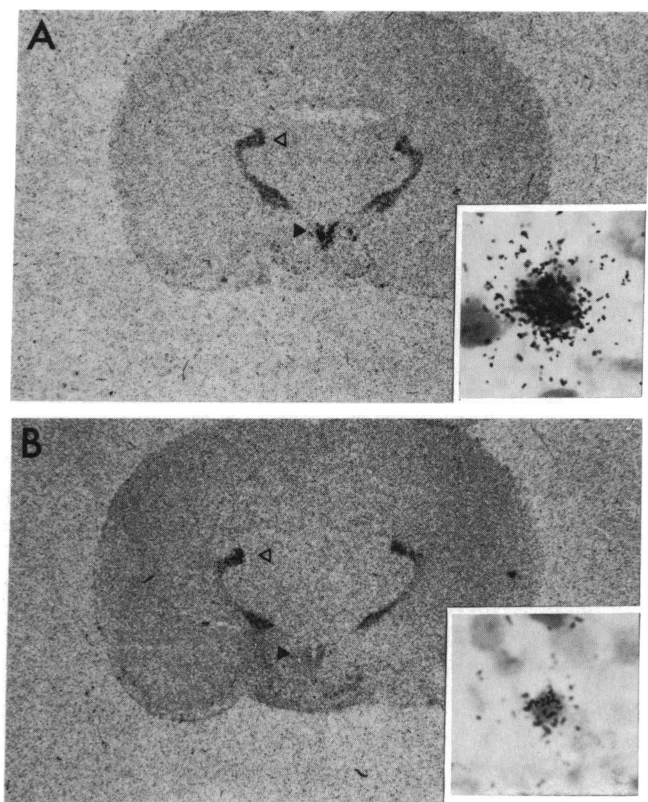

FIG. 2. *In situ* hybridization histochemistry of TRH mRNA-containing cells in the PVN of the hypothalamus and the reticular nucleus of the thalamus. The autoradiographic hybridization signal on x-ray film appears as an area of darkening over the PVN (solid arrowhead) and reticular nucleus (open arrowhead) of a rat treated with PrSur (*A*) and of a PrSur-treated rat injected with T_3 (*B*). Using NTB-3 nuclear emulsion, the hybridization appears as dense clusters of grains over individual cell bodies within the PVN of a PrSur-treated rat (*Inset A*) and of a PrSur-treated rat injected with T_3 (*Inset B*). Each inset shows the most intensely labeled cells within the respective PVN.

DISCUSSION

The specificity of the *in situ* hybridization histochemistry technique was validated in two ways. First, blot analysis of RNA from hypothalamus, as well as from microdissected PVN and supraoptic nuclei, was performed using the same 48-base probe and at the same stringency as the *in situ* studies. We found that the probe hybridized to a single band corresponding to the appropriate size for TRH mRNA (1.75 kilobases; ref. 14) in hypothalamic RNA and in RNA from PVN; no hybridization was seen with RNA from the supraoptic nuclei, an area where neither TRH precursor nor TRH mRNA has been found (16). Second, the distribution of cells labeled by *in situ* hybridization histochemistry was consistent with previous reports of the distribution of TRH precursor-containing cells in the rat brain (16).

Although there is general agreement that thyroid hormones exert a potent negative feedback effect on pituitary thyrotropin secretion, the effect of thyroid hormones on hypothalamic TRH has remained unclear (1). In the present study, we used quantitative *in situ* hybridization histochemistry, since many studies have indicated that levels of mRNA for secreted peptides and proteins reflect biosynthetic activity (15) and that sustained changes in secretion are coupled to changes in biosynthesis to compensate for loss of peptide (8–13). Thus, we reasoned that changes in TRH mRNA levels could be a good indicator of TRH activity in animals of different thyroid status. Hypothyroidism was induced by PrSur treatment:

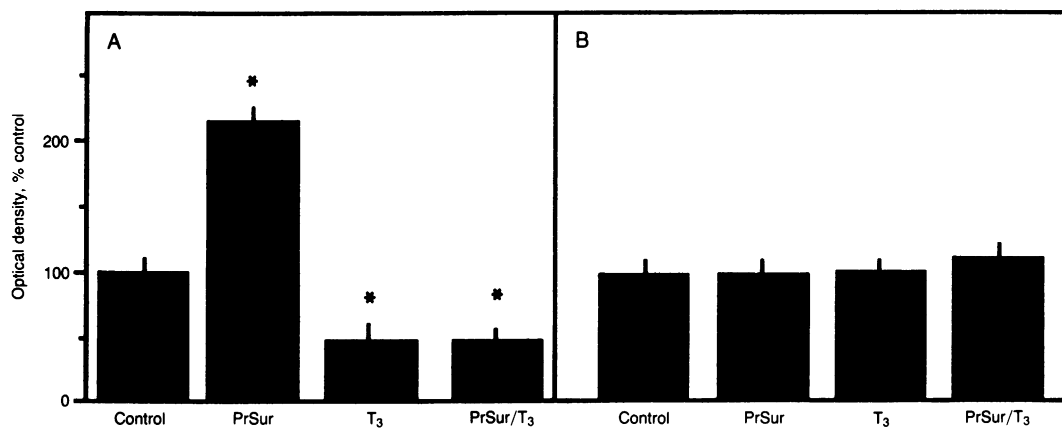

FIG. 3. Effect of PrSur treatment and T_3 replacement on TRH mRNA levels in the PVN (*A*) and reticular nucleus (*B*). Optical densities were averaged from six replicates for each animal and expressed as a percent of the level observed in control animals (normal diet/injected with saline; see *Methods*). Each bar represents the average (± SEM) of the TRH mRNA level in control rats (n = 8), T_3 (normal diet/injected with T_3, n = 8), PrSur-treated rats (diet containing PrSur/injected with saline, n = 8), and PrSur/T_3-treated rats (diet containing PrSur/injected with T_3, n = 8). Asterisks indicate $P < 0.001$ vs. control.

pituitary TSH-β mRNA was increased and serum thyroxine was decreased in the treated animals. Our results showed that TRH mRNA levels in the PVN were significantly elevated in hypothyroid animals and that injection of T_3 reduced TRH mRNA levels in PVN of both hypothyroid and euthyroid animals. Although our measurements reflect steady-state levels of mRNA and do not allow us to distinguish changes in transcriptional rate from changes in RNA stability, these results are consistent with the hypothesis that thyroid hormones exert a negative feedback effect on TRH synthesis and secretion.

TRH mRNA levels were affected by thyroid status specifically in the PVN. TRH mRNA was detected within the reticular nucleus of the thalamus, as reported previously (16), but quantitative analysis demonstrated that levels were not altered by PrSur treatment or T_3 replacement. Previous studies have shown that lesions of the PVN disrupt normal thyroid function (17), that TRH is released from the median eminence to stimulate the release of TSH from the pituitary (1), and that TRH immunoreactivity in the median eminence originates from the PVN (18). Therefore, the present results indicate that TRH mRNA-containing cells, whose somata reside within the hypothalamic thyrotropic area, mediate the thyrotropic function of the PVN and are involved in regulating pituitary–thyroid function.

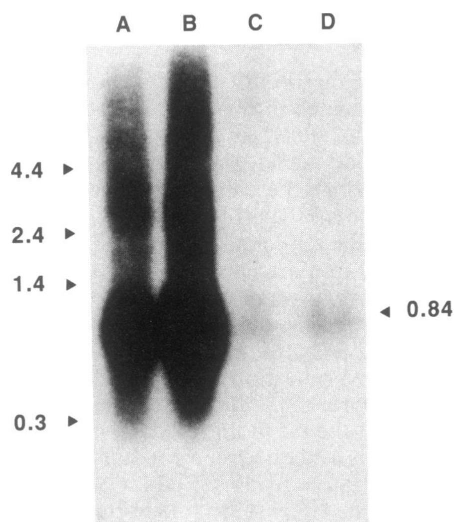

FIG. 4. Blot analysis of pituitary TSH-β mRNA. Pituitary RNA from control (lane A), PrSur-treated (lane B), PrSur/T_3-treated (lane C), and T_3-treated (lane D) rats was electrophoresed in a 1.4% agarose/2.2 M formaldehyde denaturing gel. Relative amounts of RNA loaded in lanes A–D, measured as described in *Methods*, were 1.0:1.0:1.0:1.1. After RNA was transferred to GeneScreen, the blot was hybridized with a TSH-β cDNA probe labeled to a specific activity of 6.7×10^8 dpm/μg. Sizes at left (in kilobases) refer to marker RNAs.

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