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R A Hodin, ... , M A Lazar, W W Chin

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Research Article

Thyroid hormone (T3) has been shown to regulate the level of its receptor in a number of tissues and cell lines. Recently, proteins encoded by the protooncogene c-erbA have been identified as T3 receptors. In the rat, four c-erbA gene products have been isolated, three of which, r-erbA alpha-1, r-erbA beta-1, and r-erbA beta-2, encode biologically active T3 receptors; the fourth, r-erbA alpha-2, may play an inhibitory role in T3 action. The present work examines the molecular nature of T3 receptor autoregulation using probes specific for each c-erbA mRNA. Rats were rendered hypothyroid with propylthiouracil and then treated with either saline or T3. Northern blot analyses reveal marked tissue-specific and differential regulation of the multiple c-erbA mRNAs by T3. In the pituitary the levels of r-erbA beta-1 mRNA increase, whereas the levels of the pituitary-specific r-erbA beta-2 mRNA decrease with T3 treatment. In heart, kidney, liver, and brain the levels of r-erbA beta-1 are unaffected by thyroidal status. The levels of both r-erbA alpha mRNAs decrease with T3 treatment in all tissues examined except for the brain, where there is no change. In addition, we find that changes in the mRNAs encoding specific subpopulations of T3 receptors do not always parallel changes in total nuclear T3 binding. Differential regulation of the specific c-erbA mRNA species could have important consequences for [...]

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Differential and Tissue-specific Regulation of the Multiple Rat c-erbA Messenger RNA Species by Thyroid Hormone

Richard A. Hodin, Mitchell A. Lazar,* and William W. Chin

Division of Genetics, Department of Medicine, Brigham and Women's Hospital; Howard Hughes Medical Institute; and Harvard Medical School, Boston, Massachusetts 02115; and *University of Pennsylvania School of Medicine, Endocrine Section, Philadelphia, Pennsylvania 19104

Abstract

Thyroid hormone (T_3) has been shown to regulate the level of its receptor in a number of tissues and cell lines. Recently, proteins encoded by the protooncogene c-erbA have been identified as T_3 receptors. In the rat, four c-erbA gene products have been isolated, three of which, r-erbA α -1, r-erbA β -1, and r-erbA β -2, encode biologically active T_3 receptors; the fourth, r-erbA α -2, may play an inhibitory role in T_3 action. The present work examines the molecular nature of T_3 receptor autoregulation using probes specific for each c-erbA mRNA. Rats were rendered hypothyroid with propylthiouracil and then treated with either saline or T_3 . Northern blot analyses reveal marked tissue-specific and differential regulation of the multiple c-erbA mRNAs by T_3 . In the pituitary the levels of r-erbA β -1 mRNA increase, whereas the levels of the pituitary-specific r-erbA β -2 mRNA decrease with T_3 treatment. In heart, kidney, liver, and brain the levels of r-erbA β -1 are unaffected by thyroidal status. The levels of both r-erbA α mRNAs decrease with T_3 treatment in all tissues examined except for the brain, where there is no change. In addition, we find that changes in the mRNAs encoding specific subpopulations of T_3 receptors do not always parallel changes in total nuclear T_3 binding. Differential regulation of the specific c-erbA mRNA species could have important consequences for T_3 action. (*J. Clin. Invest.* 1990. 85:101-105.) hormone regulation • pituitary gland • receptor autoregulation • thyroid hormone action • thyroid hormone receptors

Introduction

The magnitude of the cellular response to thyroid hormone (T_3)¹ is related to the number of nuclear receptors present (1). It is therefore likely that physiologic or pharmacologic alterations in receptor number may modify the response of a tissue

to T_3 . In GH₁ cells, a rat pituitary tumor-derived cell line, T_3 treatment causes a decrease in receptor number which, in turn, results in an attenuation in T_3 responsiveness, as measured by growth hormone gene transcription (2).

Numerous investigators have studied the effects of thyroidal status on rat T_3 receptors, but the results have been conflicting. Pituitary receptor levels have been reported to increase (3), decrease (4), or remain unchanged (5) in response to T_3 treatment. Similar inconsistencies exist in the literature concerning the effect of thyroidal status on receptor number in rat liver (6, 7).

T_3 receptors, encoded by the α and β c-erbA genes, are members of a superfamily of structurally related receptor proteins (8, 9). The recent identification of multiple forms of the T_3 receptor (10-18) sheds new light on the issue of receptor autoregulation. We have shown, for example, that changes in specific receptor subpopulations may not be reflected in a measurement of total receptor number in GH₁ cells (14, 18, 19).

In the rat, three putative biologically active T_3 receptors have been identified, r-erbA α -1 (10), r-erbA β -1 (13), and r-erbA β -2 (18). In addition, a related molecule, r-erbA α -2, does not bind T_3 (12, 14, 15) and may act to inhibit T_3 action (20). r-erbA α -1, r-erbA α -2, and r-erbA β -1 are expressed to varying degrees in a number of rat tissues (14, 18), whereas the expression of r-erbA β -2 is limited to the anterior pituitary gland (18).

We have examined the effects of T_3 treatment on the levels of the various c-erbA gene products in pituitary as well as a number of other rat tissues. Our results show that there is tissue-specific and differential regulation of the r-erbA mRNAs by thyroid hormone, and suggest that measurement of total nuclear T_3 binding is inadequate when examining alterations in T_3 receptors.

Methods

Adult male Sprague-Dawley rats (125-150 g) were maintained on standard chow and water supplemented with 0.05% (wt/vol) propylthiouracil (PTU) for a 6-wk period. Six daily intraperitoneal injections of either saline (PTU only or hypothyroid group) or 20 μ g/g body weight triiodothyronine (PTU + T_3 or hyperthyroid group) were then administered. During the administration of the supraphysiologic replacement dose of T_3 both groups of animals were maintained on PTU treatment. The animals were killed by decapitation and their organs were removed, immediately frozen in liquid nitrogen, and stored at -70°C. Blood was collected from each animal and assayed for T_3 to confirm thyroidal status.

The frozen tissues were homogenized and RNA was extracted using the guanidinium thiocyanate method (21). Northern blot analyses were performed by standard techniques (22). The filters were hybridized with cDNA (growth hormone, actin, and spot-14 mRNAs) or cRNA (r-erbA mRNAs) probes labeled with ³²P, typically to a specific activity of $\sim 5 \times 10^8$ cpm/ μ g DNA. The r-erbA β mRNA probes are

Dr. Hodin's present address is Beth Israel Hospital, Department of Surgery, 330 Brookline Avenue, Boston, MA 02146.

Please address correspondence to Dr. Richard A. Hodin, Thorn Research Building, Room 917, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.

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1. Abbreviations used in this paper: PTU, propylthiouracil; RA, retinoic acid; T_3 , triiodothyronine.

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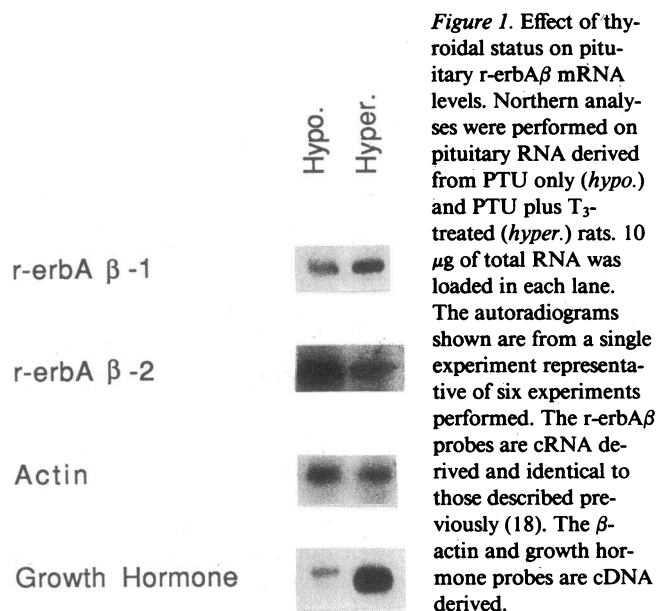


Figure 1. Effect of thyroidal status on pituitary r-erbA β mRNA levels. Northern analyses were performed on pituitary RNA derived from PTU only (*hypo.*) and PTU plus T₃-treated (*hyper.*) rats. 10 μ g of total RNA was loaded in each lane. The autoradiograms shown are from a single experiment representative of six experiments performed. The r-erbA β probes are cRNA derived and identical to those described previously (18). The β -actin and growth hormone probes are cDNA derived.

derived from the 5' region of the cDNAs and have been previously shown to be specific for either the r-erbA β -1 or r-erbA β -2 species (18). The r-erbA α probe is derived from the region common to the r-erbA α -1 and r-erbA α -2 mRNAs. We have previously determined the specificity of the 2.6- (α -2) and 5-kb (α -1) hybridizing bands (14). Conditions for hybridization were as follows: cDNA probes, 5 \times standard saline citrate (SSC)/50% (vol/vol) formamide/1% (wt/vol) SDS at 42°C; cRNA probes, 5 \times SSC/50% formamide/5% SDS at 65°C. Washing conditions were: cDNA probes, 2 \times SSC/0.1% SDS at 50°C; cRNA probes, 0.2 \times SSC/0.1% SDS at 65°C. Relative changes in mRNA levels were determined by laser densitometry of autoradiograms and normalized for β -actin mRNA. Statistical analyses were performed using the unpaired *t* test.

Nuclear extracts were prepared from either fresh or frozen tissues for the T₃ binding experiments. Tissues were homogenized in STM buffer (0.25 M sucrose, 0.02 M Tris, 1.1 mM MgCl₂, pH 7.85) and the homogenate centrifuged at 2,000 rpm at 4°C. The nuclear pellet was washed twice with STM buffer plus 0.2% (vol/vol) Triton X-100 and then with STM buffer plus 0.1 mM EDTA and 0.5 mM DTT. The nuclear pellet was then incubated for 30 min in a buffer containing 0.6 M KCl, 20 mM Hepes (pH 7.3), 20 μ M ZnCl₂, 0.2 mM EGTA, and 0.5 M DTT. After centrifugation at 27,000 rpm for 30 min the supernatant (nuclear extract) was collected. Protein content was determined using the method of Bradford (23).

2 μ g protein extract was incubated with 0.05 nM [¹²⁵I]T₃ plus varying amounts of unlabeled T₃ at 4°C for 16 h. Bound and free hormones were separated using a Sephadex G-50 column and radioactivity was measured by gamma spectrometry.

Results

r-erbA β -1 and r-erbA β -2 mRNA levels in pituitary glands of hypothyroid and hyperthyroid rats were determined using specific probes (18). A representative Northern blot analysis demonstrating that the two β forms of r-erbA are differentially regulated by T₃ in the pituitary is shown in Fig. 1. After normalization for β -actin mRNA, the r-erbA β -1 mRNA levels were shown to increase \sim 3.5-fold ($P < 0.025$), whereas the r-erbA β -2 levels decreased \sim 50% ($P < 0.005$) from the hypothyroid to the hyperthyroid state. In agreement with previous work (24), growth hormone mRNA levels increased \sim 15-fold with T₃ treatment.

In contrast to the changes seen in the pituitary, the levels of r-erbA β -1 mRNA were unchanged in brain, heart, and kidney as a function of thyroidal status (Fig. 2). In liver there appeared to be more variability in r-erbA β -1 expression. After correction for β -actin expression there was a tendency towards a decrease in r-erbA β -1 mRNA levels with T₃ treatment, although this was not statistically significant. Spot 14 mRNA was significantly increased in all livers from hyperthyroid rats (data not shown). In agreement with our previous studies (18) no hybridization corresponding to the r-erbA β -2 mRNA was detected in these nonpituitary tissues.

The r-erbA α -1 (5 kb in size) and r-erbA α -2 (2.6 kb in size) mRNAs were also studied in these various rat tissues using a probe that hybridizes to both mRNAs (14). Both the r-erbA α -1 and r-erbA α -2 mRNA levels decreased with T₃ treatment in all tissues studied with the exception of brain (Fig. 3). The magnitudes of the decreases in r-erbA α -1 were \sim 65, 69, and 22% of hypothyroid levels in heart ($P < 0.005$), kidney ($P < 0.005$), and pituitary ($P < 0.1$), respectively. The decreases in r-erbA α -2 mRNA were similar in magnitude to those seen with r-erbA α -1: 86% in heart ($P < 0.005$), 63% in kidney ($P < 0.005$), and 22% in pituitary ($P < 0.1$). The levels of both r-erbA α mRNAs remained constant in brain regardless of thyroidal status. Rat liver contains minimal amounts of r-erbA α mRNAs; hence, a quantitative analysis was not possible. A 6-kb band, which we have previously shown to hybridize to both α -1- and α -2-specific probes (14) and which may represent an unspliced heteronuclear RNA, is evident in all rat tissues examined. The level of this 6-kb band also decreases with T₃ treatment.

T₃ binding studies were performed using nuclear extracts from pituitary glands of hypothyroid and hyperthyroid animals. Fig. 4 illustrates Scatchard analyses showing no change in the affinity constant ($K_d = 0.7$ nM) between the two groups.

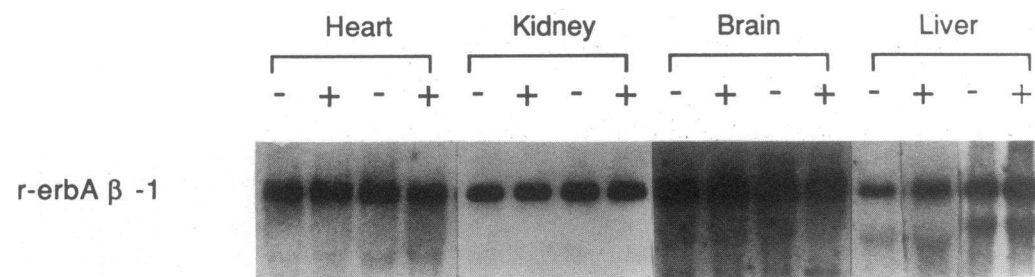


Figure 2. Effect of thyroidal status on r-erbA β -1 mRNA levels in various rat tissues. Northern analyses were performed on RNA derived from tissues of PTU only (-) and PTU plus T₃-treated (+) rats. 10 μ g of total RNA was loaded in each lane. Equal loading of RNA per lane was verified by hybridization with a β -actin probe (data

not shown). The autoradiograms shown are representative of experiments repeated at least four times in each case. The probe is identical to that used in Fig. 1.

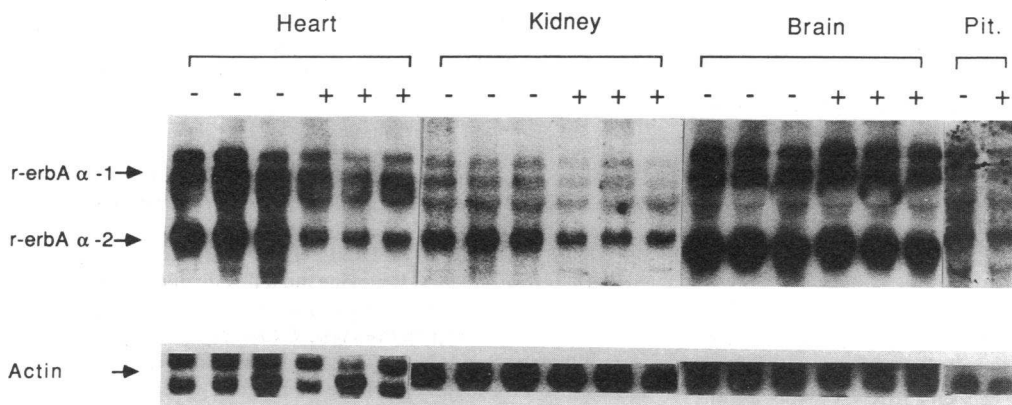


Figure 3. Effect of thyroidal status on r-erbA α mRNA levels in various rat tissues. Northern analyses were performed on RNA derived from tissues of PTU only (-) and PTU plus T₃-treated (+) rats. 10 μ g of total RNA was loaded in each lane. A cRNA probe that detects both r-erbA α -1 (5 kb) and r-erbA α -2 (2.6 kb) mRNAs was used (14). A 6-kb band, detected in all tissues examined, is discussed in the text. Faint nonspecific hybridization

with rRNA is also evident. Hybridization with the β -actin control is depicted. The r-erbA α probe is cRNA derived and includes the region common to the r-erbA α -1 and r-erbA α -2 cDNAs. The β -actin probe is a cDNA probe.

The maximum binding capacity increased slightly with T₃ treatment (0.55 ± 0.01 fmol/ μ g protein) compared with hypothyroid controls (0.42 ± 0.03 fmol/ μ g protein; $P = 0.1$). No significant changes in either affinity or maximum binding capacity were seen in nuclear extracts from hypo- and hyperthyroid liver, kidney, brain, or heart (data not shown).

Discussion

The recent identification of multiple forms of the T₃ receptor, as well as a related protein that appears to inhibit hormone action, has dictated a change in our understanding of the mechanisms of T₃ action. Since various subpopulations of T₃ receptors may be differentially regulated by T₃ (18), previous studies on receptor autoregulation in which total nuclear T₃ binding was measured may have failed to detect significant alterations in specific T₃ receptors.

Our results indicate that thyroidal status differentially affects the expression of the various r-erbA mRNAs, and possibly their products, and that these effects differ from tissue to tissue. In the pituitary gland, as in GH₃ cells (18), r-erbA β -1 levels increase, whereas r-erbA β -2 levels decrease with T₃ treatment. The increase in r-erbA β -1 mRNA appears to be greater than in the GH₃ cell model. This difference may relate

to characteristics of the GH₃ cell culture system or to differences in experimental protocol, such as the duration and magnitude of T₃ exposure. However, in kidney, heart, brain, and liver, the levels of r-erbA β -1 are unaffected by thyroidal status.

The levels of both r-erbA α species decrease with T₃ treatment in all tissues examined with the exception of brain. In each tissue, the magnitudes of the change in the two r-erbA α mRNA species were approximately the same. This downregulation of r-erbA α mRNAs is similar to what we have previously shown in GH₃ cells (14, 19). The magnitude of the r-erbA α mRNA decreases were greatest in heart and kidney and less in pituitary.

Table I summarizes the effects of thyroidal status on the r-erbA species in the rat tissues we have examined. Of note, the absence of a T₃ effect on any r-erbA mRNA in brain is consistent with its general lack of responsiveness to T₃ and may relate to the abundance of the mRNA encoding r-erbA α -2, a potential inhibitor of T₃ action (20). Table I also illustrates that the pituitary appears to be unique both in its expression of the r-erbA β -2 mRNA and the induction of the r-erbA β -1 mRNA with T₃ treatment. These data suggest a possible role for r-erbA β -2 in the regulation of r-erbA β -1 expression.

The slight increase in T₃ binding in pituitary nuclear extracts from T₃-treated rats is consistent with data from the Northern analyses, inasmuch as the changes in specific r-erbA mRNAs are in different directions. The decrease in T₃ binding seen in the GH cell model compared with the data we obtained in rat pituitary may relate to the relatively greater increase in r-erbA β -1 mRNA and more moderate decrease in r-erbA β -2 mRNA in this tissue in the whole animal. Since the total T₃

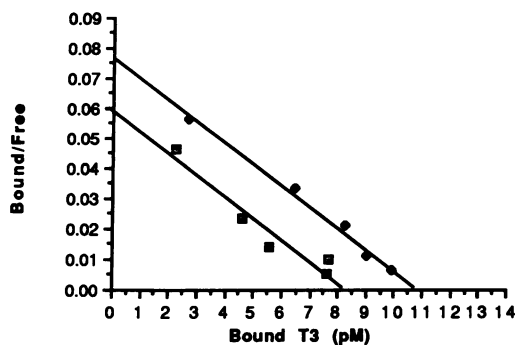


Figure 4. Effect of thyroidal status on T₃ binding in pituitary nuclear extracts. Scatchard plots from T₃ binding experiments on pituitary nuclear extracts derived from PTU only (hypothyroid, open squares) and PTU plus T₃-treated (hyperthyroid, solid squares) rats are shown. Each point represents the average of duplicate samples. The nuclear extracts are pooled from six animals in each case.

Table I. Effects of Thyroidal Status on r-erbA mRNA Levels

	r-erbA β -1	r-erbA β -2	r-erbA α -1	r-erbA α -2
Heart	No change	N/A	-65%	-86%
Kidney	No change	N/A	-69%	-63%
Liver	No change	N/A	*	*
Brain	No change	N/A	No change	No change
Pituitary	+341%	-43%	-22%	-22%

N/A, Not applicable; r-erb β -2 mRNA is not detected. *, Levels are too low to be quantitative.

receptor pool is composed of at least three c-erbA gene products, α -1, β -1, and β -2, the conflicting results in the literature on pituitary T₃ receptor autoregulation may be due to variations in experimental protocol, which could alter the effects on one or more of the receptor subpopulations. The correlation between r-erbA mRNA levels and receptor number may also be affected by posttranscriptional factors such as translational efficiency and/or protein $t_{1/2}$.

Although it is difficult to estimate relative abundances of different mRNAs by Northern analyses, it appears that r-erbA β -1 is more abundant than r-erbA α -1 in the tissues that we have examined. Thus, the lack of a difference in T₃ binding in hypo- versus hyperthyroid nonpituitary tissues correlates well with the lack of change in r-erbA β -1 mRNA levels. However, if the specific subpopulations of T₃ receptors serve different functions, such as interacting with different target genes, then the decrease in r-erbA α -1 mRNA could have great consequences for T₃ action despite the fact that total T₃ binding does not appear to change. Similarly, the regulation of the mRNA encoding the non-hormone binding r-erbA α -2 could be important for T₃ action, since the protein may function to inhibit T₃ responsiveness.

It is possible that factors other than thyroidal status influenced our results. Fasting that leads to significant weight loss, for example, has been shown to be associated with decreased hepatic T₃ receptor number (25). Although we did not specifically control for food intake, the two groups of rats in our study were of similar weight.

Autoregulation of receptors is a common biological phenomenon seen in higher organisms (26). Various members of the thyroid/steroid hormone receptor superfamily have been shown to either increase or decrease in response to treatment with their cognate hormones (27–33). Similar to what we have shown with T₃ receptors, the α and β retinoic acid (RA) receptors are differentially regulated by exposure to RA (31). Regulation of the estrogen and glucocorticoid receptors have been shown to be tissue specific (32, 33). Studies are in progress to determine if the effects of T₃ on r-erbA mRNA levels are at the transcriptional level, as is the case with the estrogen and β -RA receptors (30, 31).

Our present model of T₃ action includes the interaction of T₃ with multiple forms of its receptor, as well as a related protein that appears to inhibit T₃ action. The present studies demonstrate that experimental conclusions based on total T₃ nuclear receptor number may be misleading, since receptor subpopulations can be regulated differentially. Further work is clearly needed to determine the role that each of the r-erbA gene products plays in thyroid hormone action.

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