Effect of hypothyroidism and thyroid hormone treatment of the rat on hepatic Spot 14 and thyroxine binding prealbumin mRNAs

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Abstract. We have examined the influence of hypothyroidism and thyroid hormone replacement on hepatic levels of Spot 14 and thyroxine binding prealbumin mRNAs determined by dot hybridization and by Northern blot hybridization to specific complementary DNA probes. A marked reduction in Spot 14 mRNA was demonstrated in hypothyroidism, compared with the euthyroid state. T3 replacement of hypothyroid rats, using a wide dose range of T3 (1–20 μg) and 6 and 72 h time points, demonstrated no sustained effect at 6 h, but a dose-dependent stimulation of Spot 14 mRNA at 72 h after daily treatment with T3 was commenced. In contrast, no effect of hypothyroidism or T3 replacement on hepatic levels of thyroxine binding prealbumin mRNA was demonstrated, indicating the specificity of thyroid hormone action. T3 treatment of euthyroid rats was also associated with a dose-related stimulation of Spot 14 mRNA levels. The effect of hypothyroidism and T3 treatment of the rat on hepatic Spot 14 mRNA contrasts with divergent regulatory influences of thyroid status in the anterior pituitary and ventricular myocardium demonstrated using identical animal models, indicating the tissue specific influences of thyroid status.

Thyroid hormones exert regulatory effects on the growth, differentiation and metabolism of most tissues of higher organisms. There is overwhelming evidence that the cellular actions of thyroid hormones are mediated by nuclear receptors (1). High affinity nuclear binding sites for tri-iodothyronine have been demonstrated in most mammalian tissues and recent cloning of c-erb-A (c)DNA suggests that T3 receptors are encoded by these cellular proto-oncogenes (2). Multiple c-erb-A cDNAs have now been cloned and classified according to structure into two major groups, designated α and β, each class comprising several members (3). The function of these multiple thyroid hormone binding proteins is not clear, but differences in their behaviour and tissue distribution may provide an explanation for the tissue and gene specific regulatory actions of T3.

We have reported previously regulatory effects of thyroid status on gene expression in the rat anterior pituitary (4, 5) and ventricular myocardium (6). These studies have indicated the specificity of thyroid hormone action, with a biphasic (stimulatory then inhibitory) time-dependent effect of T3 treatment of the hypothyroid rat on thyrotrophin messenger RNAs in the pituitary, contrasting with simple stimulatory influences on growth hormone and prolaction mRNAs, and in the ventricular myocardium, opposite regulatory effects of thyroid status on α and β myosin heavy chain mRNAs.

The liver is a further important site of thyroid hormone action, with some 19 products showing selective changes in response to variations in thyroid status (7). Spot 14 is one such thyroid regulated gene expressed in the liver; the function of the 17 000 Dalton protein product of this gene has not been determined fully, but evidence points to a
role in the regulation of lipogenesis (1). Spot 14 gene expression represents a powerful model for the elucidation of thyroid hormone effects since its mRNA is stimulated rapidly by T₃ treatment of the hypothyroid rat (8). A further important gene product expressed in the liver is thyroxine binding prealbumin (TBPA). This protein represents a major thyroid hormone binding protein present in serum; the effect of thyroid status on TBPA gene expression has not been examined previously.

In the present study we have extended our observations of the regulatory influences of thyroid status in the pituitary and myocardium in a detailed investigation of the effect of hypothyroidism and T₃ treatment of the rat (at a variety of doses and time points) on hepatic levels of Spot 14 mRNA; influences on TBPA mRNA were examined in parallel to determine the gene specific actions of thyroid status in the liver.

**Material and Methods**

*Animals*

Female Wistar rats (200–500 g) were used in the study. Rats were rendered hypothyroid by treatment with ¹³¹Iodine (33 μCi/rat) and propylthiouracil (Sigma, Dorset, UK) in drinking water (60 mg/l) for 10 weeks. The development of hypothyroidism was confirmed by measurement of serum TSH concentrations (4). Hypothyroid rats were treated with T₃ (1, 2, 10 or 20 μg by daily sc injection) and rats killed by decapitation either 6 or 72 h from the time of the first injection of T₃. Results from T₃ treated animals (N = 10 in each group in dot hybridization study) were compared with those from untreated euthyroid rats (N = 10) and untreated hypothyroid rats (N = 10) using Student’s t-test for unpaired data. In addition, euthyroid rats were treated with T₃ (1, 2, 10, 20 or 100 μg) and killed 6 h later; results were compared with those from untreated controls (N = 10 in each group).

Similar treatment regimens were used in Northern blot hybridization studies, performed in order to define the specificity of hybridization of the cDNA probes used and to confirm the results of dot hybridization experiments.

**RNA preparation**

Sections of liver were removed from animals immediately after decapitation, frozen in liquid nitrogen and stored at −70°C before RNA preparation. RNA was prepared for dot hybridization by phenol chloroform extraction (9). Briefly, liver tissue was homogenized in 10 volumes of TRIS (10 mmol/l, pH 8.3), EDTA (1 mmol/l) and Nonidet (0.5% v/v). Proteinase K (30 μl, 2 g/l) was added to the homogenate before centrifugation, the supernatant (cytoplasm) transferred to an Eppendorf tube and adjusted to a final concentration of NaCl (100 mmol/l), TRIS (100 mmol/l) and sodium dodecyl sulphate (1% w/v) before extraction of RNA with phenol/isooamyl alcohol/chloroform (100/1/100 v/v/v). The final pellet was dissolved in 50 μl sterile distilled water and the concentration of RNA in each sample calculated from the absorbance at 260 nm.

Total hepatic RNA was prepared for Northern blotting studies by homogenization in isothiocyanate and centrifugation through caesium chloride (10, 11). Briefly, liver slices were homogenized in guanidinium isothiocyanate buffer (guanidinium isothiocyanate 4 mol/l, mercaptoethanol 100 mmol/l, N-lauryl sarcosine 0.5% v/v) and the homogenate, to which caesium chloride was added (to a final volume of 2.5 ml containing 1 g CsCl₂), was layered onto a 1.2 ml caesium chloride cushion (CsCl₂, 5.7 mol/l, EDTA 100 mmol/l, pH 7.0) and centrifuged at 100 000 × g overnight. The RNA pellet was resuspended in sterile distilled water, precipitated with ethanol and glyoxyalted before gel electrophoresis.

**Cytoplasmic dot hybridization**

Levels of Spot 14 and TBPA mRNAs in hepatic cytoplasm were determined by dot hybridization to radiolabelled cDNA probes using a method adapted from that of White & Bancroft (12). Samples of RNA were denatured by heating to 60°C in 6 × SSC (standard saline citrate: NaCl 900 mmol/l, trisodium citrate 90 mmol/l), 8% formaldehyde (v/v) for 15 min; 20 μl of each sample (approximately 50 μg RNA in each sample) was applied in duplicate with suction to a nylon hybridization membrane (Genescreen, NEN, Herts, UK) held in a 96-well manifold (Hybridbot apparatus, Bethesda Research Laboratories, Uxbridge, Middlesex, UK) and fixed by baking. Spot 14 and TBPA cDNAs (kindly supplied by H. Towle, MN, and P. Dickinsson, Melbourne, Australia) were labelled with [α²³P]dCTP to specific activities of 1–4 × 10⁶ cpm/μg DNA by nick translation (13). Filters were prehybridized and hybridized as described previously (14). After hybridization, the filters were washed twice in 2 × SSC at 65°C (5 min per wash), twice in 2 × SSC with sodium dodecyl sulphate (1% v/v) (1 h and 30 min washes) and twice in 0.1 × SSC (30 min per wash). Hybridization was visualised by autoradiography and quantitated by scanning densitometry. The results of densitometry were corrected for the amount of RNA applied in each dot. There was a linear relationship between hybridization determined by scanning densitometry of X-ray films and dilution of hepatic RNA samples, as in previous studies using similar techniques (14).

**Northern blot analysis**

Samples of denatured RNA (20 μg per lane) were electrophoresed through a horizontal 1.4% agarose gel in phosphate buffer (NaH₂PO₄ 125 mmol/l, Na₂HPO₄ 125...
mmol/l, pH 6.6; 80 V for 6 h) before blotting onto a hybridization membrane and hybridization with radiolabelled cDNAs as described above.

Results

Induction of hypothyroidism was associated with a marked fall in the hepatic level of Spot 14 mRNA determined by dot hybridization, when compared with the euthyroid state (Fig. 1). In animals examined 6 h after T3 treatment was initiated, marked effects of T3 replacement on Spot 14 mRNA were absent; a significant increase in Spot 14 mRNA was found 6 h after injection of T3 (2 μg) when levels were compared with those in untreated hypothyroid animals, but a similar change was not demonstrated with other doses at this time point. In contrast to the results at 6 h, there was a significant increase in Spot 14 mRNA at all doses studied 72 h after daily T3 treatment was commenced, with evidence of a dose-dependent stimulatory effect of T3. Regulatory effects of hypothyroidism and T3 replacement were specific to Spot 14 gene expression in that hepatic levels of TBPA mRNA were unaffected (Fig. 1).

The results of dot hybridization were confirmed by Northern blot hybridization of the Spot 14 cDNA to total RNA prepared from rat livers (Fig. 2). Hybridization of the Spot 14 cDNA to several species of RNA of size 1.3–1.5 kb was visualised, in agreement with previous studies (8). In accord with the results of dot hybridization experiments, a marked reduction in Spot 14 mRNA was found in hypothyroidism, compared with the euthyroid state, and stimulatory effects of T3 replacement at 72 h on Spot 14 mRNA were confirmed.

Fig. 1.
Analysis of hepatic levels of Spot 14 and TBPA mRNAs, determined by dot hybridization, in euthyroid (CON) and hypothyroid (HYPO) rats and 6 and 72 h after T3 administration (1, 2, 10 or 20 μg) to hypothyroid rats. The significance of results compared with values in euthyroid controls (▲ p < 0.05) and hypothyroid animals (★ p < 0.05, ★★ p < 0.005) is indicated.

Fig. 2.
Northern blot hybridization of hepatic RNA prepared from euthyroid (C), hypothyroid (H) rats 72 h after T3 treatment (1, 2, 10 or 20 μg) to a Spot 14 cDNA.
When the effect of T₃ treatment of euthyroid animals was examined, a dose-dependent increase in Spot 14 mRNA was evident (Fig. 3), with significant stimulatory actions of T₃ at 10, 20 and 100 μg doses. In contrast, there was no significant effect of T₃ treatment on TBPA mRNA, results again confirmed by Northern blot hybridization (Fig. 4). Northern blot hybridization of hepatic RNA to the TBPA cDNA revealed hybridization of the probe to a single species of RNA of appropriate size (15) and demonstrated no effect of T₃ treatment.

Discussion

In the present studies, marked regulatory effects of thyroid status on Spot 14 mRNA were evident, in contrast to a lack of effect on TBPA mRNA, indicating specificity of the actions of T₃. The results of dot hybridization experiments were in accord with those of Northern blot hybridization, which in turn confirmed hybridization of the cDNA probes employed to mRNA species of appropriate size (15, 16).

The observed reduction in Spot 14 mRNA in hypothyroidism is in accord with previous reports which describe an approximately 4-fold difference between relative levels of Spot 14 mRNA in the euthyroid and hypothyroid states (16). We demonstrated, in addition, stimulatory actions of T₃ replacement at 72 h on Spot 14 mRNA, but no sustained effect at 6 h. Previous studies of T₃ replacement of hypothyroid rats and Spot 14 mRNA have been confined to receptor saturating doses of T₃ (100 μg/100 g body weight or 200 μg given iv injection) (8, 16). In the present experiments, however, in which a wide dose range of T₃ was administered, marked stimulatory actions of T₃ were achieved only at 72 h after daily T₃ replacement was commenced; furthermore, a clear dose-dependent effect was evident at this time point. Stimulatory actions of T₃ treatment in the euthyroid state were also demonstrated in the present studies, in accord with a report of a 2-fold increase in Spot 14 mRNA 1.5 h after injection of T₃ (200 μg) in the euthyroid rat (17). In contrast to the absence of a sustained effect of T₃ treatment of the hypothyroid rat at 6 h, a dose-dependent stimulation of Spot 14 mRNA was induced in the euthyroid rat at this time point. The explanation for this discrepancy is not clear; differences in absorption of sc administered T₃ between groups of animals should be considered.

The demonstrated effects of thyroid status on hepatic levels of Spot 14 mRNA are consistent with direct influences on transcription of the Spot 14 gene, although effects on the half-life of Spot 14 mRNA have not been excluded. A recent report has demonstrated T₃ effects on the rate of Spot 14 gene transcription, suggesting that steady state le-

![Fig 3](image-url)

Analysis of hepatic levels of Spot 14 mRNA, determined by dot hybridization, in euthyroid rats (CON) and 6 h after T₃ treatment (1, 2, 10, 20 or 100 μg). The significance of results compared with values in euthyroid controls (* p < 0.05, ** p < 0.005) is indicated.
levels of mRNA reflect influences on transcripton, rather than mRNA stability (18).

Despite the observation that at least 19 gene products expressed in the liver are regulated by changes in thyroid status (7), with 11 gene products being stimulated by T3 administration to the hypothyroid rat, 7 inhibited, and one showing a biphasic response, no effect of hypothyroidism or T3 treatment on TBPA mRNA was evident in the present studies, suggesting that TBPA synthesis is not thyroid hormone regulated.

It is interesting to contrast the results of our studies of regulation of Spot 14 mRNA with those of our previous studies, using identical animal models, of gene expression in the anterior pituitary and ventricular myocardium. In these studies, divergent and highly specific stimulatory or inhibitory effects of hypothyroidism and T3 replacement on expression of the TSH β and α subunit, GH, prolactin, and α and β myosin heavy chain genes were evident, which contrasted with the demonstrated effects on Spot 14 and TBPA mRNAs. The explanation for these tissue and gene specific influences of thyroid status is unclear. Further characterization of the transcriptional regulatory properties and tissue distribution of the multiple c-erb-A or T3 receptor proteins described may provide insight into this fundamental question.

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**Fig. 4.**
Northern blot hybridization of hepatic RNA from euthyroid (C), and T3 treated rats (6 h after administration of T3 1, 2, 10 or 20 μg) to a thyroxine binding prealbumin cDNA.

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