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Thyroid Hormone Regulates Transcription of the Gene for Cytosolic Phosphoenolpyruvate Carboxykinase (GTP) in Rat Liver†

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ABSTRACT: Using an in vitro assay with isolated rat nuclei, we have determined that thyroid hormone causes a 4–6-fold increase in the synthesis of mRNA coding for phosphoenolpyruvate carboxykinase. Proportional changes were seen in the steady-state cytosolic mRNA levels for phosphoenolpyruvate carboxykinase. Dibutyryladenosine cyclic 3′,5′-monophosphate, which stimulates transcription of the phosphoenolpyruvate carboxykinase gene in normal rats, remained effective in hypo- or hyperthyroid animals. The effect of epinephrine on transcription of the gene for phosphoenolpyruvate carboxykinase appears to be modulated by thyroid hormone.

Phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) is a key regulatory enzyme in gluconeogenesis. The regulation of enzyme levels is complex, but increasing evidence points to gene transcription as the major point of control in governing cellular enzyme activity. Insulin (Granner et al., 1983), cAMP, and glucocorticoids (Lamers et al., 1982) have been shown to regulate the transcription of the gene for phosphoenolpyruvate carboxykinase.

It has recently been shown that altered thyroid status produces marked changes in the activity and synthesis rate of phosphoenolpyruvate carboxykinase (Sibrowski et al., 1982; Muller et al., 1982). In the present paper we deomonstrate that thyroid hormone increases the transcription rate of the phosphoenolpyruvate carboxykinase gene. This leads to elevated steady-state levels of cytosolic mRNA and hence enzyme synthesis.

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Materials and Methods

Male Sprague-Dawley rats, 200–250 g, were obtained from Zvic-Miller (Pittsburgh, PA). Thyroidectomy was performed by the supplier, and rats wer allowed to recover for 2 weeks before treatment.

Isotopes. [^{32}P]UTP (760 Ci/mmol) and [^{32}P]CTP (800 Ci/mmol) were obtained from New England Nuclear, Boston, MA.

In Vitro Transcription. Details of the in vitro transcription assay have been previously described (Lamers et al., 1982). Rats were starved 48 h, refed glucose (5 g/kg of body weight), and after 2 h injected with saline, dibutyryladenosine cyclic

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Table I: Effect of Thyroid Status on the Transcription Rate of the Gene for Cytosolic Phosphoenolpyruvate Carboxykinase[a]

treatment	tot [^{32}P]RNA synthesized (cpm × 10^{-6})/10^7 nuclei	pPCK10 (cpm)	pPCK10 (ppm)	pALB (cpm)	pALB (ppm)
euthyroid	2.55 ± 0.3	1065 ± 23	499 ± 23	360 ± 32	315 ± 37
thyroidectomized	2.59 ± 0.45	671 ± 38	353 ± 15	206 ± 104	200 ± 109
T_3 treated	3.55 ± 0.34	6006 ± 875	2089 ± 248	440 ± 76	294 ± 64

[a] Nuclei were isolated from normal rats, from animals 2 weeks after thyroidectomy, or from normal rats treated 7–9 days with thyroid hormone (100 μg/kg of body weight per day). All rats were then starved for 48 h, refed glucose (5 g/kg of body weight), and killed 2 h later. The syntheses of specific phosphoenolpyruvate carboxykinase (pPCK10) and albumin (pALB) [^{32}P]RNA were quantitated as described under Materials and Methods. (parts per million) = [cpm (cDNA) − cpm (pBR322)]/(total [^{32}P]RNA × 10^{-6}) × 1.08 × (hybridization/efficiency). Background binding of [^{32}P]UTP to pBR322 was 388 ± 33 cpm, and hybridization efficiency (determined by including a [^{3}H]cRNA in all samples) was 36% ± 1.2%. Results are means ± SE of four determinations. The value 1.08 is a factor that corrects for the size of pPCK10 vs. the full length of the message (2.8/2.6 kb).

3′,5′-monophosphate (Bt_2cAMP) (15 mg/kg, interperitoneally), or L-epinephrine (250 μg/kg, subcutaneously) (both from Sigma, St. Louis, MO). Thirty minutes after treatment, animals were killed, their livers rapidly removed and homogenized in 0.3 M sucrose, 10 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH 7.5, and 5 mM DTT, and nuclei subsequently purified by ultracentrifugation through 2.3 M sucrose. Purified nuclei were counted by phase contrast microscopy, and (1–3) × 10^7 nuclei were used per 200-μL assay. A modified transcription medium containing 12.5% glycerol, 2 mM $MnCl_2$, 0.6 mM $MgCl_2$, 0.05 mM disodium ethylenediaminetetraacetate (Na_2EDTA), 50 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (NaHepes), pH 7.5, 100 mM KCl, 4 mM DTT, 0.5 mM CTP, 0.5 mM GTP, 1.0 mM ATP, 8.8 mM creatine phosphate, and 0.04 mg/ml creatine phosphokinase was used in all studies. [^{32}P]RNA synthesis was initiated by the addition of 25 μCi of [^{32}P]UTP and allowed to continue for 20 min at 25 °C. [^{32}P]RNA was purified by treatment of the nuclei with DNase (Worthington, Freehold, NJ) and proteinase K (EM Reagents, Darmstadt, FDR) as previously described (Lamers et al., 1982). Quantitation of specific transcripts was assessed after 36–48 h of hybridization of the [^{32}P]RNA to nitrocellulose-immobilized DNA. Each hybridization vial contained separate nitrocellulose filters with 2 μg of pPCK10, a 2.6-kb phosphoenolpyruvate carboxykinase cDNA that has been previously described (Yoo-Warren et al., 1983), 2 μg of pALB20F1, a 1.0-kb rat albumin cDNA (Kioussis et al., 1979), and 2 μg of pBR322, which served as a "background" filter. All plasmid DNA had been linearized and denatured prior to binding to nitrocellulose. After washing and RNase treatment, filter-bound [^{32}P]RNA was eluted and quantitated by liquid scintillation counting.

Preparation of Poly(A)$^+$ RNA. Cytoplasmic RNA was prepared from the livers of the same animals used in the transcription studies by extraction with guanidinium thiocyanate/guanidine hydrochloride (Chirgwin et al., 1979). Polyadenylated RNA was further purified chromatographically on oligo(dT) columns (Bio-Rad, Richmond, CA) as described by Maniatis et al. (1982).

RNA Dot Blots. One to eight micrograms of poly(A)$^+$ RNA was spotted on nitrocellulose filters in 2.5× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) with the Mini Fold I apparatus. Each sample contained sufficient tRNA (Worthington) to bring the total RNA spotted to 10 μg. Samples were washed briefly with 6× SSC, and the filters were baked for 2 h at 80 °C.

pPCK10 was nick translated with [^{32}P]dCTP, essentially as described by Maniatis et al. (1982), and 2 × 10^7 cpm were hybridized for 36 h with the RNA bound to nitrocellulose filters in 50% formamide, 0.8 M NaCl, 20 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.8, 4 mM EDTA, and 0.2% sodium dodecyl sulfate. After hybridization, filters were washed successively in 2× SSC and 0.1% SDS at room temperature and finally in 0.1× SSC and 0.1% SDS at 55 °C. Phosphoenolpyruvate carboxykinase mRNA was quantitated densitometrically (Goodrich et al., 1984).

Results

Administration of T_3 for 7–9 days resulted in a 4-fold increase in the transcription rate of the phosphoenolpyruvate carboxykinase gene as compared to transcription of the gene in nuclei from livers of euthyroid animals (499–2089 ppm) (Table I). Consistent with this finding is the 35% reduction in the basal trancription rate of the gene noted in hepatic nuclei from thyroidectomized animals. There is a 6-fold induction of phosphoenolpyruvate carboxykinase gene transcription by thyroid hormones (comparing thyroidectomized vs. T_3-treated animals). These changes are not due to a general alteration in the overall rate of transcription since total [^{32}P]RNA synthesis is increased only 40% by T_3 treatment. Also, no discernible affect was seen in this parameter following thyroidectomy. With T_3 treatment, albumin mRNA synthesis is not increased above the levels noted in the livers of euthyroid rats; however, an approximate 50% decrease in albumin mRNA synthesis was also noted following thyroidectomy although there was considerable variation in this measurement. This is consistent with previous findings that thyroid hormone appears necessary for maintenance of basal transcriptional activity of several genes (Seelig et al., 1981).

Phosphoenolpyruvate carboxykinase synthesis is greatly induced by cAMP and also increased by epinephrine (Cimbala et al., 1981). We have shown that the induction of phosphoenolpyruvate carboxykinase following Bt_2cAMP treatment is due to a rapid increase in the transcription rate of the gene; maximum rates of transcription are obtained between 20 and 30 min (Lamers et al., 1982). We determined the effect of the combination of thyroid hormone and Bt_2cAMP or epinephrine on the transcription of the phosphoenolpyruvate carboxykinase gene. As expected, Bt_2cAMP stimulated the transcription of the phosphoenolpyruvate carboxykinase gene in euthyroid rats nearly 10-fold in 30 min (Figure 1). Bt_2cAMP stimulated transcription 6-fold in thyroidectomized animals, an increase comparable to euthyroid animals. However, the maximally Bt_2cAMP-induced rate of transcription was much lower in thyroidectomized rats than in normal animals (2445 vs. 4590 ppm). Bt_2cAMP was effective in inducing transcription of the phosphoenolpyruvate carboxykinase gene in T_3-treated rats to a rate similar to that noted for nuclei from the livers of Bt_2cAMP-treated euthyroid animals.

Similar results were noted for nuclei from the livers of rats of various thyroid states that had been treated with epinephrine. This catecholamine doubled the rate of phosphoenolpyruvate carboxykinase gene transcription in euthyroid animals and caused a 6-fold induction in thyroidectomized rats.

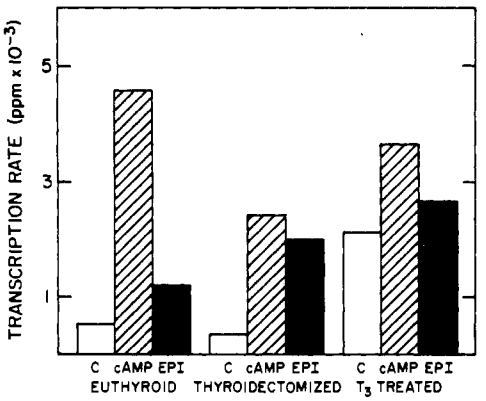

FIGURE 1: Effects of Bt_2cAMP (15 mg/kg) and epinephrine (250 μg/kg) on synthesis of phosphoenolpyruvate carboxykinase mRNA. Rats of various thyroid status were treated as described in Table I and given Bt_2cAMP or epinephrine 30 min prior to sacrifice. Results presented are the average of two to four determinations.

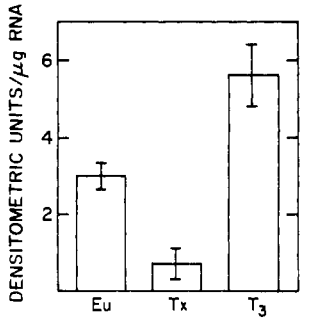

FIGURE 2: Analysis of cytosolic phosphoenolpyruvate carboxykinase mRNA. Poly(A)$^+$ RNA was prepared as described under Materials and Methods, spotted onto nitrocellulose filters, and hybridized with ^{32}P-labeled pPCK10. Phosphoenolpyruvate carboxykinase was quantitated by densitometry. The assay was linear in the range of RNA (1–8 μg) used in these studies. Results are means and SEM of eight determinations.

However, epinephrine caused only a modest, but consistent, increase (25%) in the transcription rate of the gene in T_3-treated rats. The relatively small changes in the transcription rate of the phosphoenolpyruvate carboxykinase gene noted after the administration of Bt_2cAMP or epinephrine to rats treated with T_3 may be accounted for by the much higher basal transcription rates in these animals.

Seitz and co-workers (Sibrowski et al., 1982; Muller et al., 1982) have reported that the synthesis rate of cytosolic phosphoenolpyruvate carboxykinase in rat liver is increased by thyroid hormone, suggesting an increase in enzyme mRNA. In order to determine if the increase in transcription of the gene noted after thyroid hormone administration was accompanied by a parallel increase in the levels of phosphoenolpyruvate carboxykinase mRNA, we measured the abundance of enzyme mRNA by hybridization to pPCK10, a phosphoenolpyruvate carboxykinase cDNA. Since phosphoenolpyruvate carboxykinase mRNA is polyadenylated (Iynedjian & Hanson, 1977), we performed these analyses on purified poly(A)$^+$ RNA to maximize signal intensity. T_3 caused a marked accumulation and thyroidectomy a decrease in the steady-state level of phosphoenolpyruvate carboxykinase mRNA (Figure 2). Combined with the transcription data, it seems clear that these changes are due to alterations in the synthesis of phosphoenolpyruvate carboxykinase mRNA although changes in the rate of its degradation cannot be ruled out.

Discussion

In the present study we demonstrate that thyroid hormone regulates the transcription of the gene for phosphoenolpyruvate carboxykinase. These results are consistent with earlier reports (Sibrowski et al., 1982; Muller et al., 1982) of thyroid hormone induction of enzyme synthesis. The predominant, if not exclusive, location of thyroid hormone receptors in the nucleus is suggestive of a direct effect on gene transcription. Studies demonstrating T_3 induction at the level of transcription have thus far focused on the growth hormone gene. There have been several reports demonstrating a direct effect of thyroid hormone on transcription of the growth hormone gene in cultured pituitary tumor cells (Evans et al., 1982; Spindler et al., 1983; Yafee & Samuels, 1984). The present study extends these observations with an in vivo system and a gene that is very actively transcribed, phosphoenolpyruvate carboxykinase. It is important to note that we cannot exclude the possibility that thyroid hormones administered to rats may act via an intermediate, although this seems doubtful. From the data in Figure 2 it is apparent that phosphoenolpyruvate carboxykinase mRNA increases by a factor of 5 from hypothyroid to euthyroid and by a factor of 2 between euthyroid and hyperthyroid states. The changes in transcription rates for these two transitions are 1.4 and 4.2, respectively. Thus, it remains a possibility that thyroid hormone may mediate alterations in message stability as a means of regulating levels of phosphoenolpyruvate carboxykinase.

Bt_2cAMP remained a potent inducer of phosphoenolpyruvate carboxykinase in either hypo- or hyperthyroid animals, precluding a permissive role of thyroid hormone in the effect of the cyclic nucleotide. On the other hand, induction of phosphoenolpyruvate carboxykinase mRNA synthesis by epinephrine, an action presumably mediated via α-adrenergic receptors in rat liver (Exton, 1977), was altered by thyroid status. Epinephrine caused a 5-fold stimulation in the transcription rate of the phosphoenolpyruvate carboxykinase gene in hypothyroid, a 2.5-fold increase in euthyroid rats, and 0.25-fold increase in hyperthyroid animals. Hyperthyroidism causes a marked decrease in myocardial α-adrenergic receptors (Ciaraldi & Marinette, 1977; Williams & Lefkowitz, 1979), and hypothyroidism has the opposite effect. The decreased response to epinephrine we have observed may be due to thyroid hormone mediated alterations in hepatic catecholamine receptors.

Five hormones have thus far been shown to regulate the transcription of the gene for phosphoenolpyruvate carboxykinase. Three of these hormones, glucagon, epinephrine, and insulin, act via membrane-bound receptors. The remaining two, T_3 and glucocorticoids, act via cytoplasmic and/or nuclear receptors. Is there a common feature in the phosphoenolpyruvate carboxykinase gene that is responsive to all of these affectors, or are there independent regulatory domains within the gene? We have recently determined that a 520-bp, 5′-flanking region of the phosphoenolpyruvate carboxykinase gene is responsible for cAMP inducibility (Wynshaw-Boris et al., 1984). Whether thyroid hormones alter gene expression by a receptor-mediated interaction with specific sequences within this regulatory region of the phosphoenolpyruvate carboxykinase gene is currently under investigation.

Registry No. EC 4.1.1.32, 9013-08-5; Bt_2cAMP, 362-74-3; L-T_3, 6893-02-3; epinephrine, 51-43-4.

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Kallikrein-Related mRNAs of the Rat Submaxillary Gland: Nucleotide Sequences of Four Distinct Types Including Tonin†

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ABSTRACT: We have determined the nucleotide sequence of four submaxillary gland mRNAs, designated PS, S1, S2, and S3, that encode kallikrein and kallikrein-like serine proteases. The four enzymes share between 74% and 86% amino acid sequence identity and are identical in length with the exception of single two amino acid deletions in the S2 and S3 enzymes. The PS enzyme appears to be a true tissue kallikrein. The S1 enzyme shares 86% amino acid sequence homology with the PS enzyme and retains key amino acid residues thought to be primary determinants of kallikrein cleavage specificity. The S2 enzyme is rat submaxillary tonin. The amino acid sequence of the S3 enzyme is identical with tonin at 84% of its amino acid positions and retains the same amino acid substitutions at positions likely to determine substrate cleavage preferences.

Tissue kallikrein is a generic term for a family of serine proteases of closely related structure which are found in many mammalian tissues. The proteases of this family are more specific in cleaving substrates than other simple serine proteases of the pancreatic type. Each has very low activity on substrates such as collagen and casein (Fiedler, 1979; Bothwell et al., 1979). True tissue kallikreins (EC 3.4.21.8) are family members that cleave kininogen to selectively release the vasoactive peptides bradykinin or lysylbradykinin (Seki et al., 1972; Alhenc-Gelas et al., 1981; Yamada & Erdos, 1982). The enzymes are acidic glycoproteins comprising several forms with molecular weights between 25 000 and 40 000 [reviewed by Fiedler (1979)]. Isozymes may vary among tissues but appear to differ only in their carbohydrate content (Fiedler, 1979). The vasodilatory effect produced by the kinin product of kininogen cleavage by kallikrein may play a role in the regulation of local blood flow in exocrine glands (Hilton, 1970; Hilton & Jones, 1968; Carretero & Scicli, 1981). Tissue kallikreins are distinct from plasma kallikrein, a high molecular weight, complex protease involved in the intrinsic blood coagulation system (Movat, 1979).

The extended family of kallikrein-like enzymes includes proteases of closely related structure with altered specificities and physiological roles not limited to the genesis of kinins. The murine submaxillary gland contains the highest level of kallikrein-like enzymes among the many tissues investigated (Frey et al., 1968; Brandtzaeg et al., 1976). The polypeptide substrates for these enzymes also are found in the submaxillary gland (Barka, 1980). Members of the kallikrein family include tonin, which cleaves angiotensin II from angiotensinogen (Boucher et al., 1974), the γ subunit of nerve growth factor

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