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Rapid induction of a specific nuclear mRNA precursor by thyroid hormone

(regulation of gene expression/cDNA cloning)

PREMA NARAYAN, CHEN W. LIAW, AND HOWARD C. TOWLE*

Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455

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ABSTRACT **Administration of thyroid hormone to the thyroidectomized rat results in a rapid and dramatic increase in the relative amount of hepatic mRNA coding for spot 14—a translational product with an approximate M_r of 17,500 and isoelectric point of 4.9. We have now isolated a cDNA clone containing sequences homologous to this thyroid hormone-responsive mRNA. Two distinct mRNA species that differed by 200 nucleotides in length were found to be capable of hybridizing to the cDNA probe. Both mRNA species were proportionally elevated in relative concentration in rats with increasing plasma levels of thyroid hormone. The earliest change in the levels of mature mRNA occurred at 20 minutes following thyroid hormone treatment of the thyroidectomized rat. Analysis of nuclear RNA revealed a single higher molecular weight species that was homologous to spot 14 mRNA. An increase in the nuclear level of this putative precursor occurred by 10 minutes following thyroid hormone administration, a time preceding the earliest change in mature mRNA. Thus, thyroid hormone appears to act at least in part at a nuclear level in altering the cellular concentration of this mRNA species. The rapidity of this change suggests that it may reflect a direct response to the binding of thyroid hormone to its nuclear receptor.**

Many of the physiological actions of thyroid hormones are thought to be mediated by binding of hormone to a specific nuclear receptor in target tissues (1–3). This interaction is hypothesized to result in alterations in the expression of specific genes through unknown mechanisms. Evidence for this hypothesis has come largely from studies of several specific mRNAs that are induced by thyroid hormones, including pituitary growth hormone (4–6) and hepatic malic enzyme (7, 8). Recently, we have examined the pleiotropic effects of thyroid hormone on the hepatic mRNA population of the rat and shown that 20 of 230 mRNA species detected were responsive to alterations in plasma 3,5,3′-triiodo-L-thyronine (T3) levels (9). However, in no case has the change in the concentration of specific mRNA been shown to be the direct consequence of the thyroid hormone–receptor interaction. In fact, many of the mRNAs that are affected by thyroid hormones respond after lag times of several hours following hormone administration (10) and thus could be responding to unknown intermediates that are the actual targets of the initial hormone action.

In this regard, we have focused our attention on a recently identified T3-responsive mRNA in rat liver (9–11). This mRNA encodes a translational product, arbitrarily designated spot 14, with an approximate M_r of 17,500 and isoelectric point of 4.9. By cell-free translational assay, the relative level of hepatic mRNA encoding spot 14 was found to be increased in response to either elevated plasma T3 levels or by feeding a high-carbohydrate, fat-free diet to the rat. This sensitivity to both T3 and high-carbohydrate diet is a property that spot 14 shares with several hepatic mRNAs coding for "lipogenic" enzymes, including malic enzyme mRNA (7, 8) and 6-phosphogluconate dehydrogenase mRNA (12). The induction of spot 14 mRNA by T3 has also been shown to occur in primary cultures of rat hepatocytes, indicating hormone is acting at least in part directly on the liver (13). Of particular interest was the rapidity with which spot 14 mRNA responded to thyroid hormone; a 4- to 5-fold increase was detected by 1.5 hr following T3 treatment of a thyroidectomized rat (11). This change represented the earliest measured response of a hepatic mRNA to T3. We now report that by using a more sensitive hybridization assay, a change in the amount of the nuclear precursor for this mRNA can be detected by 10 min after T3 treatment. This change precedes the earliest changes in cytoplasmic mRNA encoding spot 14.

METHODS

Treatment of Animals. Male Sprague–Dawley rats weighing 200–250 g were used in all experiments. Animals were rendered hypothyroid by surgical thyroidectomy (commercially performed) followed by administration of 0.1 mCi (1 Ci = 37 GBq) of ^{131}I and were used after cessation of weight gain for a period of 2 consecutive wk. For experiments on the time course of induction, animals were injected intravenously with a single dose of 100 μg of T3 per 100 g of body weight. Chronic hyperthyroidism was induced by intraperitoneal injection of 20 μg of T3 per 100 g of body weight per day for 7 days. Animals were fed either standard chow diet (Purina) or a high-carbohydrate (58% sucrose), fat-free diet (fat-free test diet, ICN) ad lib for 7 days.

Construction of a Plasmid cDNA Library to Rat Hepatic mRNA. Hepatic poly(A)-containing RNA was extracted from hyperthyroid rats fed high-carbohydrate, fat-free diet as described by Towle *et al.* (7). Single-stranded cDNA synthesized from this RNA was fractionated on a 5–20% alkaline sucrose gradient (14). Fractions containing cDNA greater than 500 nucleotides were pooled and used in the synthesis of double-stranded cDNA, which was subsequently treated with S1 nuclease and tailed with dCMP residues (15). This DNA was annealed with *Pst* I-cut pBR322 DNA tailed with dGMP residues (16) and used to transform *Escherichia coli* RRI by a modification of the procedure of Villa-Komaroff *et al.* (17).

Screening of cDNA Library by Differential Hybridization. Plasmid DNA was isolated from transformants by the alkaline extraction procedure of Birnboim and Doly (18). Equivalent amounts of DNA samples (0.25 μg) were fixed, in triplicate, onto two identical nitrocellulose filters by the method of Kafatos *et al.* (19). Filters were prehybridized at 65°C for 5 hr in a solution containing 0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone, 0.9 M NaCl, 0.09 M sodium citrate, 0.1% sodium dodecyl sulfate, 20 mM sodium

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Abbreviation: T3; 3,5,3′-triiodo-L-thyronine.

*To whom reprint requests should be addressed.

phosphate (pH 6.5), 50 μg of poly(A) per ml, and 50 μg of yeast tRNA per ml (20). Duplicate filters were hybridized at 65°C for 18 hr with 5×10^5 cpm of ^{32}P-labeled, single-stranded cDNA synthesized from poly(A)-containing RNA isolated from either thyroidectomized, chow-fed rats or hyperthyroid rats fed the high-carbohydrate, fat-free diet. Filters were washed as described (20) and exposed to Kodak XAR-5 film for 48 hr. Any cDNA plasmid yielding different intensities for the hybridization signals from the duplicate filters would contain sequences for an mRNA species present in altered concentrations in the two states.

Hybrid-Selected Translation. Plasmid DNA was isolated as above (18) and purified by sedimentation in a CsCl/ethidium bromide gradient (21). Plasmid DNA was linearized with *Eco*RI and 10 μg was fixed onto a 25-mm^2 piece of nitrocellulose (19). Hybrid-selection was carried out by a modification of the method of Ricciardi *et al.* (22). Poly(A)-containing RNA was hybridized to filters in a buffer containing 10 mM Pipes (pH 6.4), 0.4 M NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 1 mg of yeast tRNA per ml, and 50% deionized formamide for 20 hr at 42°C. Washing was performed by resuspending the filter in 1 ml of hybridization buffer at 42°C for 5 min and repeated 12 times. Hybridized RNA was eluted in 0.1 ml of 10 mM Tris·HCl, pH 7.6/0.1 mM EDTA at 95°C for 1 min and precipitated with ethanol in the presence of 20 μg of calf liver tRNA per ml. RNA samples were translated (12) and analyzed by two-dimensional gel electrophoresis (9).

Analysis of Cellular RNA. Total cellular RNA was isolated by a modification of the guanidine hydrochloride method of Deeley *et al.* (23). RNA samples were subjected to electrophoresis on a 1.5% agarose gel containing 5 mM methylmercuric hydroxide (24). After electrophoresis, gels were treated as described by Alwine *et al.* (25) and RNA was then transferred to nitrocellulose (26). Hybridization was performed for 24 hr at 42°C with $1–2 \times 10^7$ cpm of ^{32}P-labeled, nick-translated pS14-cl plasmid in a solution containing 50% formamide, 0.1% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone, 0.75 M NaCl, 0.075 M sodium citrate, 50 mM sodium phosphate (pH 6.5), 0.1% sodium dodecyl sulfate, 100 μg of denatured salmon sperm DNA per ml, and 100 μg of yeast tRNA per ml (20).

For measurement of changes in the mRNA levels in the various states, total cellular RNA was denatured by incubation for 15 min at 65°C in the presence of 7.4% (vol/vol) formaldehyde in 0.9 M NaCl/0.09 M sodium citrate, pH 7.0 (27). Various dilutions of RNA were fixed onto nitrocellulose and hybridized with ^{32}P-labeled pS14-cl insert DNA as described above. Radioactivity hybridized to each sample was quantified directly by cutting and counting spots.

Analysis of Nuclear RNA. Rat liver nuclei were isolated as described by Roop *et al.* (28). Nuclei were lysed at room temperature in a buffer containing 2% sodium dodecyl sulfate, 7 M urea, 0.35 M NaCl, 1 mM EDTA, and 10 mM Tris·HCl (pH 8.0). Nucleic acids were extracted with an equal volume of a mixture of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous layer was removed and the organic layer was reextracted with 0.5 vol of the lysis buffer. The aqueous layers were pooled and reextracted with an equal volume of the organic mixture. The aqueous phase was removed and extracted with 0.5 vol of chloroform/isoamyl alcohol (24:1). To separate RNA from DNA, solid CsCl was added to the final aqueous phase to a concentration of 1.4 M. This solution was layered over a 2-ml cushion of 5.7 M CsCl/0.1 M EDTA and centrifuged at 100,000 × *g* in an SW 41 rotor for 23 hr at 20°C. The RNA was dissolved in 10 mM Tris·HCl, pH 7.6/0.1 mM EDTA and precipitated with ethanol. Nuclear RNA samples were subjected to electrophoresis on 1.8% agarose gels containing 2.2 M formaldehyde (29). RNA was transferred to nitrocellulose (26) and hybridized with pS14-cl insert DNA as described above. The relative levels of nuclear precursor in the various samples were determined by scanning autoradiograms densitometrically.

RESULTS

Cloning of the cDNA for Spot 14. Poly(A)-containing RNA was extracted from the livers of rats that had been rendered hyperthyroid by T3 injection and fed the high-carbohydrate, fat-free diet. In this state, mRNA coding for spot 14 has been estimated to represent close to 0.5% of the cell-free translational activity of hepatic RNA (10). This poly(A)-containing RNA population was enzymatically converted into double-stranded cDNA and inserted into the *Pst* I site of the plasmid pBR322 by using the dG-dC tailing procedure. Following cloning, individual transformants were screened by a differential hybridization assay to identify those containing cDNA sequences homologous to mRNA species that are altered in cellular concentration following the hormonal and dietary treatment (see *Methods*). Transformants that were positive in this first assay were subsequently analyzed by the technique of hybrid-selected translation followed by two-dimensional gel electrophoresis. In this manner, a cDNA clone was identified that contained sequences homologous to an

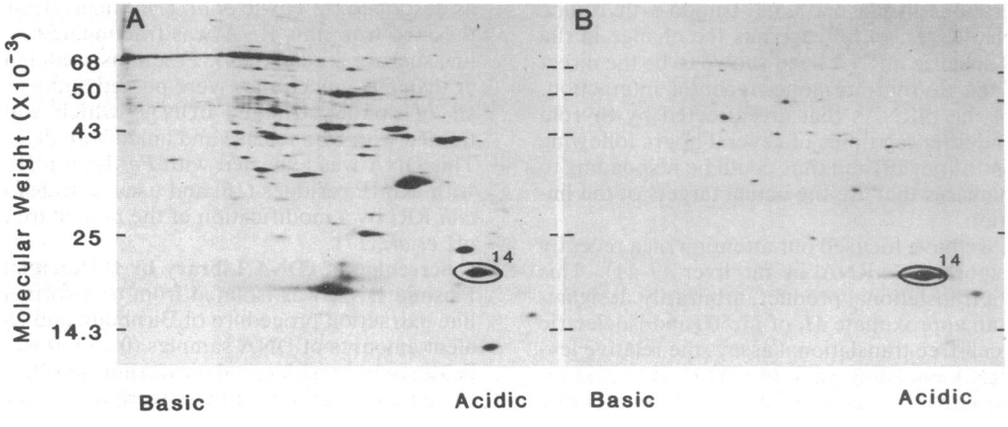

FIG. 1. Identification of a cDNA clone homologous to spot 14 mRNA by hybrid-selected translation and two-dimensional gel electrophoresis. Hepatic poly(A)-containing RNA (3 μg) from a hyperthyroid rat fed high-carbohydrate diet was hybridized to nitrocellulose-bound recombinant plasmid pS14-cl DNA (10 μg). The hybridized mRNA was eluted, translated in the mRNA-dependent reticulocyte lysate system with [^{35}S]methionine, and analyzed by two-dimensional gel electrophoresis. (*A*) Total translational products from poly(A)-containing RNA. (*B*) Translational product encoded by mRNA hybrid selected with pS14-cl. Note that the background of light spots could be largely removed by further washing of the filter before elution but was retained to help orient the position of the hybrid-selected product.

mRNA that was increased in concentration by the hormonal and dietary treatment and that coded for a translational product that migrated to the same position as spot 14 (Fig. 1). The cDNA insert of this plasmid was 720 base pairs long including dG-dC tails. This plasmid has been designated pS14-cl.

Measurement of mRNA Levels for Spot 14. To obtain an estimate of the size and quantity of mRNA coding for spot 14, electrophoretic analysis of rat liver RNA was performed. Total cellular RNA was isolated from animals of different thyroidal states (hypothyroid, euthyroid, hyperthyroid) maintained on either chow or high-carbohydrate, fat-free diet. Equal amounts of these RNA samples were then subjected to electrophoresis on an agarose gel under denaturing conditions, transferred to nitrocellulose, and hybridized to ^{32}P-labeled DNA of pS14-cl. Two distinct mRNA species were found to hybridize to pS14-cl—one of ≈1325 nucleotides in length and the other of ≈1525 nucleotides (Fig. 2). Both mRNA species were present in all of the dietary and hormonal states examined (although different exposure times were necessary to adequately resolve them in varying states). Furthermore, both mRNA species increased in concentration following either T3 treatment or switching to the high-carbohydrate, fat-free diet.

To obtain a more quantitative value for the relative amount of mRNA coding for spot 14 in each state, a second filter hybridization method (dot blot hybridization) was used. Varying amounts of total cellular RNA isolated from each of the above states were directly fixed onto nitrocellulose and hybridized to ^{32}P-labeled DNA from pS14-cl. Following hybridization, the amount of hybridized DNA was determined by direct counting and plotted as a function of RNA concentration (Fig. 3). The slope of this plot was found

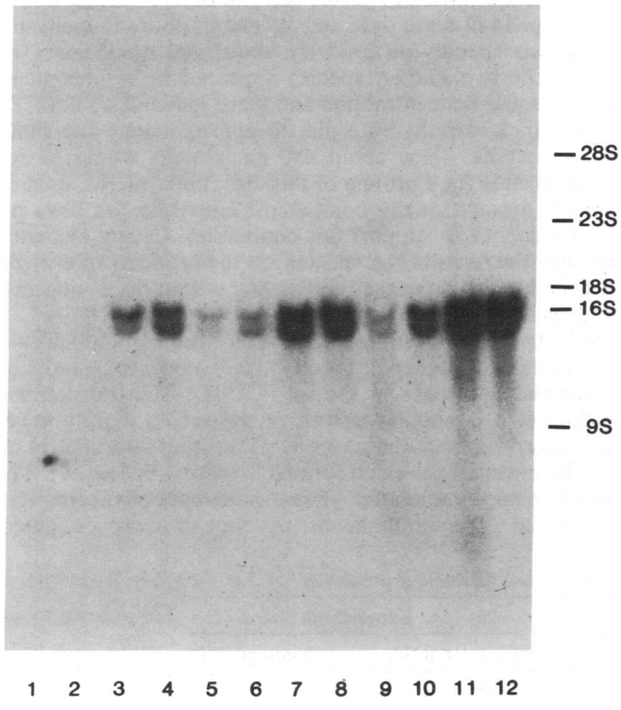

FIG. 2. Electrophoretic analysis of spot 14 mRNA. Total cellular RNA (25 μg) was electrophoresed on a 1.5% agarose/5 mM methylmercuric hydroxide gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pS14-cl DNA. Autoradiography was carried out for 29 hr. RNA was extracted from livers of rats (two per group) that were hypothyroid fed normal chow diet (lanes 1 and 2), hypothyroid fed high-carbohydrate diet (lanes 3 and 4), euthyroid fed chow diet (lanes 5 and 6), euthyroid fed high-carbohydrate diet (lanes 7 and 8), hyperthyroid fed chow diet (lanes 9 and 10), and hyperthyroid fed high-carbohydrate diet (lanes 11 and 12).

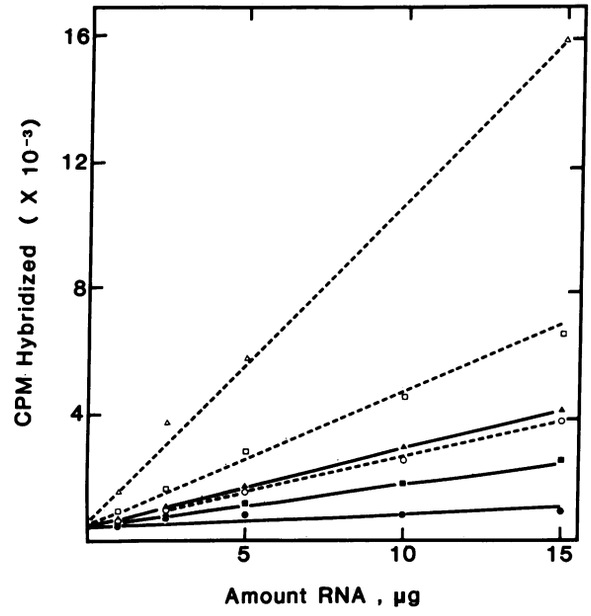

FIG. 3. Measurement of changes in the levels of spot 14 mRNA in response to thyroid hormone and diet. Total cellular RNA isolated from two animals in each experimental group was spotted in duplicate and hybridized with ^{32}P-labeled pS14-cl insert DNA. The data were quantified by direct liquid scintillation counting of individual spots. The slopes were determined by least-squares linear regression. ●, Hypothyroid, chow fed; ○, hypothyroid, fed high-carbohydrate diet; ■, euthyroid, chow fed; □, euthyroid, fed high-carbohydrate diet; ▲, hyperthyroid, chow fed; △, hyperthyroid, fed high-carbohydrate diet.

to be linear below 20 μg of RNA and should be proportional to the relative amount of specific mRNA in the sample. In animals maintained on chow diet, a 6-fold and 11-fold higher relative level of spot 14 mRNA sequences was found in euthyroid and hyperthyroid rats, respectively, compared to that present in the thyroidectomized state. Switching from the chow diet to the high-carbohydrate, fat-free diet led to increases of 9-, 3-, and 4-fold in thyroidectomized, euthyroid, and hyperthyroid animals, respectively. These relative changes in spot 14 mRNA concentration following hormonal and dietary manipulation are in reasonable agreement with the values estimated previously by cell-free translational assay (10).

Measurement of the Nuclear Precursor of Spot 14 mRNA. To follow the nuclear events leading to an increased cellular concentration of spot 14 mRNA, we examined the putative nuclear precursor for this mRNA. In particular, considering the rapidity with which spot 14 responds to T3 treatment, we were interested in determining the time course of changes in the level of nuclear precursor. Consequently, thyroidectomized rats were injected intravenously with 100 μg of T3, a dose designed to saturate the nuclear receptor for the course of the experiment (1). At varying time points following injection, nuclear RNA was isolated from the livers of these animals and analyzed by electrophoresis on a denaturing agarose gel as described above. In addition to the mature mRNA species, a single higher molecular weight band capable of hybridizing to pS14-cl DNA was found (Fig. 4).† This RNA species had an approximate size of 4750 base pairs based on ribosomal RNA standards. The presence of a single larger precursor of this size is consistent with preliminary mapping of the rat gene for spot 14, which indicates that a single inter-

†Note that in this gel system the presence of a doublet of RNA species separated by 200 base pairs cannot be excluded for the nuclear precursor.

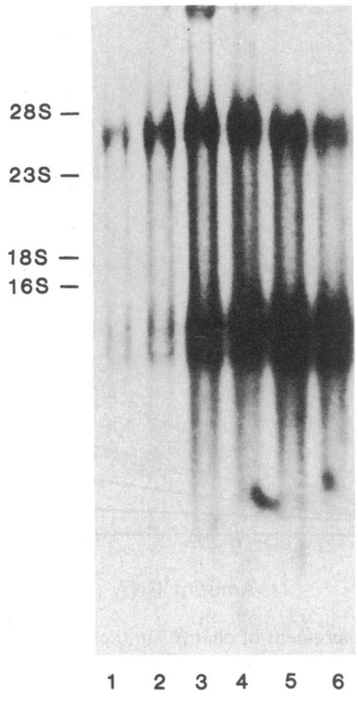

FIG. 4. Time course of induction of nuclear precursor for spot 14 mRNA. Nuclear RNA was isolated and a sample of 30 μg was subjected to electrophoresis on a 1.8% agarose/2.2 M formaldehyde gel, transferred to nitrocellulose, and hybridized with 7.5×10^6 cpm of ^{32}P-labeled pS14-cl insert DNA. Autoradiography was performed for 8 hr. Lane 1, RNA from a thyroidectomized rat injected with saline vehicle; lanes 2–6, RNA from thyroidectomized rats injected with 100 μg of T3 per 100 g of body weight and killed at 10, 20, 30, 40, and 100 min, respectively. Three animals were used per time point, of which a representative set is shown on this gel.

vening sequence of about 3250 base pairs interrupts this gene (30).

The relative amount of nuclear precursor for spot 14 mRNA was found to increase as early as 10 min following hormone treatment. To obtain a more quantitative estimate of the amounts of precursor in the varying samples, autoradiograms were scanned densitometrically and values were expressed relative to that of the control thyroidectomized rats. The results of two separate time course experiments are shown in Table 1. As can be seen, the relative levels of nuclear precursor obtained in the two experiments varied to some extent. We presume that this variability is due in large part to the difficulty of estimating the very low levels present in the thyroidectomized state. However, in all six rats that had received T3 for 10 min, there was an increase in precursor level over that found in vehicle-injected controls. Further increases in the amount of precursor were found to occur up to about 30–40 min following hormone administration. Measurements of spot 14 mRNA levels in cellular RNA in the same animals indicated no change at the 10-min time point. The earliest detectable rise in mRNA levels of about 70% occurred at 20 min following hormone treatment. Spot 14 mRNA levels continued to accumulate throughout the length of the time course studied. Thus, the rise in amount of nuclear precursor for spot 14 mRNA occurs at a time earlier than for the mature mRNA.

DISCUSSION

As initially designated, spot 14 represents an ^{35}S-labeled translational product synthesized in a cell-free system programed with hepatic mRNA (9). The relative amount of translatable mRNA coding for spot 14 was found to increase in the transition from hypothyroid to hyperthyroid states and following a switch from normal chow diet to a high-carbohydrate, fat-free diet (10). We have now isolated a cDNA clone (pS14-cl) containing sequences homologous to spot 14 mRNA. This assignment is based on two lines of evidence. First, the hepatic mRNA species that is capable of hybridizing to this cDNA clone encodes a translational product that migrates to the same position as spot 14 on two-dimensional gel electrophoresis. Second, the relative changes in tissue levels of spot 14 mRNA measured by hybridization to this cDNA clone are in good agreement with the previous results obtained by translational assay. This observation further indicates that the translational assay was detecting changes in the relative mass of spot 14 mRNA and not alterations in the translational efficiency of the mRNA following hormonal and dietary treatment.

Two distinct mRNA species capable of hybridizing to the plasmid pS14-cl were detected by electrophoretic analysis. These two species differed by about 200 nucleotides in length. The two mRNA species appeared to be present in roughly equal concentrations and were induced by both T3 and the high-carbohydrate diet to approximately the same relative extent. Since about 450 nucleotides would be required to code for a protein of this size, both mRNA species could be translationally competent. However, we have no direct evidence to support this contention. Recent evidence indicates the two mRNA species are the products of a single rat gene but are processed differently within the 3′-untranslated region (30).

Spot 14 can be easily distinguished from α_{2U}-globulin—a well-characterized hepatic protein also known to be induced by thyroid hormones in the rat (31, 32). The translational product of α_{2U}-globulin mRNA migrates to a slightly lower molecular weight and more basic pI position than spot 14 on two-dimensional gel electrophoresis (spot 15 of ref. 9). The induction of α_{2U}-globulin synthesis in hypophysectomized rats requires the simultaneous presence of androgen, gluco-

Table 1. Kinetics of the relative induction of spot 14 mRNA and its nuclear precursor

Time after T3 injection, min	Experiment 1		Experiment 2	
	Cellular RNA	Nuclear RNA	Cellular RNA	Nuclear RNA
10	1.1 ± 0.26	6.0 ± 0.26	0.9 ± 0.11	2.2 ± 0.27
20	1.6 ± 0.52	6.7 ± 2.05	1.7 ± 0.46	2.1 ± 0.66
30	ND	ND	2.3 ± 0.34	2.8 ± 0.08
40	2.4 ± 0.16	10.0 ± 3.70	2.8 ± 1.00	2.7 ± 0.05
100	ND	ND	4.1 ± 0.19	2.2 ± 0.46

Thyroidectomized animals injected with T3 intravenously were killed at the indicated times and livers were promptly removed. Values for spot 14 mRNA in cellular RNA were obtained by the dot blot hybridization method. Values for spot 14 precursor were obtained by scanning autoradiograms and quantifying area under the peak corresponding to the precursor band. All values are expressed relative to vehicle-injected thyroidectomized controls and represent the mean ± SEM of three animals per group. ND, not determined.

corticoid, thyroid and growth hormones and occurs slowly over several days. Spot 14 mRNA can be induced within minutes by thyroid hormone alone (unpublished results). α_{2U}-Globulin is encoded by a family of about 20 closely related genes (33) and is secreted from the hepatocyte, whereas spot 14 is the product of a single gene and appears to be a cytosolic protein. We have recently determined the nucleotide sequence of spot 14 mRNA (30) and it shares no homology with that of α_{2U}-globulin (34).

Using the plasmid pS14-cl as a hybridization probe, we were able to detect a single putative precursor to spot 14 mRNA in nuclear RNA isolated from liver. The size of this RNA species was roughly 4750 nucleotides; however, this value should be considered only as an approximation due to the lack of reliable RNA size markers in this range. The relative amount of this putative precursor in nuclear RNA increased following T3 treatment of thyroidectomized animals. Furthermore, the earliest increase in the precursor to spot 14 mRNA preceded the first changes in mature mRNA. Thus, T3 appears to be acting, at least in part, at the nuclear level to influence the production of spot 14 mRNA. This action could be at the level of transcription, but we cannot exclude other possible sites of action, such as the stabilization of the nuclear precursor. Recently, the transcription of the growth hormone gene in cultured rat pituitary cell lines was shown to be regulated by thyroid hormones (35, 36). Comparable experiments on the spot 14 gene must be carried out to elucidate the site of its control.

The rapid kinetics of induction of the putative precursor to spot 14 mRNA leads us to speculate that this action may be a primary response to hormone—that is, a direct consequence of the interaction of hormone with its nuclear receptor. The relative amount of the high molecular weight RNA species is increased by at least 2-fold within 10 min following hormone injection. This period must incorporate the time required for hormone to reach the nuclear receptor in the liver, the time required to transduce the signal of binding into a biochemical action, and the time necessary to accumulate an increased mass of RNA. It is hard to conceive that within this time frame T3 could induce an intermediary product that is the actual effector of expression of the spot 14 gene. Thus, the gene for spot 14 would seem to be an excellent candidate to study the mechanism by which T3 can act to alter mRNA production.

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