Transcriptional Regulation by Thyroid Hormone of an mRNA Homologous to a Protease Inhibitor

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ABSTRACT: We have previously cloned a cDNA of a rat liver mRNA, designated 4-12B, markedly induced by triiodothyronine (T_3) at a pretranslational level [Magnuson, M. A., Dozin, B., & Nikodem, V. M. (1985) *J. Biol. Chem. 260*, 5906–5912]. Here we show that this hormonal effect is due in part to an increase of the rate of transcription of the 4-12B gene. In addition, the nucleotide sequence of 4-12B cDNA has been determined, revealing significant similarity with the sequences of the superfamily of serine protease inhibitors and a very high homology with contrapsin, a mouse serum trypsin inhibitor, at the level of nucleotide and amino acid sequence (77.9 and 66.8%, respectively). The optimized alignment of the putative reactive center region of 4-12B with four related members of this superfamily revealed that lysine–serine residues are located at the reactive site or adjacent to it, thus suggesting that the triiodothyronine-regulated rat 4-12B mRNA might code for a protease inhibitor with trypsin-like specificity. Although not enough data are presently available to assign definitively antitryptic activity to this protein, the high degree of similarity with members of the superfamily of serine protease inhibitors leaves no doubt that 4-12B is a member of this superfamily.

Diverse effects of 3,5,3′-triiodothyronine (T_3)[1] on growth and development have been recognized by many investigators. Currently it is believed that the mechanism by which T_3 elicits its effects is the interaction of the T_3–nuclear receptor complex with specific DNA sequences located within thyroid hormone sensitive genes, leading to alterations in the production of specific nuclear RNAs. Recently several mRNAs have been shown to be under thyroid control: thyroid stimulating hormone subunits (Shupnik et al., 1985), α_{2u}-globulin (Kulkarni et al., 1985), growth hormone (Yaffe & Samuels, 1984), ornithine aminotransferase (Mueckler et al., 1984), malic enzyme (Towle et al., 1980; Magnuson & Nikodem, 1983; Dozin et al., 1985), phosphoenolpyruvate carboxykinase (Loose et al., 1985), and hepatic mRNA spot 14 (Narayan et al., 1984; Narayan & Towle, 1985). Nevertheless, it is likely that many other mRNAs are under T_3 control. For this reason we have cloned four mRNAs whose production varies with the thyroid status of the animal, using the technique of differential hybridization (Magnuson et al., 1985). Herein we report that one of these, 4-12B cDNA, whose cytoplasmic mRNA concentration was increased about 13-fold after thyroid hormone treatment of hypothyroid rats (Magnuson et al., 1985), is 77.9% homologous with the reported cDNA sequencing coding for mouse contrapsin (Hill et al., 1984), a serine protease inhibitor with trypsin-like specificity. In addition, T_3 regulation of this mRNA results, in part, from an alteration of the rate of transcription of the corresponding gene.

Experimental Procedures

Materials. Restriction endonucleases were obtained from New England Biolabs or Bethesda Research Laboratories and used according to the manufacturer's instructions. T_4 ligase and Klenow enzyme were obtained from Boehringer Mannheim. M13 replicative form DNAs (mp 8, 9, 18, and 19) were from Pharmacia P-L Biochemicals. All other reagents were of analytical grade.

Treatment of Animals. Surgically thyroidectomized female Sprague-Dawley rats weighing 120–150 g were obtained from Taconic Farms and placed on a low iodine diet (Teklad Diets). Hypothyroidism was confirmed after 4–6 weeks by the failure to gain weight and by elevated thyroid-stimulating hormone levels in serum (>15 ng/dL). Forty-eight hours before sacrifice, a group of hypothyroid rats was injected intraperitoneally with T_3 (200 μg/100 g, body weight) dissolved in 10 mM NaOH. Control rats were injected at the same time with an identical amount of the alkaline saline vehicle.

"In Vitro" Runoff Transcription Assays. The rate of 4-12B gene transcription was measured by the level of incorporation of [α-^{32}P]UTP into nascent RNA transcripts in isolated nuclei. Liver was perfused in situ with Dulbecco's phosphate-buffered saline containing 200 μg mL^{-1} heparin. Livers were homogenized in buffer A (10 mM Tris-HCl, pH 7.6, 40% glycerol, 10 mM $MgCl_2$, and 10 mM NaCl) containing 0.1% Triton X-100, precooled at −20 °C. Nuclei were pelleted by centrifugation at 4000*g* for 10 min at −10 °C and washed twice with the same buffer without Triton. They were then repelleted by centrifugation at 35000 rpm for 65 min in a Beckman SW 41 rotor at 4 °C, through a 2.1 M sucrose cushion in 10 mM Tris-HCl, pH 7.6, and 10 mM $MgCl_2$ and stored in buffer A at −70 °C at a concentration of (1–2) × 10^8 mL^{-1}. In vitro transcription assays were performed according to the method of Clayton and Darnell (1983) with the following modifications: nuclei were adjusted to about 50 absorbance units at 260 nm (determined in 1% sodium dodecyl sulfate), and the reaction medium contained 1 mM each of ATP, GTP, and

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[1] Abbreviations: T_3, 3,5,3′-triiodothyronine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; kDa, kilodalton.

Table I: Effect of T_3 on 4-12B Gene Transcription and Cellular 4-12B mRNA Level in Rat Liver[a]

thyroid status	total transcription (cpm × 10^{-6})	4-12B gene transcription (ppm)	ratio vs. hypothyroid	4-12B mRNA (cpm/100 μg of RNA)	ratio vs. hypothyroid
hypothyroid	24.8 ± 4.3	9.7 ± 0.5	1.0	658 ± 12	1.0
T_3 (200 μg, 48 h)	26.7 ± 6.1	30.7 ± 3.2	3.2	7766 ± 563	11.8

[a] Mean ± SD of three separate determinations. Total transcription is expressed as cpm of [^{32}P]RNA synthesized by about 50 absorbance units of nuclei; the 4-12B gene transcription is expressed as parts per million (ppm) = cmp (4-12B filter − M13 mp8 filter)/cpm × 10^{-6} in the hybridization mixture; nonspecific hybridization was typically 3 ppm. In preliminary experiments a time course of induction of the 4-12B transcription by T_3 was established in order to determine the optimal time point. After 4- and 24-h treament with T_3 (200 μg/100 g, body weight), the transcription increased 1.3- and 1.85-fold above its basal activity, respectively. Sustained stimulation for 9 days (50 μg/100 g, body weight) resulted in 3.5-fold enhancement of transcriptional rate (single determinations performed in duplicate).

CTP, 1 unit μL^{-1} of RNasin ribonuclease inhibitor (Promega Biotec), and 250 μCi of [α-^{32}P]UTP (3000 Ci $mmol^{-1}$; New England Nuclear), in a volume of 300 μL. After a 20-min incubation at 30 °C, the reaction was terminated by digestion at 37 °C for 1 h with 100 μg mL^{-1} each of DNAse I and proteinase K in the presence of 10 mM $CaCl_2$ (Tullis & Rubin, 1980). Yeast tRNA was added to 25 μg mL^{-1}, and RNA was extracted with phenol/chloroform (1:1) and precipitated with 0.5 volume of 7.5 M ammonium acetate and 2.5 volume 95% ethanol. The RNA was hybridized as described by McKnight and Palmiter (1979) to DNA immobilized on nitrocellulose filters (BA85, Schleicher & Schuell manifold). The single strand specific DNA probe was the insert sequence of the plasmid 4-12B subcloned into the *Pst*I and *Hin*cII sites of M13 mp8 (Messing & Veria, 1982). Following a 3-day hybridization at 42 °C, the filters were washed and incubated with RNases A and T_1 (1 μg mL^{-1} and 10 units mL^{-1}, respectively) (Clayton & Darnell, 1983). The [^{32}P]RNAs were eluted (Clayton & Darnell, 1983), and the radioactivity was quantitated in Beckman Ready Solv HP. The 4-12B gene transcription was expressed as parts per million (ppm) = cpm (4-12B filter−M13 mp8 filter)/cpm × 10^{-6} in the total transcription products. Nonspecific hybridization to M13 mp8 was typically 3 ppm. Hybridization to a 1800-base-pair rat albumin cDNA was included in most experiments as an internal control and 320–360 ppm whether or not the nuclei were from hypothyroid- or T_3-treated rats.

RNA Isolation and Dot-Blot Hybridization. The isolation and dot hybridization assays were performed as previously described (Magnuson et al., 1985), with liver tissues from the same animals as used for transcription assays.

DNA Sequencing. Restriction fragments of 4-12B cDNA insert were ligated into bacteriophage vector M13 (Messing & Veira, 1982) and sequenced by the chain termination method (Sanger et al., 1977) by using ^{35}S-labeled nucleotides (New England Nuclear). The region close to the poly(A) sequence [which is connected with the vector sequence (Magnuson et al., 1985)] was sequenced in the 3′–5′ direction with a 5′d(T_{12}G)3′ primer (New England Biolabs, 1303).

Results and Discussion

We have reported that the level of liver cytoplasmic mRNA for 4-12B increased about 13-fold after daily injection of T_3 (15 μg/100 g, body weight) for 10 consecutive days and was markedly elevated 48 h after a single dose of T_3 (200 μg/100 g, body weight) in hypothyroid rats (Magnuson et al., 1985). An increased concentration of a specific mRNA in the cytoplasm could be due to an increase in transcriptional activity and/or increased stability of nuclear or cytoplasmic mRNAs. To determine whether thyroid hormone can regulate 4-12B gene expression by increasing its rate of transcription, we employed an in vitro runoff transcription assay.

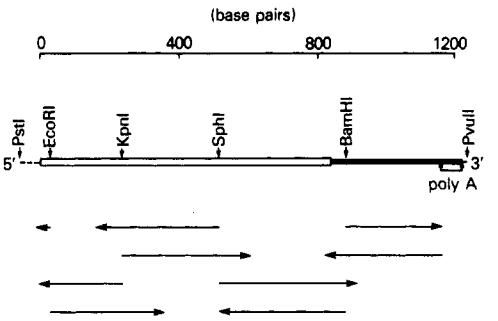

FIGURE 1: Partial restriction map of 4-12B cDNA and strategy used for sequencing. The open bar denotes the coding region and the closed bar the noncoding region; dashes indicate portions of the plasmid vector. Arrows indicate the direction and length of primed DNA synthesis on M13 subclones relative to mRNA. All regions were sequenced on both strands, except for the first 31 bases at the 5′ end, and all subcloning sites used have also been sequenced. The distal 3′ region has been sequenced in the 3′–5′ direction by a special primer hybridizing to the beginning of the poly(A) sequence.

Transcription assays were performed in vitro on isolated liver nuclei by measuring the hybridization of nascent RNA transcripts to a single-stranded 4-12B cDNA. Table I shows a comparison of the rate of 4-12B gene transcription and 4-12B mRNA level in liver of hypothyroid rats and hypothyroid rats treated with T_3. Rats were injected with 200 μg/100 g, body weight, 48 h before sacrifice: this dose and duration of treatment were shown to saturate the T_3 receptors (Oppenheimer et al., 1977) and were used to obtain maximal stimulation. In T_3-treated animals there was an increase of about 3-fold in the rate of transcription of the 4-12B gene. However, the discrepancy between the increase in the rate of transcription (about 3-fold) and the increase of the concentration of 4-12B mRNA in cytoplasm (about 12-fold) indicates the presence of additional pretranslational control mechanisms. This could be due to an effect of T_3 upon the stability or processing of the 4-12B nuclear RNA transcripts and/or the degradation of the 4-12B mRNA in cytoplasm. Work is currently under way to resolve this issue.

The restriction map of 4-12B cDNA with the strategy used for sequencing is shown in Figure 1. The cloned sequence of 4-12B mRNA accounts for about 60% of the full size determined by Northern analysis (Magnuson et al., 1985). Possible similarities were examined in the National Biomedical Research Foundation protein library by using the algorithm of Lipman and Pearson (1985) in the computer program FASTP. This computer program is more comprehensive than some because it takes account of identities as well as of conservative amino acid replacements, and its optimized alignment permits a preservation of the highest homology attainable albeit with certain penalties for insertions and deletions. The highest similarity was found comparing the 4-12B-derived amino acid sequence with the sequence of mouse contrapsin, a trypsin

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                10        20        30        40        50        60        70        80        90
   0    TTTATTGACAAAGAGCAGCCGATACTGTCAGAATTCCCAGAGAAGACAAGGGCTCTGTACCAGGCTGAGGCCTTCGTAGCTGACTTCAAGCAGTGCAATG
 100    AGGCCAAAAAGTTCATCATCGACTATGTGAGCAATCAGACCCAGGGGAAGATCGCAGAACTGTTCTCAGAACTGGATGAGAGGACATCCATGGTGCTGGT
 200    GAACTATCTCCTCTTTAAAGGCAAATGGAAGGTACCATTTAATCCCAACTCTGACACATTTGAGTCTGAGTTCTACTTGGATGAGAAGAGGTCTGTGAAG
 300    GTGCCCATGATGAAAATTAAGGATCTCACCACACCCTACATCCGGGATGAGGAGCTGTCCTGCTCTGTGCTGGAACTGAAGTACACAGGAAATGCCAGCG
 400    CCCTGTTTATCCTCCCTGACCAGGGCAAGATGCAGCAGGTGGAATCCAGCTTGCAACCAGAGACCCTCAAGAAGTGGAAGGACTCTCTGAGGCCCAGGAT
 500    TATAAGTGACGTTCGCATGCCCAAGTTCTCCATCTCCACAGACTACAACCTGGAGGAGGTCCTTCCAGAGCTGGGCATTAGGAAAATCTTCTCCCAGCAA
 600    GCTGATCTGAGTAGGATCACAGGGACCAAGAACCTGCATGTCTCTCAGGTGGTCCACAAAGCTGTGCTGGATGTGGATGAGACAGGCACAGAAGGAGCCG
 700    CTGCCACAGCAGTCACAGCAGCCCTAAAAAGTTTACCGCAAACTATACCTCTTCTGAATTTCAACCGGCCATTCATGCTGGTTATCACTGACAATAATGG
 800    TCAGTCTGTCTTCTTTATGGGCAAAGTCACTAACCCCATGTGAGTCTGAAGCTCCCCAAAATCTGACAATTCTGCCCAGGATCCTGGAACAGAGCCTGGA
 900    TGCTGATCTCTGTATATGCCCTGACATACATGCTCTGATTGGCTATTGCAAAGTTGGCTTAGACAGTGACATCAACTATCTCTATGGCTCCCATGTGCAC
1000    TGGAGCCTTTGGATTGTCAGTGTCAGGCACTTAGGACCCTTGGGAGCATCTACACATGTTTCTGAACTTGGAATCTTTCTTTATTCTTCTTCCCTGGTGA
1100    CTCCTCTTTCTGTGTTCATACCCCAAACCAAGCCATTGATAAGCCCAGTAAAGGTTCTAGA
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FIGURE 2: Partial cDNA sequence of a thyroid hormone regulated mRNA (4-12B). An arrow indicates the start of available cDNA sequence for mouse contrapsin (Hill et al., 1984).

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4-12B    :  FIDKEQPILSEFPEKTRALYQAEAFVADFKQCNEAKKFIIDYVSNQTQGKIAELFSELDE
4-12B    :  RTSMVLVNYLLFKGKWKVPFNPNSDTFESEFYLDEKRSVKVPMMKIKDLTTPYIRDEELS
m. contr.:     VVLVNYIYFKGKWKISFDPQ-DTFESEFYLDEKRSVKVPMMKMKLLTTRHFRDEELS
4-12B    :  CSVLELKYTGNASALFILPDQGKMQQVESSLQPETLKKWKDSLRPRIISDVRMPKFSIST
m. contr.:  CSVLELKYTGNASALLILPDQGRMQQVEASLQPETLRKWRKTLFPSQIEELNLPKFSIAS
4-12B    :  DYNLEE-VLPELGIRKIFSQQADLSRITGTKNLHVSQVVHKAVLDVDETGTEGAAATAVT
m. contr.:  NYRLEEDVLPEMGIKEVFTEQADLSGIIETKKLSVSQVVHKAVLDVAETGTEAAAATGVI
4-12B    :  AALKSLPQTIPLLNFNRPFMLVITDNNGQSVFFMGKVTNPM
m. contr.   GGIRK--AILPAVHFNRPFLFVIYHTSAQSILFMAKVNNPK
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FIGURE 3: Comparison of the derived amino acid sequence of 4-12B with mouse contrapsin. Amino acids are designated by the one-letter code. The optimized alignment is denoted by a colon for an identity and a dot for a conservative replacement. Gaps introduced during optimization by the computer program FASTP are shown as dashes.

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                    *
m. contr.:  G G I R K - - A I L   (66,8/217)
4-12B    :  A A L K S L P Q T I
                    *
α1 achy  :  K I T L L S A L V E   (56,1/280)
4-12B    :  T A A - L K S L P Q
                    *
α1 PI    :  E A I P M S I P - -   (42,3/279)
4-12B    :  T A A L K S L P Q T
                    *
AT III   :  V I A G R S L N P N   (34,5/287)
4-12B    :  T A A L K S L P Q T
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FIGURE 4: Computerized alignment of the amino acid sequences of the reactive center regions of mouse contrapsin (m. contr.), human α_1-antichymotrypsin ($\alpha_1$achy), human α_1-antitrypsin (α_1 PI), and human antithrombin III (AT III) with the presumptive reactive center region of rat 4-12B. Percent similarity and number of amino acids available for comparison are shown in parentheses. The reactive sites are designated by an asterisk and amino acids by the one-letter code.

inhibitor reported to be homologous to human α-antichymotrypsin (Hill et al., 1984), both being members of the superfamily of serine protease inhibitors.

The nucleotide sequence of rat liver 4-12B cDNA is shown in Figure 2. The sequence homology found between mouse contrapsin and 4-12B was 77.9% at the nucleotide level and 66.8% at the amino acid level (Figure 3). A variable but striking degree of similarity was found with other members of this superfamily: 56.1, 42.3, and 34.5% at the amino acid level with human α-antichymotrypsin, α_1-antitrypsin, and antithrombin III, respectively. A significant resemblance between the derived amino acid sequence for 4-12B and some members of the superfamily of serine protease inhibitors provides evidence that the 4-12B belongs to the same superfamily and suggests similar biological function.

The computer alignment of 4-12B-derived amino acid sequence with the related sequences allows prediction of a putative reactive center region for this protein. Figure 4 shows a schematic representation of these reactive center regions, encompassing 10 amino acid residues. It has been reported that only one amino acid position in the reactive site of these protease inhibitors determines the specificity of inhibition of the target protease (Carrell, 1984; Carrell & Travis, 1985; Takahara & Sinohara, 1982; Barth et al., 1982; Eriksson, 1965). The examination of the computer alignment revealed that the lysine residue of 4-12B coincides with the reactive site of α_1-antitrypsin and antithrombin III. In the case of α_1-antichymotrypsin and mouse contrapsin, the 4-12B lysine residue is immediately adjacent to the positions of the reactive site. It is of interest that most inhibitors of serine proteases have an amino acid at the reactive site followed by serine (Laskowski & Kato, 1980). Indeed the serine residue is found next to the lysine in the putative reactive center region of 4-12B.

There are other similar characteristics when, for example, comparing 4-12B and mouse contrapsin: (i) the molecular mass of the hybrid-selected translation product of 4-12B mRNA is about 46 kDa (Magnuson et al., 1985), which is comparable to the molecular mass of 55 kDa of mouse contrapsin, excluding a carbohydrate content of 15.1% (Takahara & Sinohara, 1982); (ii) dot-blot hybridization analysis showed the presence of a high level of 4-12B mRNA in 150 μg of total RNA preparation from liver (14 870 cpm), while the concentration of this message in kidney, heart, testes, spleen, lung, and brain was substantially lower (0, 970, 570, 60, 0, and 12 cpm, respectively). Mouse contrapsin has also been described as a liver protein (Hill et al., 1984; Barth et al., 1982) being secreted into the blood.

In conclusion, we have studied and sequenced a thyroid hormone regulated rat liver mRNA (4-12B) and showed that it is related to the superfamily of serine protease inhibitors, with the highest similarity to mouse contrapsin. The optimized alignment with the related sequences indicates that lysine–serine residues are located at the reactive site or adjacent to it. This suggests that 4-12B mRNA could code for the protein contributing to the serum trypsin inhibiting activity in rat. These protease inhibitors control proteolytic degradation responsible for immune reactions, coagulation, and inflammation. Hence, our novel finding that a protease inhibitor is transcriptionally regulated by T_3 could represent a previously

unrecognized mechanism by which thyroid hormone might affect any of several important physiological processes.

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Registry No. T_3, 6893-02-3; protease inhibitor, 37205-61-1; contrapsin, 80700-40-9.

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Interaction of Europium(III) with Phospholipid Vesicles As Monitored by Laser-Excited Europium(III) Luminescence†

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ABSTRACT: The technique of laser-excited Eu(III) luminescence was applied to monitor Eu(III) binding to a variety of phospholipids. Eu(III) excitation spectra were similar with and without the presence of neutral phospholipids, while acidic phospholipids changed the spectrum in a concentration-dependent manner. Eu(III) appears to bind to the phosphate moiety with at least a 2:1 phospholipid:metal ion stoichiometry. Analysis of luminescence lifetimes reveals that only one or two waters of hydration are removed from Eu(III) by addition of neutral phospholipids, whereas acidic phospholipids and inorganic phosphate strip off all but one or two waters. Implications with regard to fusion and use of lanthanides as probes in membrane preparations are discussed.

The interaction of metals with phospholipids has received much attention in view of the effects of metals in physiologically important membrane processes such as membrane fusion and transport. In particular, Ca(II) is proposed to mediate vesicle fusion in a number of systems, although some other divalent ions can mediate such fusion to a limited extent (Papahadjopoulos & Vail, 1978; Zimmerburg et al., 1980; Liao & Prestegard, 1980; Duzgunes et al., 1981; Ekerdt & Papahadjopoulos, 1982; Ohki, 1981; Silvius & Gange, 1984). The details of divalent cation interaction with phospholipids are therefore of interest. The luminescent lanthanide probes

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