NUCLEAR THYROID HORMONE RECEPTORS: EVIDENCE FOR ASSOCIATION WITH NUCLEOLAR CHROMATIN

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SUMMARY

When hypothyroid rat liver nuclei labeled in vivo with [125 I]L-triiodothyronine are incubated with micrococcal nuclease, the nuclear chromatin is digested and chromatin particles are released into the medium. The nuclease-treated nuclei contain intact nucleoli and a residual chromatin fraction. When this residual chromatin is purified, it contains only a small percentage of the initial nuclear DNA but is strikingly enriched in [125 I]L-triiodothyronine. This chromatin fraction has many of the characteristics of nucleolar chromatin including a high protein to DNA ratio, an abundance of nonhistone proteins, and a relatively high RNA to DNA ratio. An association of thyroid hormone receptors with a nucleolar component implicates this organelle in the early events of thyroid hormone action.

Previous investigators have established that thyroid hormone receptors are preferentially bound to the cell nucleus in rat liver and rat pituitary tumor-derived (GH_1) cells (1-5). These receptors have high affinity for triiodothyronine, number about 5,000-10,000 per nucleus (2-5) and purify with the nonhistone proteins (6). On subfractionation of nuclei, they appear to be associated with the chromatin fraction (8,9). They have a general affinity for DNA (7). There is recent evidence that in HeLa cell nuclei a T3 binding protein is associated with the slow sedimenting nucleohistone enriched in endogenous RNA polymerase activity and transcription sites (10).

In this study I report that T3 receptors are localized to a nuclease-resistant fraction of chromatin that remains after brief digestion of [125 I]-T3 labeled rat liver nuclei with micrococcal nuclease. This chromatin fraction has many of the characteristics of nucleolar chromatin.

MATERIALS AND METHODS

[125 I]L-T3 (Abbott Labs, Chicago, Illinois) specific activity 100 mCi/mg contained less than 3% iodide. L-T3 (sodium salt) was obtained from Sigma and

ABBREVIATIONS USED. T3, L-3, 5, 3' triiodothyronine; EDTA, disodium ethylenediamine tetraacetate; SDS, sodium dodecyl sulfate; TCA, trichloracetic acid.

micrococcal nuclease, SA = 10,000 u/mg was obtained from Worthington Corp.

Male albino Sprague-Dawley rats, thyroidectomized at 90-100 gms were obtained from Hormone Assay Labs, Chicago and were maintained on a low iodine diet. They were not used until at least 4 weeks after thyroidectomy.

Rat Liver Nuclei:

Fasted hypothyroid rats weighing about 110-140 gms were injected IV with 25-35 μCi [125 I]L-T3 (1 nmole) in an 0.9% saline solution containing 0.1% bovine serum albumin. Thirty minutes later the animals were decapitated and rat liver nuclei were isolated at 4°C by a method as modified from Chauveau et al. (11). Rat livers were homogenized in a Dounce homogenizer in 5 volumes of 0.25 M sucrose buffer A (100 mM NaCl, 10 mM Tricine, 8 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.8) and then adjusted to 2.2 M sucrose buffer A with 2.65 M sucrose buffer A. The homogenate was layered over 8 ml of 2.3 M sucrose buffer A and centrifuged for 1 hour at 27,000 rpm in an SW-27 rotor of a Beckman L5-65 centrifuge. The nuclear pellets were resuspended in 0.25 M sucrose buffer A and centrifuged at 2,000 xg for 10 minutes. The washed nuclear pellet was resuspended in 0.25 M sucrose, 5 mM $CaCl_2$, 10 mM Tris, pH 7.6 and centrifuged at low speed.

Isolation of Chromatin:

Chromatin was isolated from purified nuclei. Purified nuclei, prepared from 10 gms liver were resuspended in 40 ml of 10 mM Tris, 4 mM EDTA, pH 7.5, and centrifuged at 15,000 xg for 10 minutes. The crude chromatin pellet was resuspended in 10 mM Tris, 1 mM EDTA, pH 7.5 and the washing procedure was repeated. The chromatin pellet was then resuspended in 15 ml of 10 mM Tris, pH 7.5 and recentrifuged at 25,000 xg x 10 minutes. This procedure was repeated at least three times. The purified chromatin was resuspended and swollen in distilled H_2O and sheared in a French pressure cell (AMINCO) at 8,000 psi and membrane and other nuclear structural components were removed by centrifuging at 35,000 xg for 30 minutes. The sheared chromatin in the supernatant fraction was used immediately.

Biochemical Analysis of Chromatin:

Chromatin was sheared after resuspension in distilled water and fractionated into histone and nonhistone components using 0.4 N H_2SO_4 (final concentration). The acid insoluble proteins were removed by centrifuging for 1 hour at 35,000 xg. The acid soluble proteins were precipitated with one volume 10% TCA containing 0.5% sodium tungstate, pH 2.0 (12) and the precipitated protein centrifuged at low speed and dissolved in 0.2 N NaOH at 37°C for 1 hour. Alternately 1% SDS and 1% β-mercaptoethanol (final concentration) were added to the acid soluble proteins and they were dialyzed overnight against 0.1% SDS, 0.1% β-mercaptoethanol and then analyzed by SDS acrylamide gel electrophoresis at pH 7.0, according to the method of Bhorjee and Pederson (13), and stained with Coomassie Blue after fixation with TCA (14). Total chromatin and acid insoluble proteins were dissolved in 1% SDS and 1% β-mercaptoethanol and analyzed by SDS acrylamide gel electrophoresis. The protein concentration of the various fractions was determined by the Lowry procedure (15). DNA was determined by a modified diphenylamine reaction (16). RNA was determined as perchloric acid soluble OD_{260} after 0.3 N KOH hydrolysis for 60 minutes at 37°C (17).

RESULTS

Purified nuclei labeled in vivo with [125 I]-T3 were incubated at 2°C with micrococcal nuclease, the reaction was stopped with EDTA and the nuclei were pelleted. Under the conditions used, there is a rapid release of partially digested chromatin particles from the nuclei. As shown in Figure 1, within 10 minutes, 64% of the OD_{260} is released from the nuclei into the supernatant fraction and

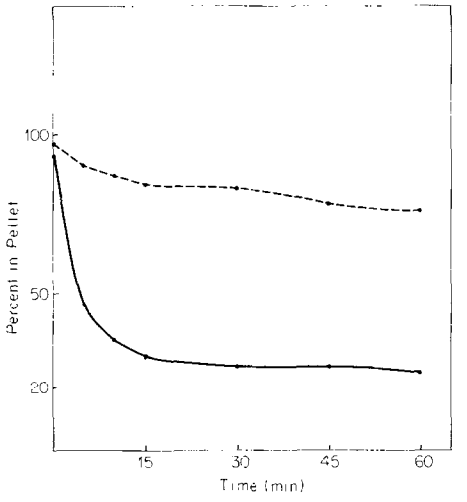

Figure 1: Purified nuclei labeled in vivo with [125 I]-T3 were resuspended to an OD_{260} of 10 in 0.25 M sucrose containing 1 mM $CaCl_2$, 10 mM Tris, pH 7.6. They were incubated at 2°C with 20 µg/ml micrococcal nuclease with gentle stirring. At various time intervals aliquots were withdrawn and the reaction stopped with 4 mM EDTA (final concentration). The nuclei were centrifuged at 3,000 xg for 10 min. The supernatant fraction was assayed for OD_{260} and radioactivity (Nuclear Chicago gamma spectrometer). The pellet was resuspended in 4 mM EDTA, .01 M Tris, pH 7.5 and sonicated for 1 min. at 30 watts (Sonifier W185). The insoluble nuclear components were spun down and the OD_{260} and radioactivity determined on this sonicated supernatant fraction.

●——● OD_{260}; ●- - -●-cpm.

within 30 minutes this increases to 73%. Only 4% of the released OD_{260} is perchloric acid soluble at 30 minutes. In contrast to this rapid release of partially digested chromatin from nuclei, there is a striking retention of [125 I]-T3 within the nucleus. At the 30 minute time point when 73% of the OD_{260} is released, only 17% of the T3 radioactivity is released.

The above results suggest that T3 receptors are preferentially associated with a part of the nucleus relatively resistant to digestion by micrococcal nuclease. This observation was pursued by isolating a residual chromatin fraction from the nuclease-treated nuclei. This relatively nuclease-resistant (Core) chromatin fraction was prepared from nuclei digested for 5 to 30 minutes with micrococcal nuclease, using the standard techniques for chromatin isolation as described in METHODS. Core chromatin was found to be markedly enriched with [125 I]-T3 receptors compared to control chromatin similarly isolated from un-

TABLE 1

T3 BINDING TO CORE CHROMATIN FRACTIONS

Chromatin Preps	[125 I]-T3 Binding Femtomoles/mg DNA	% Nuclear DNA
1. Control	69.1	65.
2. 5 min. core	332	7.7
3. 30 min. core	996	2.1

[125 I]-T3 labeled nuclei were incubated with micrococcal nuclease for various times as described in Figure 1 and chromatin was isolated as described in METHODS. Control chromatin represents total nuclear chromatin isolated from nuclei that were not incubated with nuclease. The % nuclear DNA represents the % yield of DNA in the chromatin preparation based on the initial DNA content of the isolated nuclei.

treated nuclei. This is illustrated in Table 1. As the nuclei are incubated with micrococcal nuclease for increasing periods of time there is a corresponding increase in the [125 I]-T3 binding per mg core chromatin DNA. Core chromatin prepared from 30 minute nuclease-treated nuclei was further analyzed by glycerol gradient ultracentrifugation. As shown in Figure 2, a majority of the [125 I]-T3 counts are associated with the more rapidly sedimenting chromatin peak. This demonstrates that a majority of the [125 I]-T3 counts remains bound to chromatin during the shearing process and subsequent ultracentrifugation.

An analysis of the chromatin fractions prepared from control and nuclease-treated nuclei is presented in Table 2. Notice the high protein to DNA weight ratios of the core chromatin fractions. This is due to their very high nonhistone protein content. In addition, core chromatin has a high RNA to DNA ratio compared to control chromatin. The proteins present in core chromatin were also analyzed by SDS acrylamide gel electrophoresis as illustrated in Figure 2. The gels demonstrate the striking enrichment of core chromatin with nonhistone proteins compared to control chromatin, as well as the presence of the usual histone fractions. Acid soluble proteins were also analyzed by SDS acrylamide gel

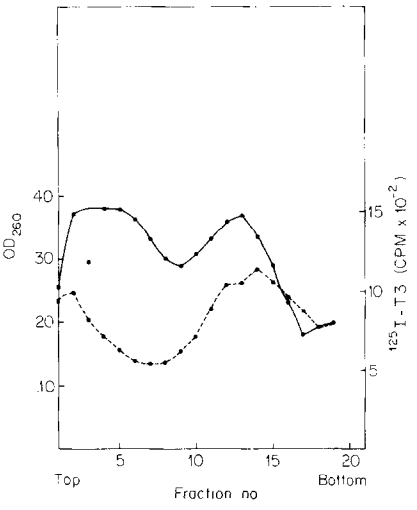

Figure 2: [125 I]-T3 labeled nuclei were incubated with micrococcal nuclease for 30 min. as described in Figure 1. After removal of the supernatant digestion products, the residual nuclear pellet was resuspended in 10 mM Tris, 1 mM EDTA, pH 7.5, and chromatin was isolated as described in METHODS. The isolated chromatin was resuspended in 10 mM Tris, pH 7.6 at 10-20 OD_{260}/ml and sheared twice in a French pressure cell at 5,000 psi. The nuclear membrane fragments and unsheared chromatin were removed by centrifuging at 35,000 xg x 30 min. and the supernatant core chromatin fraction analyzed by layering onto a 10 ml 10-75% glycerol gradient containing 1 mM EDTA, 0.1 mM dithioerythritol and 5 mM sodium phosphate buffer, pH 7.0, and centrifuging in an SW41 rotor at 35,000 rpm for 15 hours. The gradient was then fractionated and aliquots removed for determination of OD_{260} and radioactivity.

●——● OD_{260}; ●- - -●-cpm.

TABLE 2

CHEMICAL COMPOSITION OF CHROMATIN FRACTIONS

Chromatin Fraction	Protein:DNA	Nonhistone:DNA	RNA:DNA
1. Control	2.0	0.9	0.08
2. 5 min. core	4.3	2.9	.30
3. 10 min. core	4.5	3.3	.37
4. 30 min. core	5.9	3.6	.38

Chromatin was prepared from control or nuclease-treated nuclei as described in Figure 2, resuspended in distilled water and sheared in a French pressure cell at 8,000 psi. After removing nuclear membrane fragments and unsheared chromatin, the supernatant chromatin fraction was analyzed for DNA, RNA, total protein, and acid soluble ("histone") and acid insoluble ("nonhistone") protein as described in METHODS. This data represents the average of 3 separate determinations and is expressed as weight ratios. Histone:DNA ratios were not shown because of a significant contamination of the H_2SO_4 supernatant with nonhistone proteins. This is especially true in the 30 min. core chromatin fraction.

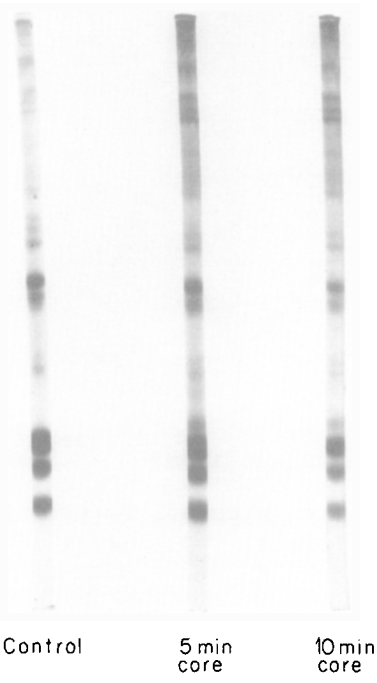

Figure 3: SDS polyacrylamide gel electrophoresis of chromatin proteins. Chromatin fractions were prepared from untreated (control) and 5 and 10 minute nuclease-treated nuclei as described in Figures 1 and 2. Approximately 75 μg of total chromatin protein was applied to each gel with conditions of electrophoresis and staining described in METHODS. Acid soluble and insoluble proteins were similarly analyzed by acrylamide gel electrophoresis (not shown).

electrophoresis and showed the usual histone fractions and a small amount of nonhistone proteins in both control and core chromatin fractions (18).

DISCUSSION

Evidence has been presented that T3 receptors are preferentially associated with a particular chromatin fraction relatively resistant to nuclease digestion. This core chromatin is isolated from nuclei that have been incubated with micrococcal nuclease at low temperatures to minimize proteolysis. This chromatin fraction is markedly enriched in nonhistone proteins and RNA. These characteristics of core chromatin are strikingly similar to those found in chromatin isolated from purified rat liver nucleoli by Wilhelm *et al.* (19). In both cases the purified chromatin fractions have a protein to DNA ratio of 4-5:1 and RNA to

DNA ratios of 0.3-0.4:1. Also, in both instances, a marked increase in the quantity of nonhistone proteins was found.

Nuclei incubated with micrococcal nuclease at 2°C have intact nucleoli which stain with toluidine blue, and they have been visualized by electron microscopy after fixation with glutaraldehyde, post fixation with osmium tetroxide and staining with uranyl acetate (18). Studies are now in progress to isolate nucleoli and their associated chromatin by established procedures to more directly demonstrate the association of T3 receptors with nucleolar chromatin.

The binding of T3 receptors to a chromatin preparation with many of the characteristics of nucleolar chromatin is of interest in view of the recent observation that in rat liver parenchymal cells the nucleolus enlarges within 3 hours following the injection of T3 together with heparin, amino acids and dibutyryl cyclic AMP and theophylline (20). This nucleolar enlargement precedes by several hours the onset of DNA synthesis which is stimulated by these substances (20).

Thyroid hormones have been previously shown to cause an increase in the rate of synthesis of rapidly labeled nuclear RNA in hypothyroid rat liver (21). Some of this newly synthesized RNA is preribosomal RNA (45S), which is specifically synthesized in the nucleolus (22), and as a consequence, there is a striking increase in the ribosomal RNA content per cell (21). In addition, nucleolar chromatin of HeLa cells has been shown to be the only chromosomal fraction to contain the specific ribosomal DNA sequences (rDNA) that are transcribed in the synthesis of preribosomal RNA (23,24). Therefore, the association of T3 receptors with a nucleolar chromatin fraction which presumably contains rDNA cistrons would suggest a regulatory role for this hormone binding protein at the level of ribosomal RNA transcription.

The nature of this regulatory role is unknown, but it is possible that nucleolar chromatin contains DNA acceptor sites for thyroid hormone receptors which are involved in the regulation of transcription of rDNA cistrons. This is reminiscent of the finding of Crippa et al. (25) that in *Xenopus* oocytes there is a protein factor with regulatory properties specific for the transcription of rDNA

cistrons. In any case, the association of thyroid hormone receptors with nucleolar components would suggest a major role for the nucleolus in the early events of thyroid hormone action.

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