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Association of the Thyroid Hormone Receptor with Rat Liver Chromatin†

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ABSTRACT: We have investigated the association of the triiodothyronine (T_3) nuclear receptor with rat liver chromatin by the use of selective endonuclease digestion and differential solubilization. The T_3 receptor was found in a fraction of chromatin having some of the characteristics of active chromatin: (a) It is highly sensitive to DNase I and micrococcal nuclease digestion; (b) it is enriched in nonhistone proteins and depleted of histone 1 (H-1). Micrococcal nuclease and pancreatic DNase I excised two receptor-containing fragments from chromatin, a minor (12–14 S) form and a major (5.5–6.0 S) form. The latter structure has a Stokes radius of 42 ± 2 Å and an estimated molecular weight of 95 400 when a partial specific volume of 0.725 cm^3/g for protein is used. It contains DNA but no histones. Similar receptor-containing fragments were excised from chromatin of other rat tissues, including brain, kidney, and heart. Both the 5.5–6 S and the 12–14 S receptor-containing chromatin structures are converted by 0.5 M KCl to the 3.5 S form (R_0 35 Å molecular weight 50 500). Titration with micrococcal nuclease and pancreatic DNase I revealed that the 5.5–6 S form is preferentially excised by endonucleases. Neither receptor occupancy nor thyroidal status had an apparent effect on the susceptibility of chromatin to endonucleolytic digestion nor did they influence the distribution of T_3 receptors in chromatin. Our results suggest that T_3 receptors are not tightly associated with nucleosomes, the basic subunit of chromatin, but are associated with the DNA-linking nucleosomes in structurally modified regions of chromatin in rat liver nuclei. The T_3 receptor-containing fragment may well represent a higher order of structural complexity necessary for T_3 action at the cellular level.

Recently, three laboratories independently reported that micrococcal nuclease excised a triiodothyronine (T_3)[1] receptor-containing fragment from chromatin as a 5.0–6.5 S complex in GH_1 pituitary cells (Samuels et al., 1980) and rat liver (Jump & Oppenheimer, 1980; Groul, 1980). Our studies indicated (Jump & Oppenheimer, 1980) that T_3 receptors in euthyroid hepatic nuclei are associated with a fraction of chromatin which is highly sensitive to both pancreatic DNase I and micrococcal nuclease digestion and suggested that T_3 receptors were associated with the expressed region of chromatin in euthyroid liver nuclei. These inferences were based on the work of others who had demonstrated the selective digestion of transcriptionally active genes by DNase I (Weintraub & Groudine, 1976; Garel & Axel, 1977) and micrococcal nuclease (Bellard et al., 1978; Bloom & Anderson, 1978; Levy-Wilson et al., 1979).

Because of the importance of receptor occupancy in mediating T_3 effects on nuclear function (Oppenheimer, 1979), we were interested in determining whether thyroidal status or receptor occupancy influences the structure and composition of chromatin, especially those regions which contain the T_3 receptor in rat liver nuclei. In this report, we have used two methods, endonuclease digestion of intact nuclei and differential solubility, to fractionate chromatin. The analysis examines the organization of chromatin neighboring the T_3 receptor binding site, as well as the distribution of T_3 receptors in isolated chromatin fragments. We were also interested in determining whether T_3 receptors in other receptor-containing tissues are also associated with chromatin structures similar to those in rat liver.

Materials and Methods

Animals. Male Sprague-Dawley rats (150–250 g) were used in all experiments. Animals were rendered hypothyroid by surgical thyroidectomy followed by administration of 100 μCi of ^{131}I. Animals were used when no further weight gain was evident, i.e., 4–5 weeks following radioablative treatment. Euthyroid and hypothyroid animals receiving in vivo administered [^{125}I]T_3 (20–50 ng/100 g of body weight, 450–550 μCi/μg; Abbott Laboratories) were injected via the tail vein and killed 30 min later unless otherwise stated. During the period, the metabolism of injected tracer T_3 is negligible, and nuclear radioactivity is in the form of [^{125}I]T_3 (Oppenheimer et al., 1974a). Greater than 95% of the nuclear [^{125}I]T_3 is specifically bound to receptors on the basis of studies in which [^{125}I]T_3 (50 ng/100 g of body weight) is injected with and without 10 μg of unlabeled T_3.

Preparation of Hepatic Nuclei. Rat liver nuclei were prepared as previously described (Oppenheimer et al., 1974b) with minor modifications (Jump & Oppenheimer, 1980). Crude nuclear pellets obtained by centrifugation through 2.4 M sucrose were resuspended in 6 mL of buffer C (0.25 M sucrose, 10 mM Tris, pH 8.0, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM DTT, and 0.4 mM PMSF) per 3 g equiv of tissue. The nuclear suspension was adjusted to 1% Triton X100, and nuclei

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[1] Abbreviations used: T_3, triiodothyronine; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid.

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were pelleted at 700*g* for 10 min. Nuclei were resuspended in buffer C which was adjusted to 0.14 M NaCl. Nuclei were sedimented as before and resuspended in endonuclease digestion buffer to 3 g equiv of tissue/mL. Micrococcal nuclease digestion buffer (buffer D) consisted of the following: 0.25 M sucrose, 10 mM Tris, pH 8.0, 25 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM DTT, 0.14 mM spermidine hydrochloride, and 0.4 mM PMSF. Pancreatic DNase I digestion buffer was composed of 0.25 M sucrose, 10 mM Tris, pH 8.0, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, and 0.4 mM PMSF.

The preparation of nuclei from rat brain, kidney, and heart was as previously described (Oppenheimer et al., 1974b) with the modification noted above for the preparation of rat liver nuclei for nuclease digestion studies.

Endonuclease Digestion of Nuclei. Micrococcal nuclease (1–20 units/A_{260}) or pancreatic DNase I (0.1–5 units/A_{260}) was added to nuclear suspensions at 0 °C. An aliquot of nuclei not exposed to exogenous nuclease was included in all nuclease digestion studies. Nuclei (60–80 A_{260} units/mL) were transferred to a 30 °C water bath for 1 min and returned to 0 °C for 5 min. Nuclease digestions were stopped by addition of EDTA to 10 mM. The extent of DNA hydrolysis by endonucleases in samples taken before and after digestion was determined in 1.0 M perchloric acid–1.0 M NaCl (pH 8).

Following nuclease digestion, nuclei were lysed by dialysis in TEDP (10 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, and 0.4 mM PMSF) for 12 h. Soluble chromatin (S_1) was separated from insoluble material (P_1) by sedimentation at 3000*g* for 10 min at 0 °C. The nuclease-soluble chromatin (S_1) was fractionated further by its differential solubility in 0.1 M NaCl. The chromatin remaining soluble in 0.1 M NaCl was designated S_2 and the 0.1 M NaCl insoluble chromatin designated P_2. The partitioning of chromatin and T_3 receptors in the various chromatin fractions is presented in Table I. The procedure described above has been used by others (Levy-Wilson et al., 1979; Scott & Frankel, 1980) to obtain nuclear subfractions enriched in transcriptionally active chromatin.

Velocity Sedimentation of Chromatin. Chromatin fragments of varying complexity were separated by velocity sedimentation. Chromatin (10–15 A_{260} units) was applied in 0.5-mL aliquots to 5–20% sucrose (w/v) gradients containing TEDP with 0.1 M NaCl, except where noted, and sedimented in an SW 41 rotor at 38000 rpm from 15 h (2 °C). Gradients were fractionated at 2 °C as previously described (Jump & Oppenheimer, 1980). During gradient fractionation, the samples passed through an ISCO turbulence-free UV flow-through cell to monitor the distribution of chromatin DNA (A_{260}) in the gradients. Radioactivity was quantitated by γ scintillation counting. Velocity sedimentation markers including catalase (11.3 S), bovine serum albumin (4.4 S), and myoglobin (2.0 S) were obtained from Sigma.

Quantitation of T_3 Receptors. Nuclear T_3 receptors were extracted from nuclease-soluble chromatin with 0.5 M KCl at 0 °C (Silva et al., 1977). Nuclei prepared as described above were digested briefly with micrococcal nuclease (2 units/A_{260}, 60 s, 30 °C). The preliminary nuclease shearing of chromatin increases the yield of in vivo labeled T_3 receptors from 50–60% (without nuclease shearing) to 80–90% recovery.

Soluble nuclear receptors were quantitated as previously described (Silva et al., 1977). Salt-extracted nuclear receptors were incubated with [^{125}I]T_3 [(0.2–20) × 10^{-10} M] in 0.5 mL of TEDP + 0.4 M KCl at 0 °C for 20 h for the receptor extracted from hypothyroid liver nuclei. Bound [^{125}I]T_3 was separated from unbound ligand by treatment of samples with an equal volume of Dowex AG-1X8 resin (160 mg/mL; TEDP + 0.4 M KCl) for 15 min at 0 °C with intermittent mixing. [^{125}I]T_3 remaining resistant to resin uptake was considered bound to protein. Nonspecific binding of [^{125}I]T_3 was determined by including 10 μM T_3 (nonradioactive) in a tube containing 2 nM [^{125}I]T_3. Data were analyzed by the method of Scatchard to obtain a maximum binding capacity. Quantitation of nuclear receptors in isolated chromatin fragments was carried out as described above in buffers containing 0.4 M KCl.

In Vitro Analysis of T_3 Receptor Distribution in Isolated Chromatin Fragments. Chromatin fragments isolated by velocity sedimentation and fractionated as described above were incubated with 0.2 nM [^{125}I]T_3 under the conditions (temperature and time) described above for nuclear receptors extracted from hypothyroid animals. Samples were treated with Dowex AG-1X8 resin to separate bound from free ligand. Nonspecifically bound radioactivity (≤1.5%) in isolated chromatin fragments, which was found to be uniform in all gradient fractions, was subtracted from the total radioactivity assayed.

Electrophoretic Analysis of Nuclear Proteins. Total nuclear proteins were prepared by digesting nuclei with a mixture of micrococcal nuclease (20 units/A_{260}), pancreatic DNase I (5 units/A_{260}), and pancreatic RNase A (1.0 unit/A_{260}) for 5 min at 30 °C in buffer C. Acid-soluble nuclear proteins were extracted from whole nuclei in 0.4 N H_2SO_4 at 4 °C. Proteins were precipitated with 5 volumes of absolute ethanol overnight (−20 °C) and collected by sedimentation in an SW 50.1 rotor at 25000 rpm for 30 min (Jump et al., 1980). Electrophoresis of nuclear proteins was carried out by using the procedure of Laemmli (1970) in slab gels (0.15 × 14 × 16 cm). Following electrophoresis, gels were then fixed in 25% Cl_3CCOOH, stained in 0.2% Coomassie blue R-250, 50% methanol, and 7.5% acetic acid, and then destained in 20% methanol and 7.5% acetic acid. Protein molecular weight markers obtained from Bio-Rad, Inc. included the following: myosin (M_r 200000), β-galactosidase (M_r 116500), phosphorylase B (M_r 94000), bovine serum albumin (M_r 68000), and ovalbumin (M_r 43000). Chymotrypsinogen A (M_r 23650), myoglobin (M_r 16890), and cytochrome *c* (M_r 13400) were purchased from Schwarz/Mann. Calf thymus histones (H_2a, H_2b, H_3, H_4, H_1) were purchased from Sigma.

Protein and DNA Determination. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. DNA analysis of total nuclei used the method Burton (1956). DNA analysis of isolated chromatin fragments used the absorbance at 260 nm (20 A_{260} units = 1 mg of DNA/mL).

Gel Filtration. Chromatin fragments generated by micrococcal nuclease digestion of in vivo [^{125}I]T_3 labeled liver nuclei were separated by gel filtration. Chromatin (20–30 A_{260} units) was applied in 1.0-mL aliquots (TEDP + 0.1 M NaCl) to columns of AcA-34 (LKB) (70 cm × 1.6 cm). Proteins were eluted (10 mL/h) with TEDP + 0.1 M NaCl, and fractions (2.0 mL) were collected for scintillation counting. Gel filtration columns had been previously calibrated with standard proteins of known molecular weight (M_r) and Stokes radius (R_0): ferritin (M_r 440000, R_0 61.5 Å), catalase (M_r 243000, R_0 51.3 Å), aldolase (M_r 156000, R_0 45.1 Å), albumin (M_r 66700, R_0 35.9 Å), ovalbumin (M_r 45000, R_0 28.6 Å), chymotrypsinogen (M_r 25000, R_0 20.9 Å), myoglobin (M_r 17600, R_0 20.1 Å), cytochrome *c* (M_r 12500, R_0 17.9 Å) (Sherman et al., 1980). Linear regression analysis of the plot K_{av} vs. R_0 or K_{av} vs. M_r for the standard proteins yielded an *r* value of

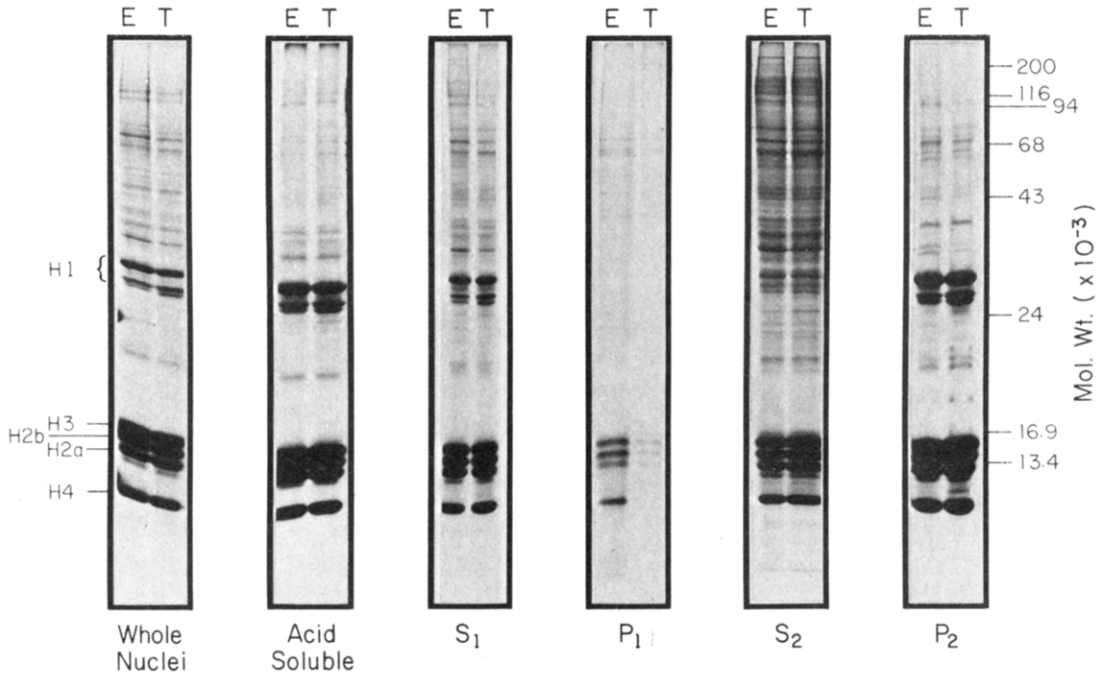

FIGURE 1: Electrophoretic analysis of total nuclear proteins, acid-soluble nuclear proteins, and proteins in various chromatin fractions. Proteins were prepared from euthyroid (E) and hypothyroid (T) nuclei and electrophoresed in polyacrylamide gels under denaturing conditions as described under Materials and Methods and Table I. Whole nuclei (80 μg), acid (0.4 N H_2SO_4) soluble nuclear proteins (80 μg), S_1 chromatin (80 μg), P_1 chromatin (25 μg, E; 15 μg, T), S_2 chromatin (100 μg), P_2 chromatin (100 μg). Molecular weight protein markers (see Materials and Methods) were electrophoresed in adjacent wells of the same slab gel.

0.96–0.98. Four independent measurements were made for the Stokes radius of the T_3 receptor and the nuclease-generated receptor-containing fragment. Measurements using agarose (Bio-Rad) A-0.5 M yielded results similar to those found with AcA-34. The molecular weight (M_r) and frictional coefficients (f/f_0) were calculated by assuming a partial specific volume of 0.725 cm^3/g as described by Siegel & Monty (1964).

Results

Differential Solubility of Rat Liver Chromatin in 0.1 M NaCl. In vivo labeled euthyroid rat liver [^{125}I]T_3 receptors are associated with chromatin structures which are soluble in 0.1 M KCl (Jump & Oppenheimer, 1980). To investigate whether labeled T_3 receptors are associated with similar structures in isolated liver chromatin from hypothyroid animals, we compared the partitioning of T_3 receptors in various chromatin fractions following micrococcal nuclease digestion of nuclei from the two thyroidal states (Table I).

Following 5–6% digestion of rat liver nuclei by micrococcal nuclease, 85% of the chromatin DNA and 90–95% of the [^{125}I]T_3 receptors are recovered in the nuclease-soluble fraction (S_1). Whereas addition of NaCl (0.1 M) precipitates only 6–7% of the chromatin-associated [^{125}I]T_3 receptors, 35% of the chromatin DNA is recovered in the P_2 fraction. A comparison of the specific activity of receptor-bound T_3 ([^{125}I]T_3/mg of DNA) in the S_2 (0.1 M NaCl soluble) and P_2 (0.1 M NaCl insoluble) fractions illustrates a 6.2–7.5-fold difference in T_3 receptor concentration. In addition, both the P_1 and P_2 fractions are depleted (3–5-fold) of receptors compared to receptor-specific activity in whole nuclei. Thus, T_3 receptors are not uniformly distributed to all chromatin fractions in rat liver nuclei. Moreover, no significant difference in the partitioning of T_3 receptors in the various chromatin fractions (S_1, P_1, S_2, and P_2) is detected in the two thyroidal states.

The protein composition of the nuclear fractions from hypo- and euthyroid nuclei was characterized further by NaDod-

Table I: Differential Solubility of Rat Chromatin in 0.1 M NaCl[a]

fractions		total DNA (mg)	% of total nuclear-bound [^{125}I]T_3	[^{125}I]T_3 (cpm/mg of DNA)
whole nuclei	E	6.40	100	10 463
	T	6.60	100	24 380
micrococcal nuclease soluble chromatin (S_1)	E	5.54	97	11 740
	T	5.60	94	26 950
residue (P_1)	E	0.49	3	3 937
	T	0.69	6	14 478
0.1 M NaCl soluble chromatin (S_2)	E	3.70	90	16 224
	T	3.58	87	40 243
0.1 M NaCl insoluble chromatin (P_2)	E	1.92	7	2 608
	T	1.98	6	5 083

[a] Details are given under Materials and Methods. Liver nuclei were isolated from euthyroid (E) and hypothyroid (T) animals and resuspended in buffer D to give 64 A_{260} units/mL, 10 464 cpm/mg of DNA and 66 A_{260} units/mL, 24 380 cpm/mg of DNA, respectively. Nuclei were digested with micrococcal nuclease (5 units/A_{260}) for 90 s at 30 °C; digestions were stopped with EDTA (10 mM) and dialyzed against TEDP at 0 °C for 12 h. The extent of digestion as measured by percent chromatin DNA hydrolyzed and soluble in perchloric acid (0 °C) was 5.8% and 4.6% for euthyroid and hypothyroid nuclei, respectively. The various chromatin samples, S_1, S_2, P_1, and P_2, were obtained as described under Materials and Methods. The experiment is representative of two separate studies involving pools of three animals in each group.

SO_4–polyacrylamide gel electrophoresis (Figure 1). The profile of chromosomal proteins recovered in the S_1 fraction consistng of both histone (acid-soluble) and nonhistone proteins appears identical with that observed with whole nuclear proteins. The P_1 fraction, which represents nuclear material remaining insoluble after micrococcal nuclease digestion, contains less than 5% of [^{125}I]T_3 receptor and residue chro-

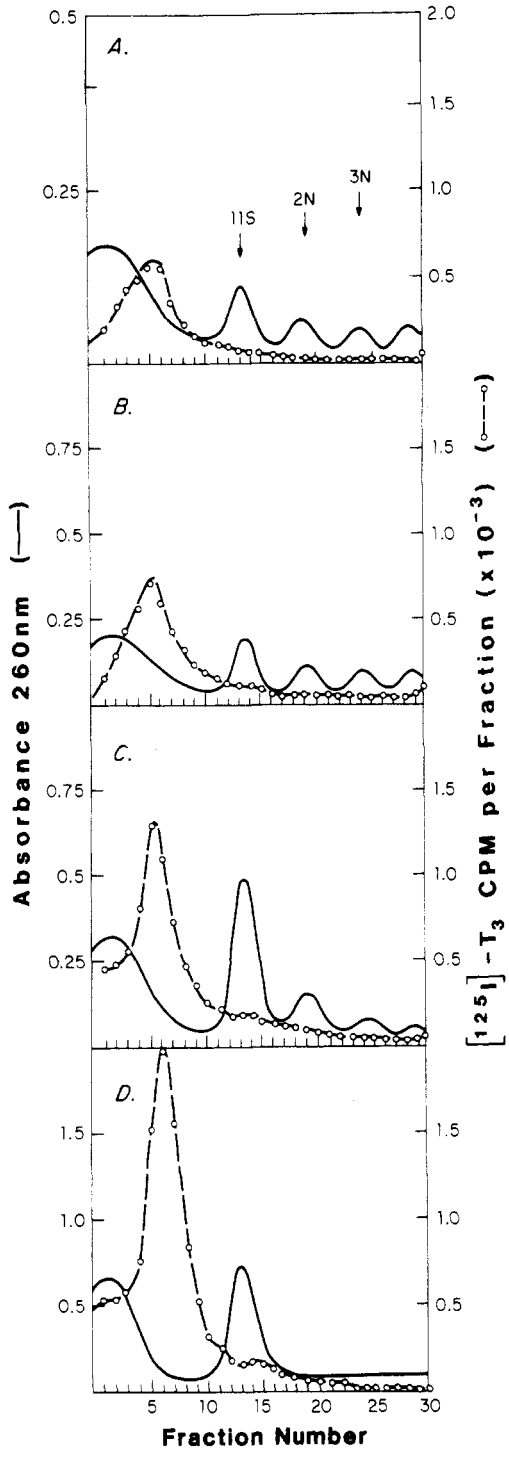

FIGURE 2: Velocity sedimentation analysis of chromatin-associated T_3 receptors from euthyroid hepatic nuclei digested with micrococcal nuclease. Hepatic nuclei were isolated from euthyroid rats injected with [^{125}I]T_3 (50 ng/100 g of body weight) 30 min before killing and digested with micrococcal nuclease (0.8, 2.5, 5.0, and 20.0 units/A_{260}) to obtain 1.9, 3.7, 7.2, and 15.9% digestion (panels A–D, respectively). Following digestion, S_2 chromatin was prepared as described under Materials and Methods. The fraction of chromatin DNA remaining in the soluble S_2 fraction was 33, 54, 47, and 39% while 35, 52, 82, and 85% of the total [^{125}I]T_3 radioactivity bound to nuclei was made soluble (panels A–D, respectively). Soluble chromatin obtained from the four digestion points was layered on 5–20% sucrose gradients containing TEDP + 0.1 M NaCl and chromatin fragments separated by velocity sedimentation (SW 41 rotor, 38 000 rpm, 15 h, 2 °C). Gradient fractions were collected and analyzed for the absorbance at 260 nm (—) and ^{125}I radioactivity (o—o). 11 S, migration of catalase and mononucleosomes; 2N, dinucleosomes; 3N, trinucleosome.

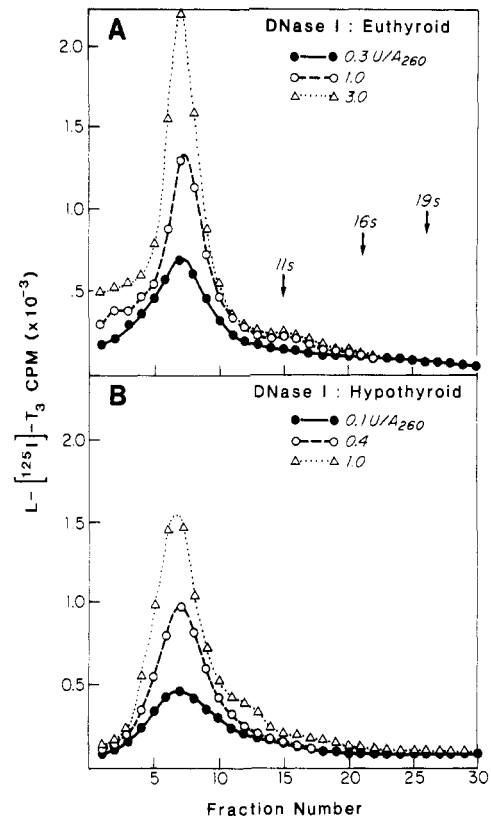

FIGURE 3: Velocity sedimentation analysis of T_3 receptor-containing fragments generated by pancreatic DNase I digestion of hepatic nuclei. Euthyroid (upper panel) and hypothyroid (lower panel) hepatic nuclei were isolated from animals injected with [^{125}I]T_3 as described. Nuclei (80 A_{260} units/mL) were digested with pancreatic DNase I for 60 s at 30 °C: (A) 0.3 (●), 1.0 (○), and 3.0 (△) units/A_{260}; (B) 0.1 (●), 0.4 (○), and 1.0 (△) units/A_{260}. Nuclei were lysed, and S_2-soluble chromatin was prepared and fractionated on 5–20% sucrose gradients containing TEDP + 0.1 M NaCl as described in Figure 2. 11 S, mononucleosomes; 16 S; dinucleosomes; 19 S, trinucleosomes.

matin (i.e., nucleosomal histones but no detectable histone H-1). In addition, this fraction also contains a specific subset of nonhistone proteins (M_r 40 000–70 000) which corresponds in size and distribution to rat liver nuclear matrix proteins (Berezney and & Coffey, 1977). The S_2 fraction contains a high ratio of nonhistone protein to histones when compared to whole nuclei and no detectable histone H-1. In contrast, P_2 chromatin has a lower nonhistone protein to histone ratio and contains nearly all histone H-1. Therefore, more than 80% of the T_3 receptor activity (Table I) is found in fractions enriched in nonhistone protein and depleted of histone H-1 (Figure 1).

Digestion of Rat Liver Nuclei with Micrococcal Nuclease and Pancreatic DNase I. We further characterized the association of receptors with chromatin by digesting rat liver nuclei from in vivo labeled euthyroid and hypothyroid animals to varying extents with micrococcal nuclease (Figure 2) or pancreatic DNase I (Figure 3).

With increasing levels of micrococcal nuclease digestion of euthyroid liver nuclei, a predominant T_3 receptor-containing structure (5.5–6.0 S, Figure 8) migrating slower than mononucleosomes accumulates as a result of oligonucleosomal degradation to mononucleosomes (Figure 2). A minor [^{125}I]T_3-labeled fragment, representing 7.7% of the total nuclear [^{125}I]T_3 radioactivity (Figure 2D) and migrating coincident with "heavy mononucleosomes" (12–14 S), became evident after more extensive nuclease digestion.

The generation of the receptor-containing entity (5.5–6 S) does not appear to represent the adventitious aggregation of

the T_3 receptor with nuclear constituents released from nuclei during digestion for the following reasons. (1) Sonic shearing of chromatin which disrupts higher order folding of chromatin does not produce the 5.5– 6S structure; (2) DNase I or micrococcal nuclease treatment of the partially purified 5.5–6 S structure generates the 3.5 S T_3 receptor (data not shown); (3) redigestion of oligomeric chromatin containing the T_3 receptor generates the 5.5–6 S structure; (4) treatment of sham digested nuclei with 0.1 M NaCl does not release either the receptor (3.5 S) or the receptor-containing fragment (5.5–6 S); (5) velocity sedimentation is carried out in 0.1 M NaCl, which should disrupt weak ionic associations between the receptor and chromatin. Taken together, these results suggest that the 5.5–6 S T_3 receptor-containing fragment represents the receptor binding site excised from chromatin by endonucleolytic cleavage of DNA neighboring the receptor.

Our studies indicate that chromatin containing the T_3 receptor is highly sensitive to endonucleolytic digestion. The isolated mononucleosomal structures displayed in Figure 2A represent 2–3% of the total nuclear DNA while 28% of the total nuclear radioactivity is recovered in a predominant T_3 receptor-containing fragment. Thus, DNA adjacent to T_3 receptors is approximately 14-fold more sensitive to endonucleolytic digestion than DNA neighboring nucleosomes in bulk chromatin.

The sedimentation characteristics of nuclease-generated receptor-containing fragments were identical in both euthyroid and hypothyroid liver nuclei, as well as in several other receptor-containing rat tissues (Figures 7 and 8). However, our analysis did not resolve a periodic distribution of T_3 receptors paralleling the distribution of isolated chromatin (Figures 2, 7, and 8). When the distribution of receptors in S_1 chromatin was analyzed in gradients containing no NaCl (0.1 M) (Figure 5), a periodic distribution of receptors was detected. The observation that the apparent periodicity can be eliminated by including 0.1 M NaCl in gradients indicates that weak ionic forces play a role in the interaction between receptor-containing structures and chromatin. Whether these interactions are of physiological significance remains to be determined.

Pancreatic DNase I was also found to excise a predominant (5.5–6.0 S) and a minor (12–14 S) receptor-containing fragment from euthyroid and hypothyroid liver nuclei (Figure 3) with increasing levels of digestion. Although a significant fraction of the DNase I solubilized chromatin sedimented slower than 11 S, a small fraction sedimented to the mono-, di-, and trinucleosomal positions. We conclude from these studies that both micrococcal nuclease and pancreatic DNase I appear to recognize similar structural features close to the receptor binding site in rat liver nuclei since each endonuclease excises receptor-containing fragments with similar sedimentation characteristics.

Endonuclease Titration of the Sensitivity of the T_3 Receptor Binding Site in Euthyroid and Hypothyroid Hepatic Nuclei. We next undertook a study designed to determine whether the increased nuclear activity (Dillman et al., 1978; Towle et al., 1979; Barsano et al., 1980; Coleoni & DeGroot, 1980) in euthyroid liver nuclei might be reflected by enhanced sensitivity of the T_3 receptor binding site to endonuclease digestion. Accordingly, we measured the fraction of the total nuclear receptor excised from chromatin migrating to 5.5–6.0 S (see Figures 2 and 3) as a function of the extent of nuclear DNA digestion.

Increasing degradation of nuclear DNA by either micrococcal nuclease (Figure 4 left panel) or pancreatic DNase I (Figure 4, right panel) results in the release of an increasing fraction of the nuclear T_3 receptor in the 5.5–6.0 S form (Figures 2 and 3). Extensive digestion (>40% perchloric acid solubility) results in a loss of [^{125}I]T_3 receptor-containing fragments. Since we have shown that the T_3 receptor-containing fragment contains DNA, these results suggest that the receptor-containing fragment is further degraded and aggregates with insoluble complexes generated during digestion. Thyroidal status has no apparent effect on the susceptibility of DNA neighboring the T_3 receptor binding site to either micrococcal nuclease or pancreatic DNase I digestion. It should be noted, however, that only 2% degradation of nuclear DNA by either endonuclease releases 30% of the T_3 receptors from chromatin as a receptor-containing fragment (5.5–6.0 S). These results indicate that a significant number of T_3 receptors are bound to a limited fraction of chromatin. Neither enzyme allowed the detection of major structural changes in chromatin-containing receptors or in chromatin structure within nuclei. Our findings thus lend no support to the possibility that T_3 action is mediated by modification of higher ordered folding of chromatin within liver nuclei (Figure 4).

Occupied and Unoccupied T_3 Receptors Are Associated with Identical Chromatin Structures. In the foregoing studies, we assayed the T_3 receptor with radioactively labeled T_3 administered in vivo. We therefore considered the possibility that a class of receptors might exist which is not readily accessible to plasma hormone. Accordingly, we undertook studies to compare the distribution of in vivo labeled T_3 receptors with the distribution of T_3 receptors labeled in vitro following the isolation of micrococcal nuclease generated fragments by velocity sedimentation.

Hypothyroid liver nuclei were isolated from animals receiving no in vivo [^{125}I]T_3 and from animals injected with [^{125}I]T_3 (20 ng/100 g of body weight, 30 min before killing). We then treated nuclei from the two sources with micrococcal nuclease until 6% of the DNA was digested. Soluble chromatin fragments (S_1) were isolated, and the distribution of the in vivo labeled T_3 receptors was determined. The distribution of unoccupied receptors in chromatin fragments isolated from animals receiving no T_3 in vivo was determined by the in vitro method of analysis described under Materials and Methods. The distribution of in vivo labeled occupied receptors in the isolated chromatin fragments is similar to the distribution of unoccupied receptors which are measured in vitro (Figure 5).

Measurement of the T_3-binding capacity in the three regions of the gradient specified in Figure 5 indicates that 42.1, 20.7, and 19.3% of the total nuclear receptors were recovered in regions A, B, and C, respectively. These values correspond to 48, 22, and 16% of the in vivo labeled T_3 receptor, which sediments to the same position. The similarity in distribution thus strongly suggests that there are no sites which are unavailable to circulating T_3 in vivo. Moreover, these studies demonstrate that over 80% of T_3 nuclear receptor activity is found in chromatin fractions (5–19 S) following mild micrococcal nuclease digestion whereas only 20% of the total nuclear DNA is recovered in the same fraction. Thus, there was a 4.4-fold enrichment in T_3-binding capacity per milligram of DNA from 404 pg in whole nuclei to 1.78 ng in isolated chromatin.

Protein Composition of Chromatin Fragments Containing T_3 Receptors. We sought further to characterize the chromatin fractions containing receptors by analyzing the protein composition in the 5.5–6.0 S, mononucleosome, and dinucleosome regions obtained from S_2 chromatin following digestion of 4.8–5.6% of euthyroid and hypothyroid rat liver

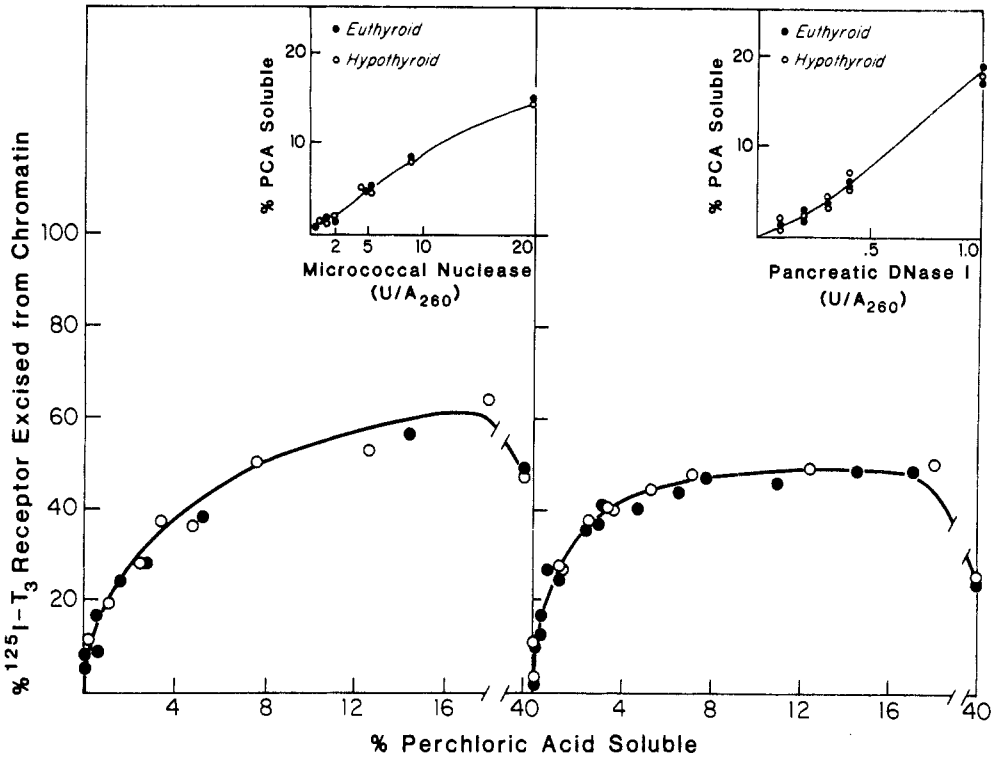

FIGURE 4: Preferential release of the 5.5–6.0 S T_3 receptor-containing fragment from hypothyroid and euthyroid chromatin by micrococcal nuclease and pancreatic DNase I digestion. Hepatic nuclei were isolated from hypothyroid (○) and euthyroid (●) rats injected with [^{125}I]T_3 (50 ng/100 g of body weight) 30 min before death. *Micrococcal nuclease.* Nuclei were resuspended in buffer D to 80 A_{260} units/mL, and micrococcal nuclease (0.2–20 units/A_{260}) was added at 0 °C. A tube receiving no exogenous nuclease was included in all studies. Tubes were transferred to 30 °C for 60 s and returned to 0 °C for 5 min. The digestion was terminated with the addition of EDTA to 10 mM. The extent of digestion was determined as described under Materials and Methods. Nuclei were lysed by dialysis against TEDP (2 °C) for 2 h. The fraction of the receptor liberated from chromatin was determined by velocity sedimentation in a 5–20% sucrose gradient containing TEDP as described in Figure 2. An alternate method which yielded identical results involved diluting the nuclear suspension 10-fold with TEDP (2 °C). The resultant lysed nuclei were sedimented in an SW 65 rotor for 3 h at 65000 rpm (2 °C). Chromatin >11 S will pellet under these sedimentation conditions. Insert: Hepatic nuclei were digested with varying concentrations of micrococcal nuclease, and the extent of digestion was measured as described under Materials and Methods (PCA, perchloric acid soluble). *Pancreatic DNase I.* Pancreatic DNase I (0.1–5 units/A_{260}) was added at 0 °C. A tube receiving no exogenous endonuclease was also included. Nuclei were digested with DNase I at 50–80 A_{260} units/mL by using the schedule and the fractionation procedure for the measurement of the receptor fragment (5.5–6.0 S) liberated from nuclei as described above for micrococcal nuclease. Insert: Hepatic nuclei were digested with varying concentrations of pancreatic DNase I, and the extent of digestion was measured as described previously.

nuclei by micrococcal nuclease (Figure 6). A heterogeneous array of nonhistone proteins sediments to the 5.5–6.0 S position following mild micrococcal nuclease digestion. Many of these proteins are not loosely bound to nuclei since the spectrum and quantity of protein migrating to this position after sham digestion of nuclei sharply differ from those illustrated in Figure 6. No histones are detected in this fraction. In addition, the mononucleosome and dinucleosomes contain no detectable histone H-1. This was expected from our previous finding that S_2 chromatin contains little or no histone H-1 (Figure 1). Although considerable difference in the protein composition is apparent in the various chromatin fractions isolated from euthyroid and hypothyroid liver nuclei, i.e., 5.5–6 S (M_r 10000–24000), mononucleosomes (M_r 14000–17000), and dinucleosomes (M_r 12000–38000), further study will be required to fully understand the significance of these differences.

Association of the T_3 Receptor with Chromatin in the Various Receptor-Containing Tissues. We were interested in determining whether T_3 receptors in other receptor-containing tissues were associated with similar nuclease-sensitive structures as found in liver. In vivo labeled T_3 receptor-containing fragments generated by micrococcal nuclease digestion of hypo- and euthyroid rat brain, kidney, heart, and liver nuclei have essentially identical sedimentation characteristics (Figure 7). A predominant receptor-containing fragment sedimenting slower than mononucleosomes and a minor 12–14 S fragment are seen in all T_3 receptor-containing tissues examined. A periodic distribution of T_3 receptors with dinucleosomes, trinucleosomes, or larger structures was not detected in these studies. The nuclease-generated fragment sediments to 5.5–6.0 S, whereas the salt-extracted receptor obtained from these tissues sediments to the 3.5 S position (Figure 8). On the basis of our studies with the rat liver T_3 receptor, we suggest that 0.5 M KCl disrupts the electrostatic interaction between the T_3 receptor and DNA and perhaps other proteins within the particle to convert the 5.5–6 S structure to the 3.5 S form of the receptor. Thus, T_3 receptors in the various receptor-containing rat tissues are associated with internucleosomal DNA structures sensitive to micrococcal nuclease digestion.

Gel Filtration of the T_3 Receptor-Containing Fragment. Additional information on the T_3 receptor-containing fragment was obtained by gel filtration. The predominant nuclease-generated receptor-containing structure from rat liver has a Stokes radius of 42.3 ± 1.5 Å. Perlman et al. (1981) reported the predominant nuclease-generated fragment (6.5 S) from GH_1 pituitary cells has a Stokes radius of 66 Å. The discrepancy between the two values is not apparent to us. The Stokes radius of the T_3 receptor (33.7 ± 2 Å) was found to be comparable to values (35 Å) reported previously (Latham et al., 1976). The molecular weight (M_r 95400) and frictional coefficient (f/f_0 = 1.39) of the predominant T_3 receptor-containing fragment were calculated by assuming a partial specific volume of 0.725 cm^3/g for protein (Siegel & Monty, 1966) and an s value of 5.5 S (Figure 8). The receptor-con-

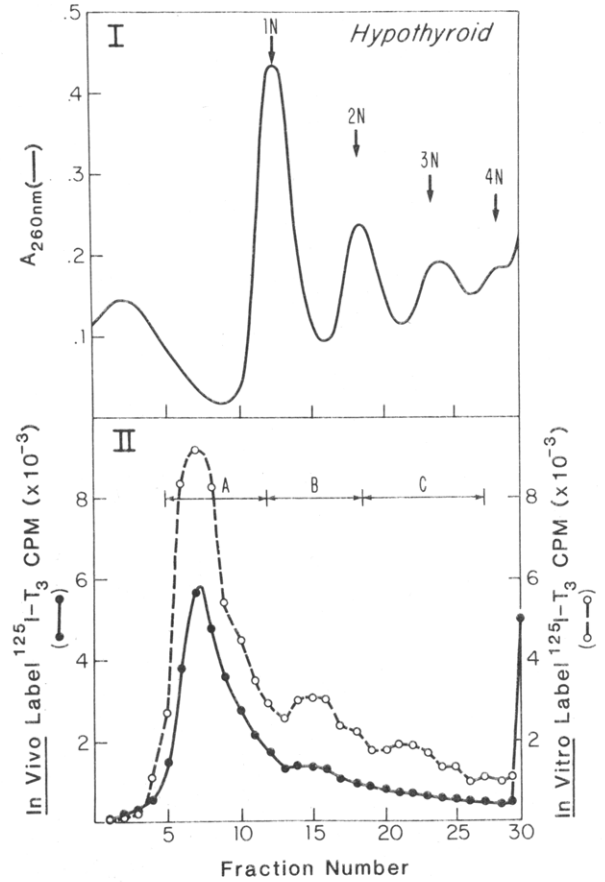

FIGURE 5: Distribution of T_3 receptors in isolated hypothyroid hepatic chromatin fragments determined by in vivo and in vitro analysis. Hepatic nuclei were isolated from hypothyroid rats either injected with [^{125}I]T_3 (50 ng/100 g of body weight) (●) or receiving no [^{125}I]T_3 in vivo (○). Isolated hypothyroid hepatic nuclei were digested with micrococcal nuclease to 6.0% digestion. Soluble S_1 chromatin (20 A_{260}/units gradient) was fractionated on a 5–20% sucrose gradient (TEDP) as described in Figure 2. During fractionation, gradients were scanned for the absorbance at 260 nm (—) (I), and the distribution of in vivo radioactivity was measured (●) (II). Gradient fractions containing isolated chromatin from animals receiving no in vivo label were assayed for the presence of T_3 receptors (○) as described under Materials and Methods. The apparent periodicity of T_3 receptors in chromatin is observed at low ionic strengths and is eliminated by including NaCl or KCl (0.1 M) in buffers during fractionation. No difference in the chromatin absorbance profile (A_{260}) was detected in the two digests from hypothyroid nuclei, and therefore, only one profile is presented. Regions A–C represent pools of chromatin in which the binding capacity for T_3 was measured (see text). 1N, mononucleosome; 2N, dinucleosome; 3N, trinucleosome; 4N, tetranucleosome.

taining structure is asymmetric as is the T_3 receptor (f/f_0 = 1.4). The differences in molecular weight between the T_3 receptor (M_r 50 500) and the receptor-containing fragment (M_r 95 400) indicate that other chromatin constituents are associated with the receptor in the fragment. In the analysis, we have not measured the fractional composition of DNA and proteins and therefore are unable to determine with precision either the molecular weight or the contributions from DNA and other proteins.

Discussion

Although the binding of T_3 by a chromatin-bound nuclear receptor is generally believed to be essential for thyroid hormone action (Oppenheimer, 1979; Baxter et al., 1979), neither the structure of chromatin adjacent to the T_3 receptor binding site nor the nuclear processes mediated by T_3 are well understood. The recent independent description of receptor-

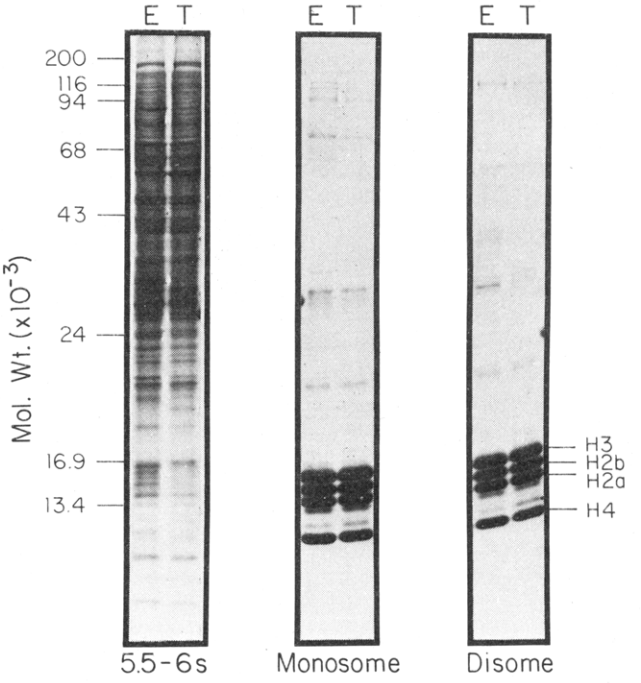

FIGURE 6: Electrophoretic analysis of proteins migrating to the 5.5–6.0 S, mononucleosome, and dinucleosome positions. Euthyroid (E) and hypothyroid (T) nuclei were digested to 5% by micrococcal nuclease, and the S_2 chromatin obtained was fractionated by velocity sedimentation as described in Figure 2. Proteins recovered from regions of the S_2 chromatin gradient migrating to 5.5–6.0 S (80 μg), mononucleosome (25 μg), and dinucleosomes (25 μg) were precipitated with absolute ethanol and electrophoresed under conditions described under Materials and Methods.

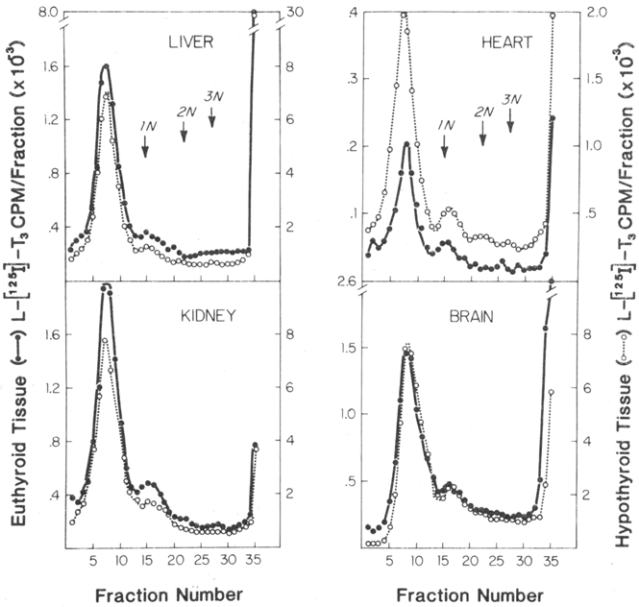

FIGURE 7: Micrococcal nuclease excises a discrete [^{125}I]T_3-labeled complex from various rat tissues. Nuclei were isolated from liver, heart, and kidney of hypothyroid (○) and euthyroid (●) animals 60 min following an intravenous injection of [^{125}I]T_3 (50 ng/100 g of body weight). Brain nuclei were isolated (Oppenheimer et al., 1974b) from animals 3 h following an injection of [^{125}I]T_3 (200 ng/100 g of body weight). Nuclei were resuspended in buffer D and digested to 15–20% perchloric acid solubility. Following dialysis against TEDP (6 h, 0 °C), soluble S_1 chromatin was separated on 5–20% sucrose gradients (TEDP) and fractionated, and radioactivity was quantitated as previously described in Figure 2. The location of mononucleosomes (1N), dinucleosomes (2N), and trinucleosomes (3N) is indicated in the figure.

containing fragments by three laboratories (Samuels et al., 1980; Jump & Oppenheimer, 1980; Groul, 1980) raises the

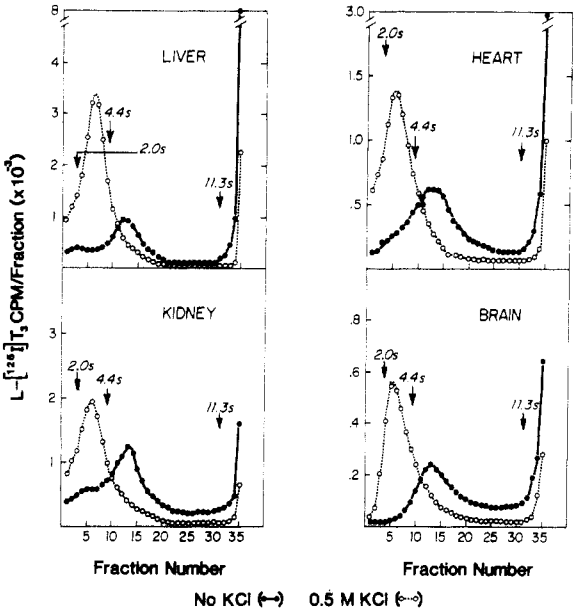

FIGURE 8: Nuclease-generated [^{125}I]T_3 receptor complex is larger than the salt-extracted form of the receptor in all rat tissues. Micrococcal nuclease solubilized chromatin containing in vivo labeled [^{125}I]T_3 receptor was obtained from liver, heart, kidney, and brain nuclei of hypothyroid rats as described in Figure 7. One-half of the soluble S_1 chromatin obtained following dialysis was adjusted to 0.5 M KCl. Soluble chromatin (●) or 0.5 M KCl treated chromatin (○) was sedimented in 4–8% sucrose gradients containing TEDP and TEDP + 0.5 M KCl, respectively. Sedimentation conditions were 38 000 rpm for 22 h at 2 °C in an SW 41 rotor. Gradients were fractionated as previously described. Sedimentation markers were run in parallel gradients containing TEP + 0.1 M KCl: myoglobin, 2.0 S; bovine serum albumin, 4.4 S; and catalase, 11.3 S. The salt-extracted T_3 receptor migrates to 3.5 S, and the micrococcal nuclease generated T_3 receptor-containing fragment migrates to 5.5–6.0 S.

possibility that this unit may well represent the next higher order of structural complexity of the receptor involved in the initiation of thyroid hormone activity. This fragment is generated as a result of the use of micrococcal nuclease and pancreatic DNase I, specific endonucleolytic probes which have been successfully used in previous studies to elucidate the structure of chromatin (Felsenfeld, 1978).

The preliminary characterization described in the present paper demonstrates that the T_3 receptor-containing fragment has a sedimentation coefficient between 5.5 and 6.0 S, a Stokes radius of 42 Å, and an approximate molecular weight of 95 400. In contrast, the T_3 receptor has a sedimentation coefficient of 3.5 S, a Stokes radius of 35 Å, and a molecular weight of 50 500 (Latham et al., 1976). Since extensive digestion of the receptor-containing fragments with endonuclease yields the receptor, it appears likely that the fragment also contains DNA. Nevertheless, it is obvious that until the partial specific volume is directly determined, neither the molecular weight nor the protein and DNA composition of the fragment can be assigned with precision. The present studies also demonstrate for the first time that other receptor-containing tissues in the rat, including brain, kidney, and heart, contain what appear to be identical fragments. In addition to the predominant 5.5–6.0 S fragment, a more complex but minor component with a sedimentation coefficient of 12–14 S can also be identified in all receptor-containing tissues.

Micrococcal nuclease is effective in excising over 90% of the T_3 receptors from rat liver nuclei. These are partitioned between the 5.5–6.0 S structure and the oligonucleosomal structures. Further digestion of oligonucleosomal chromatin with micrococcal nuclease results in the generation of the receptor-containing fragment. The nuclear fraction containing the residual 3–6% T_3 receptor also contains nucleosomal histones and several nonhistone proteins corresponding in size and distribution to rat liver nuclear matrix proteins (M_r 45 000–70 000) (Berezney & Coffey, 1977). In a recent study, Barrack & Coffey (1980) reported that approximately 50% of the dihydrotestosterone receptors in rat ventral prostate and estradiol receptors in chick liver were associated with the nuclear matrix structures. Our findings thus point to what may be the important difference between steroid and thyroid hormone with respect to subnuclear localization.

The present studies reemphasize the high degree of susceptibility of the receptor-containing chromatin to the endonuclease digestion in comparison to the susceptibility of bulk chromatin. This finding has prompted the suggestion that the receptor is situated in "active" chromatin (Jump & Oppenheimer, 1980), an inference made on the basis of studies by others with specific nucleic acid probes which have indicated that genes undergoing transcription are more susceptible to endonuclease digestion (Weintraub & Groudine, 1976; Stalder et al., 1980; Bellard et al., 1978; Bloom & Anderson, 1978). Since several studies have established that the rate of poly(A) RNA formation in the euthyroid state exceeds that in hypothyroidism by a factor of 1.7 (Dillmann et al., 1978; Towle et al., 1979), we might anticipate differences between hypothyroid and euthyroid nuclei, either with respect to the susceptibility of the T_3 receptor-containing chromatin to endonuclease digestion or with respect to the distribution of the receptor in chromatin. No such differences, however, could be demonstrated. Moreover, there were no differences in chromatin localization between occupied sites measured after fractionation.

Our results thus fail to demonstrate a marked influence by T_3 on gross chromatin structure and fail to provide evidence for an alteration in the relationship between the receptor and contiguous chromatin. The possibility that thyroid hormone can affect more limited genomic modifications, however, cannot be excluded. It should also be considered that thyroid hormone exerts no effect on the accessibility of specific genes to transcription but that the hormone acts in some other fashion to accelerate the rate of formation of specific mRNA sequences coding for target genes.

Since micrococcal nuclease preferentially digests the 40–60 base pairs of DNA linking adjacent nucleosomes (Whitlock & Simpson, 1976), it appears reasonable to postulate that the T_3 receptor is associated with this structure. At the same time, however, it is possible that the receptor is associated with linker DNA adjacent to structurally modified nucleosomes that are highly sensitive to nuclease action. The 12–14 S form of the receptor may in fact represent this form of receptor–chromatin interaction. Several investigators have shown that modification of the nucleosome structures through histone acetylation (Simpson, 1978) or interaction with high mobility group proteins (Weisbrod & Weintraub, 1979; Weisbrod et al., 1980) renders DNA around the nucleosome more sensitive to endonuclease digestion. Such modifications have been observed in transcriptionally active chromatin (Levy-Wilson et al., 1979). If the T_3 receptor were associated with the core nucleosomal histones, however, one might anticipate a periodic distribution of the receptor at some level of nuclease digestion. Such periodicity, though characteristic for estradiol receptors in chromatin particles isolated from chick oviduct (Massol et al., 1978; Senior & Frankel, 1978) and MCF-7 mammary carcinoma nuclei (Scott & Frankel, 1980), has not been found for the T_3 receptor in the present studies. The failure to detect

a periodic array may reflect the heterogenous nonhistone protein composition of receptor-containing chromatin, resulting in smearing of the receptor profile in velocity gradients. The lack of periodicity in chromatin has also been reported for dihydrotestosterone receptors in rat ventral prostate (Rennie, 1979). In contrast to T_3 receptor-containing chromatin (Jump & Oppenheimer, 1980), however, dihydrotestosterone receptors are associated with linker DNA in regions not preferentially digested by micrococcal nuclease. Thus, differences between the reported association of T_3 receptors and steroid receptors with chromatin are apparent. Whether these reflect inherent differences in receptor association with chromatin or are attributable to specific methods of analysis used will require further study.

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