Thyroid Hormone Receptor–Containing Fragment Released from Chromatin by Deoxyribonuclease I and Micrococcal Nuclease

Abstract. *Limited deoxyribonuclease I and micrococcal nuclease digestion of hepatic nuclei from euthyroid rats injected with ^{125}I-labeled triiodothyronine ([^{125}I]T_3) releases a discrete [^{125}I]T_3-labeled chromatin fragment (5.8S) which is larger than the T_3 receptor (3.5S). These results suggest the T_3 receptor is associated with a restricted fraction of hepatic chromatin that has a nuclease sensitivity characteristic of transcriptionally active chromatin.*

Evidence suggests that specific nuclear receptors for triiodothyronine (T_3) are the site of initiation for thyroid hormone action (*1*). The receptor is a nonhistone acidic protein of 3.5*S* and 50,500 daltons (*2*). Occupation of the nuclear sites is followed by a many-fold increase in messenger RNA (mRNA) sequences known to code for proteins induced by T_3, including growth hormone in pituitary cells (*3*), and α_{2U}-globulin (*4*) and malic enzyme (*5*) in rat liver. In addition, T_3 administration to hypothyroid animals results in a generalized 1.8-fold increase in total hepatic polyadenylate-containing RNA (*6*). These observations suggest a possible role for nuclear receptors in augmenting transcription. We have therefore analyzed the association of the T_3 receptor complex with chromatin. We took advantage of recent reports that pancreatic deoxyribonuclease I (DNase I) (*7, 8*) and micrococcal nuclease (*9*) digest sequences of chromatin that are active in transcription. Several investigators have indicated that certain proteins dissociated from chromatin as a direct result of DNA digestion by these enzymes are directly involved in transcription (*10, 11*). Accordingly, we analyzed the partition of receptors after limited digestion of chromatin with micrococcal nuclease and DNase I and characterized the sedimentation properties of a T_3 receptor chromatin fragment produced by such treatment.

Hepatic nuclei were rapidly isolated from rats injected with [^{125}I]T_3 and digested with varying concentrations of micrococcal nuclease as described in the legend to Fig. 1. The sedimentation profiles of soluble chromatin in Fig. 1 show chromatin fragments of varying complexity. The [^{125}I]T_3 receptor was not uniformly distributed in chromatin but was associated with the smaller particles preferentially liberated by micrococcal nuclease. With more extensive digestion resulting in the degradation of large oligonucleosomal structures to smaller particles, all [^{125}I]T_3 receptors accumulated in a single peak sedimenting slower than the 11*S* mononucleosomes. Virtually all radioactivity in this peak was specifically bound since intravenous injection of 10 μg of T_3 obliterated all radioactivity in the T_3 peak. When nuclear suspensions were incubated in the absence of added micrococcal nuclease, as described in Fig. 1, 1 to 2 percent of the hepatic chromatin and 8 to 9 percent of the total chromatin-bound radioactivity was released. The labeled material migrated to the same position as chromatin fragments in the preparations treated with micrococcal nuclease. These results probably reflect the activity of endogenous Ca^{2+}-dependent endonuclease (*12*).

To obtain additional information about the fraction of chromatin containing the receptor we used hepatic nuclei labeled in vivo with [^{125}I]T_3 and digested with DNase I under conditions reported to selectively degrade sequences corresponding to transcriptionally active DNA (*7*). We compared the fraction of [^{125}I]T_3 receptor complex liberated from chromatin both after DNase I and micrococcal nuclease treatment and related this fraction to the extent of nuclease digestion as determined by perchloric acid solubility. The results (see Fig. 2) show that 50 to 60 percent of nuclear receptor is liberated as a 5.8*S* particle after only 5 percent of

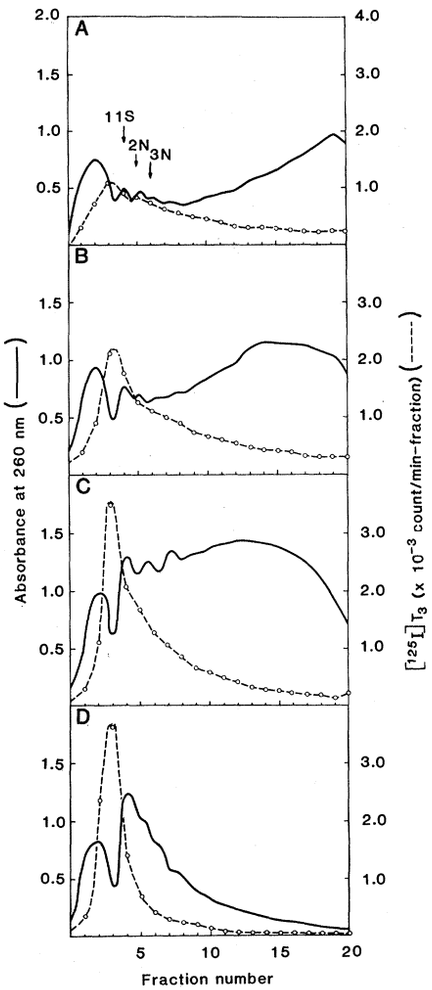

Fig. 1. Distribution of the T_3 receptor in chromatin fragments of varying complexity. Euthyroid Sprague-Dawley rats (300 to 350 g) were injected in the tail vein with [^{125}I]T_3 50 ng/100 g body weight (478 μCi/μg, Abbott) 30 minutes before they were killed. During this 30-minute period the metabolism of the injected T_3 is negligible and nuclear radioactivity is in the form of T_3 (*16*). Liver nuclei were isolated (*16*) and washed in buffer A [0.25*M* sucrose; 10 m*M* tris, *p*H 8.0; 1 m*M* $CaCl_2$; 1 m*M* $MgCl_2$; 1 m*M* DTT (dithiothreitol)] containing 1 percent Triton X-100 and subsequently, in buffer A plus 0.14*M* NaCl. Nuclei were resuspended to 50 A_{260} per milliliter (in micrococcal nuclear digestion) in buffer B (10 m*M* tris, *p*H 8; 0.9 m*M* $CaCl_2$; 0.9 m*M* $MgCl_2$; 1 m*M* DTT; 0.14 m*M* spermidine; 25 m*M* KCl) (*17*). Micrococcal nuclease (P-L Biochemicals, Inc.) was added to the nuclear suspension at concentrations of 0.37, 1.6, 4.7, 20.3 unit/A_{260} at 2°C in panels A to D, respectively. The temperature was raised to 30°C for 1 minute and returned to 2°C for 10 minutes. The reaction was terminated by the addition of EDTA to 10 m*M*, and the extent of digestion was measured in 1*M* perchloric acid and 1*M* NaCl as described by Stradling (*17*). Digested nuclei were dialyzed against a mixture of 10 m*M* tris, *p*H 8; 20 m*M* $NaHSO_3$ (neutralized); 0.1*M* KCl, 1 m*M* DTT, and 1 m*M* EDTA for 12 hours at 2°C for nuclear lysis (*17*). Insoluble chromatin was removed by sedimentation (4000*g*, 10 minutes, 4°C). Soluble chromatin was fractionated on 10 to 30 percent sucrose gradients in buffer C (10 m*M* tris, *p*H 8, 2 m*M* EDTA, 0.12*M* KCl, 1 m*M* DTT for 7 hours at 35,000 rev/min in a SW-41 rotor at 2°C. Fractionation of chromatin digested as described above yields 24, 57, 63, and 58 percent of the total hepatic chromatin and 61, 76, 79, and 78 percent of the total [^{125}I]T_3 radioactivity bound to nuclei in panels A through D, respectively. When 0.1*M* KCl was omitted from the dialysis buffer, 80 to 90 percent of hepatic chromatin was solubilized by nuclease at 2 percent perchloric acid solubility. Addition of KCl to 0.1*M* percipitates a significant fraction of chromatin, but has no effect on the nuclease solubilized T_3 receptor. The percentage of perchloric acid solubility attained in the four digestion points was 1.0, 2.6, 5.0, and 12.4 as shown in panels A to D, respectively. Direction of sedimentation is from left to right. Fractionation of gradients was monitored for absorbance at 260 nm (——) by use of an ISCO turbulence-free flow through cell. Individual fractions (0.6 ml) were analyzed for absorbance at 260 nm and for ^{125}I radioactivity (- - -) by gamma scintilation counting. The position to which the mononucleosome (11*S*) migrated was determined by sedimenting catalase (11*S*) in parallel gradients (2*N*, dinucleosome, 3*N*, trinucleosome).

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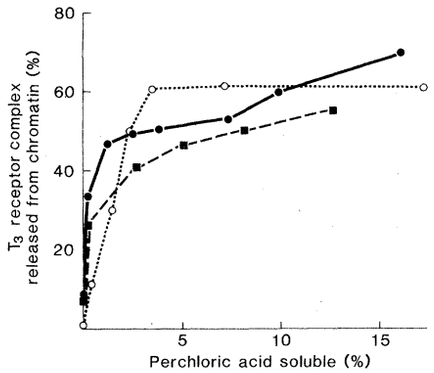
chromatin is solubilized by either enzyme. Approximately 40 percent of the hepatic receptor appears to be insensitive to nuclease digestion, suggesting that this fraction of receptor is either associated with a domain of chromatin resistant to nuclease digestion or, after being released by nuclease treatment, is redistributed to an insoluble chromatin complex.

We found no apparent differences between hypothyroid and euthyroid animals with respect to the kinetics of digestion of chromatin or the sedimentation profile of chromatin labeled with endogenously administered T_3. Thus, hypothyroidism per se does not appear to reduce the susceptibility of chromatin containing the T_3 receptor to endonuclease digestion. Since these experiments were carried out only with receptors labeled after intravenous injection of T_3, it is impossible to be certain about the distribution of receptor entirely devoid of T_3.

We compared the sedimentation characteristics of the nuclease-solubilized chromatin fragment and the salt-extracted nuclear T_3 receptor (Fig. 3). The sedimentation profile of the chromatin fragment and the salt-solubilized receptor in the 5 to 10 percent sucrose gradients containing 0.12*M* KCl is demonstrated in Fig. 3A. The two nucleases generate a receptor-containing fragment with the same sedimentation characteristics (5.8*S*), whereas the salt-extracted T_3 receptor is a smaller species with a sedimentation coefficient of 3.5*S* as previously reported (*2*). When each of the preparations was treated with 0.5*M* KCl and sedimented in gradients containing 0.5*M* KCl all protein-bound radioactivity migrated at the expected 3.5*S* position (Fig. 3B). In other experiments we observed that limited (1 to 2 percent perchloric acid–soluble) and more extensive (15 to 20 percent perchloric acid–soluble) digestion for both nucleases yield fragments with the same sedimentation value (5.8*S*). Thus, the fragment generated by either of the two nucleases appears to be relatively resistant to further nuclease digestion but complexed to other chromatin constituents by electrostatic interaction.

The results reported here indicate that thyroid hormone receptor is (i) nonrandomly distributed in euthyroid hepatic chromatin, (ii) binds to chromatin in a domain particularly sensitive to DNase I and micrococcal nuclease, and (iii) is complexed with a subnucleosomal structure of 5.8*S* which can be generated by limited digestion with DNase I, micrococcal nuclease, and probably the Ca^{2+}-dependent rat liver endonuclease. Since the T_3 receptor complex is rapidly released by micrococcal nuclease and does not comigrate with mononucleosomes, the T_3 receptor is probably associated with the linker region of chromatin. Our findings further support the concept that the T_3 nuclear receptor complex is associated with transcriptionally active chromatin. This inference is based on recent studies demonstrating the preferential digestion of transcriptionally active sequences by DNase I (*7, 8*) and micrococcal nuclease (*9*) and the selective release of specific nuclear proteins such as high mobility group (HMG) proteins believed to be important in transcription (*10, 11*). Since the nonhistone HMG proteins are much more abundant (10^6 per cell) (*11*) than nuclear receptors (4000 per cell) (*1*) our findings suggest that the T_3 nuclear receptors modify a more re-



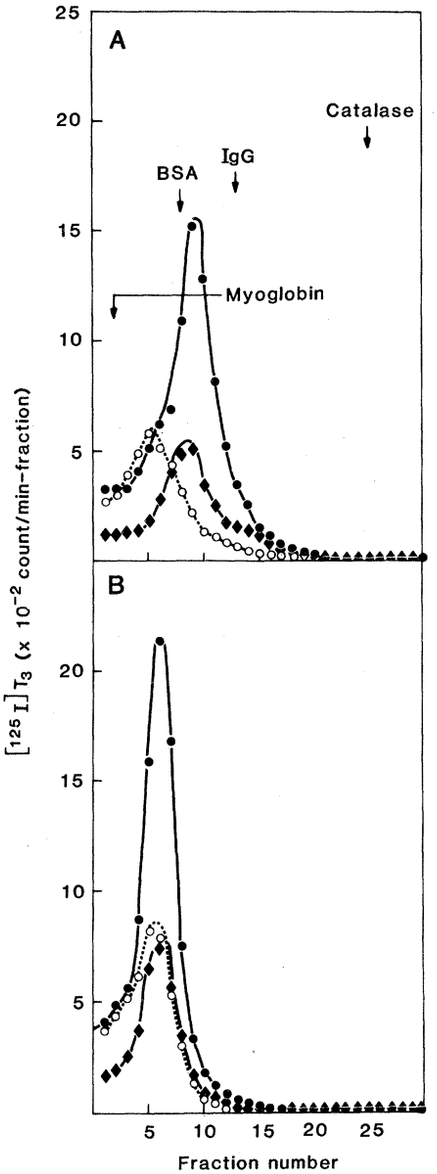


Fig. 2 (left). Preferential liberation of 5.8*S* $[^{125}I]T_3$ receptor complex from hepatic nuclei by pancreatic DNase I and micrococcal nuclease. Pancreatic DNase I: hepatic nuclei labeled in vivo with $[^{125}I]T_3$ were prepared as described in Fig. 1 and digested with pancreatic DNase I with modification to the procedure described by Weintraub and Groudine (*7*). Nuclei were resuspended to 35 A_{260} per milliliter in reticulocyte stabilization buffer (RSB) + DTT (10 m*M* tris, *p*H 8, 10 m*M* NaCl, 3 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 1 m*M* DTT), and digested with varying concentrations of pancreatic DNase I (Worthington) (0 to 3.0 units per A_{260}) according to the digestion schedule in Fig. 1. Nuclei were sedimented at 4000*g* for 10 minutes and the supernatant retained. The nuclear pellet was resuspended and lysed in 0.2 m*M* EDTA and sedimented as before. Supernatants were pooled, applied to 5 to 20 percent sucrose gradients in buffer C and fractionated (SW-41 rotor at 35,000 rev/min, 15 hours, 2°C). Of the radioactivity applied to these gradients 80 to 90 percent migrated as a single peak less than 11*S*. Fractions containing the 5.8*S* $[^{125}I]T_3$ receptor fragment were pooled and the ^{125}I radioactivity was quantitated. (○) The percentage of total nuclear $[^{125}I]T_3$ release from liver nuclei by DNase I. Micrococcal nuclease: hepatic nuclei isolated from animals injected with $[^{125}I]T_3$ were digested with micrococcal nuclease and the soluble chromatin was obtained as described in Fig. 1 and was fractionated in 5 to 20 percent sucrose gradients as described above. Regions of the gradient corresponding to the 5.8*S* $[^{125}I]T_3$ fragment were pooled and quantitated as described above for the DNase I study. Two separate experiments (● and ■) are reported for the fraction of $[^{125}I]T_3$ receptor excised from liver nuclei as a 5.8*S* fragment by micrococcal nuclease. Fig. 3 (right). Velocity sedimentation of $[^{125}I]T_3$ nuclear receptor complex liberated from chromatin by nuclease. Hepatic nuclei obtained from euthyroid rats injected with $[^{125}I]T_3$ were digested with micrococcal nuclease (●) or pancreatic DNase I (◆) to 15 and 17 percent perchloric acid solubility, respectively, as described in Figs. 1 and 2. Nuclear receptor (labeled with $[^{125}I]T_3$ in vivo) was extracted from chromatin with 0.5*M* KCl (*18*). The nuclease-generated receptor fragment and salt-extracted receptor (○) were adjusted to either 0.12*M* KCl (A) or 0.5*M* KCl (B) and sedimented in 5 to 10 percent sucrose gradients [10 m*M* tris, *p*H 8, 2 m*M* EDTA, 0.2 m*M* PMSF (phenylmethylsulfonylfluoride), 1 m*M* DTT] containing the corresponding KCl concentration (SW-41 rotor, 35,000 rev/min for 24 hours, 2°C). Protein markers are indicated in the figure and were sedimented in parallel gradients containing 0.1*M* KCl. Gradients were fractionated (0.4 ml) and radioactivity determined as described.

stricted set of genes than do the HMG proteins.

Previous efforts to correlate the subnuclear localization of the T_3 receptor with nuclear functions have yielded conflicting results. Charles *et al.* (*13*) first demonstrated an enrichment of the receptor in slowly sedimenting sheared rat liver and HeLa cell chromatin containing high RNA polymerase II activity, and suggested on the basis of this observation that the T_3 nuclear receptor was associated with transcriptionally active chromatin. Subsequent reports from this laboratory (*14*), however, have indicated a failure to obtain an enrichment of the T_3 receptor in transcriptionally active chromatin in pituitary tumor cells of the GH line by means of DNase II. In contrast, Samuels *et al.* (*15*), using DNase II effected a threefold enrichment of the receptor in transcriptionally active chromatin from GH_1 pituitary cell nuclei. The basis for these discrepancies is not apparent to us.

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19. After submission of this manuscript, Samuels *et al.* [H. H. Samuels, F. Stanley, W. Casanova, T. C. Shau, *J. Biol. Chem.* **255**, 2499 (1980)] demonstrated that micrococcal nuclease digestion of rat pituitary GH_1 cell nuclei excises the T_3 receptor as a predominant 6.5*S* form. These authors also reported that a less abundant 12.5*S* form of receptor is released from chromatin by nuclease. We have observed that, under velocity sedimentation conditions different from those described herein, micrococcal nuclease liberates the T_3 receptor as a predominant 5.8*S* form and minor 12*S* to 12.5*S* form from euthyroid rat liver nuclei.
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Evidence for L-Glutamate as the Neurotransmitter of Baroreceptor Afferent Nerve Fibers

Abstract. *Microinjection of L-glutamate into the intermediate nucleus tractus solitarii in anesthetized rats elicits hypotension, bradycardia, and apnea, simulating baroreceptor reflexes. Ablation of the nodose ganglion results in selective reduction of high-affinity uptake of L-glutanate in the nucleus tractus solitarii. L-Glutamate may be the neurotransmitter of afferent nerve fibers from arterial baroreceptors.*

Primary afferent fibers of arterial baroreceptors projecting through the ninth and tenth cranial nerves terminate in the medulla oblongata on intrinsic neurons within the middle third of the nucleus tractus solitarii (NTS) (*1*). These neurons relay signals from baroreceptors to other areas of brainstem and spinal cord, thereby eliciting hypotension, bradycardia, and apnea, the cardinal responses to baroreceptor stimulation (*2*).

The baroreflex may be modulated centrally by neurotransmitters of several classes, including the catecholamines norepinephrine and epinephrine (*3*), γ-aminobutyric acid (GABA) (*4*), and several neuropeptides (*5*). These transmitters, however, seem to be contained in processes of neurons whose cell bodies reside within the central nervous system (CNS). The identity of the neurotransmitter synthesized, stored, and then released into NTS by the primary baroreceptor afferents is unknown.

We have examined the possibility that the amino acid L-glutamate, a putative transmitter in several intrinsic pathways of the CNS (*6*) and of dorsal root afferent fibers (*7*), may be the neurotransmitter released from baroreceptor afferents within the NTS.

We first examined the physiological effects of microinjecting L-glutamate into the NTS. Rats were anesthetized with halothane, and a cannula was inserted into the aorta through the ventral tail artery for recording arterial pressure and heart rate. The animals were placed in a stereotaxic frame, and the dorsum of the medulla was exposed. Drugs were dissolved in 0.9 percent saline and injected directly into the NTS through a glass micropipette with a tip 50 to 100 μm in outside diameter. Exactly 0.1 μl of each substance was injected in 1 second by a microinfusion pump. In some rats, the location of the cannula was verified by injecting drugs mixed with methylene blue. The dye did not alter the response to the injected agent. After the experiment, the animals were killed, the brains sectioned, and the injection sites identified.

L-Glutamate injected unilaterally resulted, after a latency of 1 to 5 seconds, in the development of hypotension, bradycardia, and apnea (Fig. 1A). The cardiovascular responses depended on dosage (Fig. 1B), appearing at a dose of 5 ng (30 pmole) and reaching a maximum at approximately 1000 ng. The average response in eight animals after a unilateral injection of 1000 ng was to lower mean arterial pressure by 37 ± 3.5 mm-Hg from a baseline level of 84 ± 2.0 mm-Hg and to slow the heart rate by 53 ± 10.9 beats per minute from a baseline level of 344 ± 21.4 beats per minute. Saline (0.9 percent) injected into the NTS usually had no cardiovascular effect, although the average effect in 32 rats was a fall of arterial pressure by 5 ± 1.2 mm-Hg and of heart rate by 6 ± 1.9 beats per minute. Within a dosage range of 5 to 100 ng, the responses to L-glutamate could be elicited with each subsequent injection. At higher doses (> 500 ng), they could not be repeated for at least 30 minutes after injection.

The hypotensive effects of L-glutamate were anatomically specific and restricted to the intermediate third of the NTS, as verified histologically. Injections of L-glutamate as high as 100 ng into sites adjacent to the NTS (including the area postrema, median raphe nuclei, and external cuneate nucleus) and into the dorsal medullary tegmentum failed to elicit any cardiovascular responses. Injection of the L-glutamate analog kainic acid

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