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AUTORADIOGRAPHIC DETECTION OF TRIIODOTHYRONINE IN NUCLEI FROM NORMAL AND HYPOTHYROID RAT LIVER

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SUMMARY. Nuclear triiodothyronine was visualized by light and electron microscope autoradiography of liver nuclei isolated after intraperitoneal injection of [^{125}I] triiodothyronine in rats. The nuclear hormone, essentially bound to the putative nuclear triiodothyronine receptor, was found mostly associated with the border of condensed chromatin.

After in vivo injection, thyroid hormones rapidly penetrate the cellular compartment where several binding components have been described. Particularly one set of specific high affinity binding sites for 3,5,3'-triiodo-L-thyronine (T_3) was found in nuclei of several responsive tissues (1-3) and was shown to belong to the class of acidic chromatin proteins (4-6). Nuclear T_3-binding protein(s) (NTBP) has been partly characterized after in vivo or in vitro binding of [^{125}I] T_3 (T_3^*) (7-8) and partially purified preparations have been obtained (9). Several properties of NTBP (collected in ref. 10) suggest that it could act as a receptor mediating the effects that thyroid hormones probably exert in regulating genomic transcription (11-15). NTBP is a DNA-binding protein (8, 16), but little is known as to its location in chromatin. Data from nuclease digestion studies suggested that the T_3 receptor could be associated with nucleolar chromatin (17) or with both transcriptionally active and inactive fractions of the chromatin, more than 80 % being confined to the latter (18). Since many chromatin proteins are capable of translocation during sub-nuclear fractionation, direct visualization of T_3 binding sites should be of importance in order to avoid possible erroneous interpretations. We attempted to fix the hormonal amino acid T_3 and its receptor in situ using conventional electron microscopic methods following several controls in the light microscope. Results obtained with rat liver nuclei isolated after T_3^* injection are reported here. To our knowledge, this is the first autoradiographic indication that the T_3-NTBP complexes are probably mostly associated with the loosened chromatin in the perichromatin region

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MATERIALS AND METHODS

Male Sprague-Dawley rats, normal (100-200 g) or severely hypothyroid (about 80 g) (19), were injected intraperitoneally (i.p.) with 10-100 ng/100 g body weight of [^{125}I] T_3 (about 500 mCi/mg, from Abbott Radiopharmaceuticals). Liver nuclei were prepared 2 hr later as previously described (6), washed once with 0.25 % Triton X-100, suspended in 0.32 M sucrose - 1 mM $MgCl_2$ and immediately processed for : (a) solubilization of NTBP with 0.4 M KCl in TEM (20 mM Tris-Cl, 2 mM Na_2EDTA, 1 mM $MgCl_2$ pH 7.95) in order to check protein-bound T_3^* and in vitro binding properties for T_3 (apparent affinity constant, Ka and maximum binding capacity, MBC, in saturation analyses using either T_3 or T_3^*) as already described (6) and (b) histological autoradiographic preparation.

Light microscope analysis : nuclei were either spread and immediately frozen (- 70°C) on emulsion precoated slides or fixed with glutaraldehyde (3 % in 0.1 M Na phosphate pH 7.2 - 0.25 M sucrose - 1 mM $MgCl_2$, for 15 min at 0°C with resuspension) or formaldehyde (0.75 % in 0.1 M triethanolamine pH 7.8 - 0.25 M sucrose - 1 mM $MgCl_2$, for 1 hr at 0°C). Fixed nuclei were washed 4-times in buffered sucrose, spread on slides, air-dried and coated with emulsion. Slides were coated by dipping into Ilford L_4 emulsion (dilution 1 : 4) and left for 3-20 days at - 20°C (frozen nuclei) or 2°C (fixed nuclei) with a dessiccant. Frozen nuclei were fixed in methanol just before development. After development (Kodak D 19, 5 min, 18°C), fixation (Amfix rapid fixer 1 : 3, 30 sec) and 3 washings in distilled water, nuclei were stained with Giemsa solution. Silver grain counting was carried out on a minimum of 400 nuclei distributed on several parts of each slide. Background counts (< 10 % of nuclear grain counts) were measured in several areas of equal surface surrounding the counted nuclei and subtracted.

Electron microscope analysis : the nuclear pellet (from 0.5 g liver) was fixed in 3 % glutaraldehyde as above, post-fixed in 2 % osmium tetroxide (15 min, 0°C), dehydrated in a graded series of acetone and embedded in Epon 812. Ultrathin sections from several parts of each block were stained with uranyl acetate and lead citrate, coated with a thin layer of carbon and dipped into Ilford L_4 emulsion (dil. 1 : 4) as previously described (20). After 6-12 weeks exposure, the preparations were developed in Microdol-X, fixed, washed and examined in a Siemens Elmiskop 101 at 80 KV (20). Silver grains were counted on a least 300 nuclear sections in each preparation. Background counts estimated on areas of Epon devoid of nuclei amounted to less than 2 % of count values from equivalent nuclear areas.

Radioactivity, protein and DNA estimations were performed as previously described (6, 8). The authenticity of T_3 was checked in ethanol extracts of nuclei by thin-layer chromatography on cellulose plates in the tert. pentanol-dioxane- 1 M NH_4OH (2 : 2 : 1) system (21). Values are expressed as mean ± S.E.M.

RESULTS

General features of nuclear T_3 binding

Two hours after T_3^* i.p. injection, a time chosen as being close to that of maximum hormonal uptake in rat liver nuclei (3), liver contained 0.88 ± 0.1 % (n = 9) and 2.1 ± 0.4 % (n = 6) of the injected dose per gram tissue in normal (N) and hypothyroid (H) rats, respectively. Nuclear radioactivity amounted to 7.7 ± 0.6 % and 16.8 ± 1.3 % of liver radioactivity in the same N and H series, taking into account the DNA recovery during nuclear preparation (50 to 70 %). Triton X-100 washing solubilized 6.4 ± 0.5 % (9 N rats) and 5.2 ± 0.6 % (6 H rats) of nuclear radioactivity and more than 60 % in N rats receiving in addition to T_3^* a load of 10 µg T_3, in accordance with Surks et al (4) who suggested a retention of excess T_3 on the outer nuclear membrane. Nuclear radioactivity (more than 98 % as authentic T_3) could be solubilized by 0.4 M KCl in TEM (81.4 ± 1.6 %

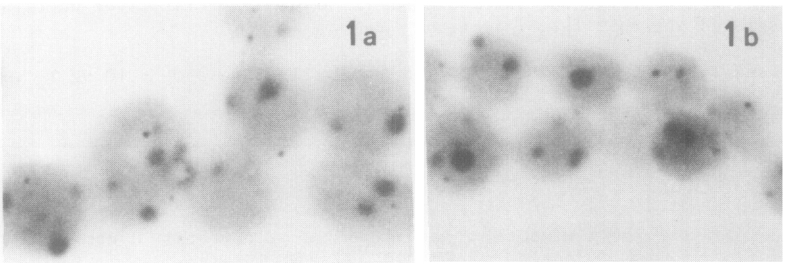

Figure 1. Light microscope autoradiograph of rat liver nuclei isolated after [^{125}I] T_3 injection. a : frozen nuclei; b : glutaraldehyde fixed nuclei. x 2200.

in 8 N rats and 79.1 ± 2.5 % in 5 H rats). No significant difference was detected between N and H rats when solubilization was performed at various KCl concentrations and at pH 7.95 and 7.4. More than 80 % of the extracted radioactivity was protein-bound and could be exchanged during a subsequent incubation of the nuclear extract with a 200-fold excess of unlabeled T_3, in agreement with previous results (6, 8). Ka and MBC values were 1.6 ± 0.3 x 10^{10} M^{-1} and 358 ± 26 pg T_3/mg DNA in 7 N rats, as compared to 1.8 ± 0.3 x 10^{10} M^{-1} and 324 ± 43 pg T_3/mg DNA in 4 H rats. From MBC values of about 1 pmole T_3/g liver equivalent nuclei, not significantly different between N and H rats as already reported (19), we can assume the presence of about 2000 T_3 binding sites per nucleus on the basis of 3 x 10^8 nuclei per g liver (22).

Light microscope analysis

Since T_3 dissociation from NTBP is slow (T 1/2 about 100 hr at 0°C (8)) and T_3-NTBP complexes are tightly bound to chromatin (4), T_3^*-NTBP complexes are likely to be maintained *in situ* during freezing of freshly prepared nuclei. Glutaraldehyde fixed 95.9 ± 0.2 % (n = 13) of nuclear T_3^* while subsequent washings never extracted more than 1 %. The same nuclear retention of T_3^* was observed : (1) when fixed nuclei were maintained in suspension for 1 week at 0°C; (2) when fixed nuclei were heated at 60°C for 5 min, conditions leading to NTBP denaturation and solubilization of free T_3; (3) when fixed nuclei were treated with 0.4 M KCl, suggesting a covalent binding of T_3-NTBP complexes within the nucleus. Formaldehyde fixation was less complete (79.9 ± 1.2 % T_3^* retained in 7 nuclear preparations) although stable during washings.

As shown in Fig. 1, nuclei were essentially pure, both well preserved and individualized, with a slightly reduced size after fixation. Table I shows grain count values obtained in a typical experiment out of 6 series of assays. Up to 50 % of nuclei were labeled with 1-2 grains. Both the proportion of labeled nuclei and the mean grain number per labeled nucleus increased with increasing

Table I. Light microscope autoradiographic analysis of rat liver nuclei isolated after [^{125}I] T_3 injection.

Injected T_3^* : 25 µCi/100 g B.W. in N and H rats, the same + 10 µg T_3 in N + T_3 rat.
Exposure time : 6 days.

Rats	Nuclei	Labeled nuclei % of total nuclei	Mean grain no per labeled nucleus	Peripheral grains % of total grains	Nuclear T_3^* dpm/mg DNA
N	Frozen	22.0	1.1	66.0	37 200
	Glutaraldehyde	28.0	1.2	78.1	
H	Frozen	35.0	1.2	66.6	95 400
	Glutaraldehyde	45.3	1.3	75.8	
N + T_3	Frozen	6.0	1.0	80.0	2 130
	Glutaraldehyde	13.0	1.0	83.4	

nuclear radioactivity. Maximum values obtained with highly labeled nuclear preparations (see exp. 2 and 3 from table III) were 60 % of nuclei labeled with 1.8 grains, some having up to 5 grains. Furthermore, whatever the method used, grains appear not to be concentrated on a particular structure but about 75 % were located in the peripheral part of the nucleus (nuclear border and the outer third of the apparent nuclear surface). No difference between N and H rats was observed. Assuming 3 x 10^8 nuclei/g liver, nuclear radioactivity in the experiment shown in table I leads to calculating about 5 decays per nucleus in H rat, 2 decays in N rat and 0.1 decay in control N rat over the 6 day period of exposure. As sensitivity for ^{125}I detection decreases with increasing dose and sample thickness (23), our results obtained with whole nuclei spread over or under the emulsion layer fit rather satisfactorily with the known ^{125}I content of nuclei.

Electron microscope analysis

About 80 % of T_3^* was retained within nuclei after double fixation, acetone dehydration and embedding (Table II). The labeled material released in acetone was detected in several peaks as shown by TLC, the largest being iodide and a front peak. Less than 20 % migrated as T_3.

Most nuclear sections showed the typical condensed chromatin figures essentially along the nuclear periphery and an almost complete absence of outer nuclear membrane. In 3 experiments (Table III), 10 to 25 % of nuclear sections were generally labeled with 1 grain, rarely 2 or 3. In both N and H rats, more

Table II. Retention of T_3 radioactivity during preparation for electron microscope autoradiography.

	Normal	Hypothyroid
	% of nuclear ^{125}I T_3	
Glutaraldehyde	4.1 ± 0.4 (8)[$]	4.7 ± 0.6 (4)
Sucrose-Na phosphate	0.9 ± 0.1 (8)	1.4 ± 0.2 (4)
Osmium tetroxide	1.2 ± 0.2 (8)	1.5 ± 0.3 (4)
Acetone 70-90 % (combined)	11.8 ± 1.5 (5)	13.1 ± 2.7 (3)
Acetone 100 % (3 combined)	0.8 ± 0.3 (5)	0.9 ± 0.3 (3)
Epon-acetone	1.2 ± 0.5 (3)	1.1 ± 0.4 (3)
Residual nuclei	80.0	77.3

[$] number of determinations in parentheses.

Table III. Electron microscope autoradiographic analysis of rat liver nuclei isolated after [^{125}I] T_3 injection.

Injected T_3* : 15 µCi/100 g B.W. in exp. 1; 60 µCi/100 g B.W. in exp. 2 and 3.
Exposure time : 12 weeks in 1; 8 weeks in 2 and 3.

Exp.	Rat	Nuclear T_3* dpm/mg DNA	Grain no per 100 nuclear sections	Labeled nuclear sections per 100 sct.	Grains straddling dense chromatin border per 100 grains
1	N	19 900	12.8	10.1	67.9
	H	35 400	20.2	19.7	68.1
2	N	64 020	11.1	10.5	74.4
	H	204 060	27.6	24.5	67.1
3	N	68 780	12.0	11.3	72.9
	H	203 650	23.5	19.9	66.3

than two thirds of total grains were found straddling the border of dense chromatin where the latter is in continuity with euchromatin. Most of these grains displayed a peripheral location consistantly distinct from the inner membrane of the nuclear envelope (Fig. 2). Grains were rarely found over the nuclear membrane and the nucleolus (< 5 %); remaining grains were dispersed. Grains found on partly decondensed nuclear sections were counted as dispersed. With a mean diameter of 8 µm, each nucleus could have yielded about 50 sections of equal area.

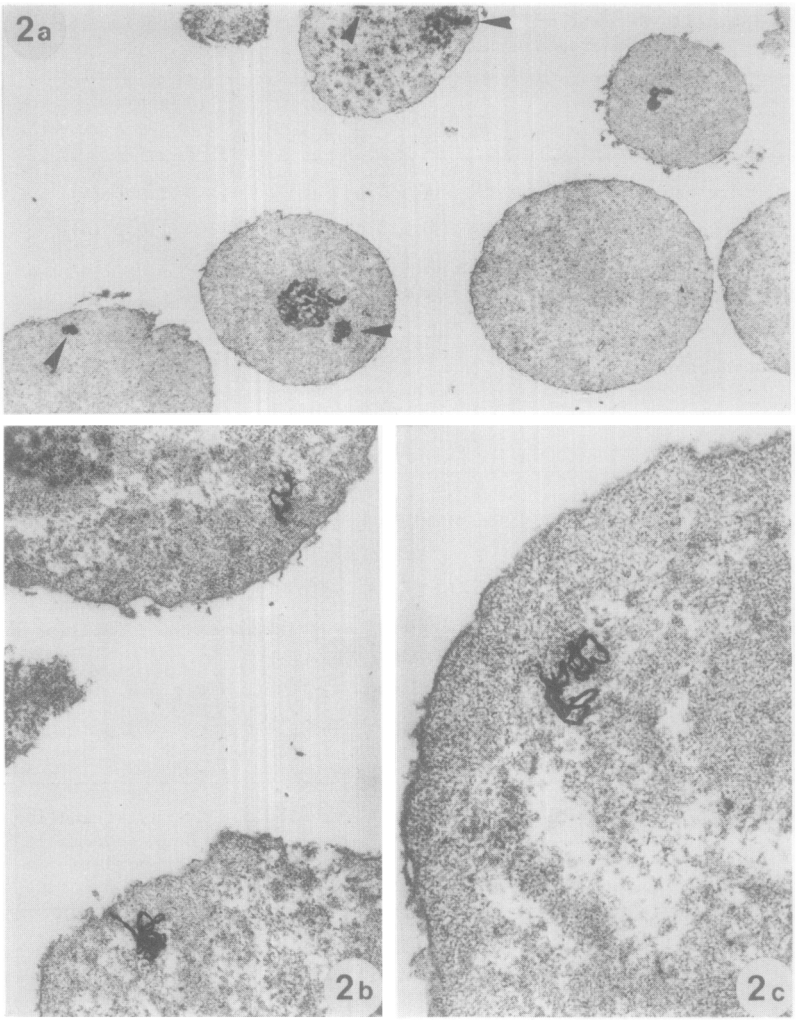

Figure 2. Electron microscope autoradiograph of rat liver nuclei isolated after [^{125}I] T_3 injection. a : x 4500 ; arrows indicate silver grains ; b : x 16 500 ; c : x 18 500. Higher magnification in b and c shows grains at the border of condensed chromatin.

In exp. 2 H and 3 H (table III), this leads to an approximate estimation of 13 grains per nucleus while after 2 months exposure about 75 decays per nucleus could be postulated from the nuclear radioactivity.

DISCUSSION

Under conditions which, on the basis of T_3^* retention and light microscope controls, are likely to have preserved T_3-NTBP complexes on their chromatin site an autoradiographic detection could be obtained in the electron microscope in

liver nuclei isolated after T_3^* injection in rat. Most nuclei had the classical aspect of hepatocyte nuclei with the largest part of dense chromatin in close contact with the inner membrane of the nuclear envelope. We reproducibly found higher than 2/3 of silver grains at the junction of dense chromatin and euchromatin. Although our data do not at present constitute strictly quantitative estimations, they are in good agreement with values extrapolated from nuclear radioactivity. Such results are the first EM autoradiographic attempt at nuclear localization of T_3 since the report of Bar-Sella et al. (24). These authors studied T_3^* localization in posterior pituitary tissue, after a higher T_3 dose injection, and preferentially found it on every cellular membrane including nuclear ones.

Our findings are of particular interest when compared to the results of Fakan et al. (25) who visualized the perichromatin fibrils in close proximity to condensed regions as the most active sites for extranucleolar transcription in rat liver. Since nuclear-bound T_3^* represents essentially T_3 bound to NTBP, a preferential location of the T_3 - "receptor" at the border of dense chromatin near active transcription sites suggests its participation in regulating genomic transcription.

No difference was detected between normal and hypothyroid rats except for a higher level of labeling in H rats due to a lesser dilution of T_3^* with endogenous unlabeled T_3 and a lesser degree of T_3-binding sites saturation. NTBP binds T_3 with the same Ka and MBC and is apparently similarly bound to chromatin whatever the thyroid status (26, these results). The only observable differences at present are : (1) the level of NTBP saturation with T_3; (2) small physicochemical changes of the NTBP molecule after T_3 binding (9, 27); (3) a slight decrease of Ka for T_3 when NTBP is chromatin-bound (26). In this study only T_3-NTBP complexes were visualized and these were seen in the same position in N and H rat nuclei. However, it is possible that T_3 binding to NTBP could displace the receptor molecule on another chromatin site. A different location of unoccupied NTBP might be visualized when specific anti-NTBP antibodies will become available.

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