Characterization of a thyroid-hormone-binding site on nuclear envelopes and nuclear matrices of the male-rat liver

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Nuclear envelopes and nuclear matrices were isolated from the male-rat liver. Incubation of ^{125}I -labelled 3,3',5-tri-iodothyronine (T_3) with the nuclear-envelope fraction resulted in specific binding of T_3 to the membranes. Maximum specific binding occurred at 30°C after 2 h incubation. Storage for 1 week at -80°C resulted in no loss of binding. Scatchard analysis revealed a class of binding sites with K_D 86 nM. 3,3',5'-Tri-iodothyronine was as effective a competitor of $^{125}\text{I}]\text{T}_3$ binding to nuclear envelopes as was L- T_3 itself, and tri-iodothyroacetic acid was 70% as potent as T_3 . L- and D-thyronine did not compete for $^{125}\text{I}]\text{T}_3$ binding. Incubation of nuclear envelopes with 0.6 M-NaCl before addition of T_3 resulted in the complete loss of specific binding sites, whereas exposure of the membranes to 2.0 M-NaCl after incubation with T_3 did not extract binding sites. Nuclear matrices, after incubation with $^{125}\text{I}]\text{T}_3$ under the same conditions, were shown to possess a class of binding sites with a similar K_D but with approx. 30% of the maximum binding capacity. Nuclear envelopes from hypothyroid animals may possess slightly lower numbers of binding sites compared with nuclear envelopes from the intact animal, whereas nuclear matrices from hypothyroid animals have the same number of binding sites as do nuclear envelopes from the intact animal. In conclusion, nuclear envelopes and nuclear matrices have a class of binding sites with relatively high affinity for T_3 . It is distinct from nuclear and cytosolic binding sites.

Much evidence suggests that thyroid hormones (Oppenheimer, 1979) must enter the nucleus before exerting their action. Clearly the hormones must traverse the nuclear-envelope barrier during translocation from the cytoplasm into the nucleus. It is possible that modulation of thyroid-hormone action occurs via interaction of the hormones with nuclear-envelope components during their passage across the nuclear envelope. To investigate this, we have characterized an L- T_3 binding site on a male-rat liver nuclear-envelope preparation. In addition, as some components of the nuclear envelope are also present in nuclear matrices, we have investigated whether the T_3 -binding site is also present on nuclear matrices. We report here that a T_3 -binding site having similar affinity is present on both nuclear envelopes and nuclear matrices.

Materials and methods

Materials

L- $^{125}\text{I}]\text{T}_3$ (3800 $\mu\text{Ci}/\mu\text{g}$) was purchased from New England Nuclear (Montreal, Canada). T_3 , 3,3',5-tri-iodothyroacetic acid, deoxyribonuclease I, Triton X-100 and protease Type VI were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). r T_3 was purchased from Henning-Berlin (Berlin, Germany). L- T_4 and D- T_4 were generously given by Dr. N. Henderson (Department of Biology, University of Calgary).

Animals

Male Sprague-Dawley rats, weighing 200–300 g (average wt. 340 g), were obtained from Charles River Canada Inc. (Montreal, Canada) and maintained on a diet of Wayne Lab Blox (Allied Mills, Chicago, IL, U.S.A.) and tap water *ad libitum*. The rats were killed by decapitation. The livers were

Abbreviations used: T_3 , 3,3',5-tri-iodothyronine; r T_3 , 3,3',5'-tri-iodothyronine; T_4 , thyroxine.

quickly removed, rinsed in 0.32M-sucrose containing 3mM-MgCl₂, stripped of connective tissue, weighed, and placed on ice.

In two experiments, athyreotic animals (male Sprague-Dawley rats, average wt. 310g, 16–18 weeks old) were used. Surgical thyroidectomy was performed by the supplier. On receipt in the laboratory, thyroidectomized animals were fed on a diet supplemented with calcium lactate (2%, w/v) for 2 weeks and then injected intraperitoneally with 100 μ Ci of Na¹³¹I. The animals were killed 6 weeks after injection. The serum T₃ concentration of these rats was undetectable (less than 2 ng/100 ml) as measured by radioimmunoassay.

Preparation of nuclear envelopes

All procedures were carried out at 4°C. Nuclei were isolated by modifications of the procedure of Widnell & Tata (1964). Tissue (50–60g) in 250 ml of 0.32M-sucrose containing 3mM-MgCl₂ was homogenized with a Polytron (Brinkman Instruments, Rexdale, Ont., Canada) at setting 4 for 1 min. The homogenate was passed through four layers of cheesecloth. The filtrate was then diluted to 1 litre and centrifuged at 4000g for 20 min. The pellet was resuspended in 2.4M-sucrose containing 1mM-MgCl₂ (adjusted to pH 7.4 with NaHCO₃), and this suspension was centrifuged at 50000g for 60 min. The resulting pellet of purified nuclei was then used to prepare nuclear envelopes by modifications of the procedure of Kay *et al.* (1972). The nuclei were resuspended in 0.25M-sucrose containing 1mM-MgCl₂ (adjusted to pH 7.4 with NaHCO₃) and centrifuged at 750g for 5 min. The pellet was resuspended in digestion buffer (10mM-Tris/HCl, 0.30M-sucrose, 0.1mM-MgCl₂, 5mM-2-mercaptoethanol, pH 8.5) to which freshly dissolved deoxyribonuclease I was added to a concentration of 10 μ g/ml. The suspension was incubated at room temperature for 20 min. After centrifugation at 12000g for 5 min, the crude nuclear envelopes were resuspended in 10mM-Tris/HCl, pH 7.4, and 2 ml was layered on a discontinuous sucrose gradient of 5 ml of 0.25M-sucrose and 10 ml each of 1.5M-, 1.8M- and 2.0M-sucrose (all sucrose solutions made up in 10mM-Tris/HCl, pH 7.4). The gradients were centrifuged at 100000g for 90 min. The major band of purified nuclear envelope was removed from the gradients with a Pasteur pipette and washed twice in 10mM-Tris/HCl, pH 7.4, by centrifugation at 33000g for 5 min.

Preparation of nuclear matrices

Nuclear matrices were prepared by the method of Barrack & Coffey (1980). Nuclei were sequentially extracted with Triton X-100 (1%, w/v), deoxyribonuclease (200 μ g/2mg of protein), low-Mg²⁺ buffer (0.25mM-MgCl₂) and 2M-NaCl. The

insoluble residue remaining after these extractions was the nuclear matrix.

Protein determinations

Protein content of subcellular fractions was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Polyacrylamide-slab-gel electrophoresis

Nuclear-envelope and nuclear-matrix proteins were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in 12%-acrylamide gels (Laemmli, 1970).

Assay of T₃ binding to nuclear envelopes and nuclear matrices

Stock solutions of T₃ and analogues were made in 0.1M-NaOH. Working solutions were made by diluting 0.02 ml of the stock solution in 9.98 ml of 20mM-Tris/HCl, pH 7.4. NaOH was added to each assay tube to a concentration of 40 μ M. [¹²⁵I]T₃ (0.1 ml) was diluted to 10.0 ml in 0.1M-NaOH and then further diluted 3.33-fold in 20mM-Tris/HCl, pH 7.4.

Nuclear envelopes or nuclear matrices isolated from male-rat liver (5–20 μ g of protein/assay tube) were incubated in a final volume of 0.25 ml of 20mM-Tris/HCl, pH 7.4, containing [¹²⁵I]T₃ (46500 d.p.m./assay tube) for the times and at the temperatures specified. Each sample was assayed in triplicate. To identical tubes 10 μ M-T₃ was also added to determine non-specific binding. The reaction was terminated by centrifugation in a Beckman Microfuge B and removal of the supernatant with a Pasteur pipette. The pellet was washed twice by addition of 0.25 ml of 20mM-Tris/HCl, pH 7.4, centrifuged again and the supernatant was removed. Radioactivity was assayed in a Tracor Analytic model 1185 Gamma System (counting efficiency 86%). Specific binding was defined as that binding obtained by subtracting the radioactivity in the tube containing excess unlabelled T₃ (non-specific binding) from that tube containing only labelled T₃ (total binding).

Studies of the effects of NaCl on T₃ binding were done in two ways. In one series of experiments, nuclear envelopes (20 μ g of protein/assay tube) were preincubated with 0.3 ml of 0.6M-NaCl in 20mM-Tris/HCl, pH 7.4, for 30 min at 20°C. The tubes were then centrifuged and the resulting pellet was washed once in 20mM-Tris/HCl, pH 7.4. As described above, the pellet was then incubated in the assay mixture to determine T₃ binding to the extracted pellet. Control nuclear envelopes were preincubated with 20mM-Tris/HCl, pH 7.4, instead of NaCl. In another series of experiments, nuclear envelopes were incubated with T₃ and then the nuclear envelopes obtained by centrifugation

were washed. Then the pellet was incubated with 0.3 ml of NaCl at concentrations of 0.6M, 1.0M and 2.0M in 20mM-Tris/HCl, pH 7.4, for 30 min at 20°C. The nuclear envelopes were then pelleted and washed once again before being assayed for radioactivity. Control nuclear envelopes were incubated with 20mM-Tris/HCl, pH 7.4, instead of NaCl.

Results

Characterization of T_3 binding to male-rat liver nuclear envelope

The yield of nuclear-envelope material was 1.90mg of protein/rat or 1.16mg of protein/g of starting tissue. The major band of purified nuclear envelopes usually formed at the 1.5M/1.8M-sucrose interface, but occasionally was positioned at the 1.8M/2.0M-sucrose interface. Agutter & Gleed (1980) have reported that the density of sheep liver nuclear-envelope preparations varies according to the concentrations of nuclei during the lysis stage. This preparation of rat liver nuclear envelopes consists of relatively intact nuclear envelopes, which retain the inner and outer membranes with morphologically well-preserved nuclear-pore complexes (Kay *et al.*, 1972). We obtained intact ghosts and some torn membrane sheets, as monitored with phase-contrast microscopy.

We studied conditions for optimal binding of T_3 to nuclear envelopes. Fig. 1 shows that this binding was temperature-dependent. Little or no specific binding occurred at 4°C. At 20°C and 30°C very

Fig. 1. Effect of time and temperature on the binding of T_3 to male-rat liver nuclear envelopes

Nuclear envelopes (20 μ g of protein/assay tube) were incubated in triplicate with [125 I] T_3 (0.07 nM) and 0.5 nM- T_3 in the presence and absence of 10 μ M- T_3 at 4°C (●), 20°C (Δ), 30°C (■) and 37°C (○) for 0–2 h. Specific binding was calculated from the difference in binding in the presence and absence of 10 μ M- T_3 .

little specific binding occurred for the first 15 min of incubation. With increasing lengths of incubation, increasing binding occurred until 1 h, at which a plateau was reached. At 30°C there was a slight decrease in binding thereafter to 2 h, whereas binding remained constant at 20°C. Longer incubations, for 4 h, 8 h and 24 h, at 30°C resulted in decreasing binding of 20%, 35% and 43% respectively (results not shown). T_3 binding at 37°C reached a maximum at 15 min and remained at that value to 30 min. Thereafter a rapid decrease in binding occurred, so that at 60 min no specific binding was detectable. As the highest extents of T_3 binding to nuclear envelopes occurred at 30°C after 2 h incubation, these conditions were chosen for further experiments. At each temperature, specific binding represented approx. 25% of the total binding.

Fig. 2 demonstrates that, after 2 h incubation at 30°C, a linear relationship exists between T_3 bind-

Fig. 2. Effect of increasing concentrations of nuclear-envelope protein on the binding of T_3 to male-rat liver nuclear envelopes

Nuclear envelopes (5–40 μ g of protein/assay tube) were incubated in triplicate with [125 I] T_3 (0.07 nM) and 0.5 nM- T_3 in the presence (■) and absence (●) of 10 μ M- T_3 for 2 h at 30°C. Specific binding (▲) was calculated from the difference in binding of [125 I] T_3 in the presence and absence of unlabelled T_3 .

ing and membrane protein concentrations to $40\mu\text{g}/\text{assay tube}$. Fig. 2 also shows that there is considerable non-specific binding of T_3 to the membrane preparation.

The effect of storage of nuclear envelopes at -80°C on the binding of T_3 was investigated. Storage at -80°C for 7 days did not result in decreased T_3 binding. After 2 weeks storage, however, T_3 binding to two nuclear-envelope preparations decreased to 39.8% and 55.0% of the binding observed with the fresh preparation. For routine work, nuclear-envelope preparations were stored in liquid N_2 and used within 1 week of preparation.

Fig. 3(a) shows the saturation plot of T_3 binding to nuclear envelopes from male-rat liver. Scatchard (1949) analysis of these data (Fig. 3b) showed that the K_D for specific binding could be calculated as $86 \times 10^{-9}\text{M}$, with an apparent number of binding sites of $18 \times 10^{-9}\text{M}$ or 223 pmol/mg of protein. We have not yet ruled out the existence of more than one class of binding sites, especially in view of the fact that there is considerable non-specific binding.

Specificity of T_3 binding to the male-rat liver nuclear-envelope preparation was examined by competition experiments in which analogues of T_3 were included in incubation mixtures. Fig. 4 shows that L- T_4 and D- T_4 are not effective in inhibiting binding of $[^{125}\text{I}]\text{T}_3$. The concentration of unlabelled L- T_3 required to inhibit the binding of $[^{125}\text{I}]\text{T}_3$ by 50% was 72.5% the concentration of unlabelled tri-iodothyroacetic acid required to inhibit the binding of $[^{125}\text{I}]\text{T}_3$ by 50%. Therefore unlabelled tri-iodothyroacetic acid was 72.5% as potent as L- T_3 . On the other hand, r T_3 was as effective in inhibiting the binding of $[^{125}\text{I}]\text{T}_3$ as unlabelled L- T_3 . $[^{125}\text{I}]\text{T}_3$ binding at 0% inhibition was 2200 fmol/mg of protein, and binding at 100% inhibition was 650 fmol/mg of protein.

Two kinds of experiments were performed to investigate whether salt extraction of nuclear envelopes caused any change in binding of T_3 to nuclear envelopes. When nuclear envelopes were preincubated with 0.6M-NaCl, 73% of the specific T_3 binding to nuclear envelopes preincubated in 20mM-Tris/HCl, pH 7.4, was lost. On the other hand, nuclear envelopes extracted with 0.6M-, 1.0M- and 2.0M-NaCl after incubation with T_3 retained 98.5%, 97.9% and 100% respectively of the T_3 -binding sites.

We have also characterized T_3 binding to nuclear matrices prepared from male-rat liver. Rat liver nuclear matrices were verified to be matrices by phase contrast microscopy. Fig. 5 shows peptide profiles of nuclear envelopes and nuclear matrices prepared from male-rat liver. Although the matrix and the nuclear envelope consist of many proteins, and many dissimilarities exist between the two

Fig. 3. Saturation analysis of T_3 binding to male-rat liver nuclear envelopes

Nuclear envelopes ($20\mu\text{g}$ of protein/assay tube) were incubated in triplicate for 2 h at 30°C with increasing amounts of T_3 (0.1–300.0 nM) and $[^{125}\text{I}]\text{T}_3$ (0.07 nM) with (■; to measure non-specific binding) and without (▲; to measure total binding) $10\mu\text{M}$ -unlabelled T_3 . Specific binding (●) was calculated from the difference in retained radioactivity in the absence and presence of unlabelled T_3 (a) and plotted according to Scatchard (b). The line of best fit was determined by linear-regression analyses, and the correlation coefficient was 0.86 ($P > 0.005$).

peptide profiles, both preparations are primarily composed of three major polypeptides, ranging in M_r from 60000 to 68000. Table 1 shows that Scatchard analysis of binding to nuclear matrices reveals a class of binding sites having a K_D of $118 \times 10^{-9} M$ and a capacity of $5 \times 10^{-9} M$ or 65 pmol/mg of protein. The affinity of T_3 for nuclear matrices is similar to the affinity of T_3 for nuclear envelopes. However, the number of binding sites is approx. 70% less.

We also investigated binding of T_3 to nuclear envelopes and nuclear matrices from thyroidectomized animals. Scatchard analysis (Table 1) revealed that the affinity of T_3 for these prepara-

tions was similar to affinities of fractions prepared from intact animals. The number of binding sites on nuclear envelopes, however, may be slightly decreased in the thyroidectomized animal compared with the intact animal.

Fig. 4. Effect of unlabelled L- T_3 , L- T_4 , D- T_4 , tri-iodothyroacetic acid and r T_3 on the specific binding of L-[^{125}I] T_3 . Nuclear envelopes (20 μg of protein/assay tube) were incubated for 2 h at 30°C with 0.5 nM- T_3 and [^{125}I] T_3 (0.07 nM) with unlabelled L- T_3 ($\square$), L- T_4 ($\blacksquare$), D- T_4 ($\bullet$), tri-iodothyroacetic acid ($\circ$) or r T_3 ($\blacktriangle$) at concentrations of 0.5, 5.0, 50.0, 500, 5000 and 10000 nM. Two triplicate experiments were performed. The values represent the means.

Fig. 5. Electrophoretic analysis of nuclear-envelope and nuclear-matrix peptides. A sample (200 μg of protein) of each preparation was applied to the polyacrylamide gel and, after separation of peptides, gels were stained with Coomassie Blue. Lanes: a, M_r markers; b, nuclear-envelope protein; c, nuclear-matrix protein.

Table 1. Scatchard analyses of T_3 binding to nuclear-envelope and nuclear-matrix preparations obtained from intact male rats and thyroidectomized rats

Scatchard analyses were performed as described in the legend to Fig. 3. Values are the means of triplicates from two preparations. The values in parentheses are the results obtained in each experiment.

Preparation	K_D (nM)	No. of binding sites (pmol/mg of protein)
Nuclear envelopes from intact male	86 (73, 99)	223 (225, 221)
thyroidectomized male	90 (105, 75)	133 (96, 170)
Nuclear matrices from intact male	118 (99, 137)	65 (85, 45)
thyroidectomized male	140 (100, 180)	72 (60, 82)

Discussion

Present evidence strongly suggests that thyroid hormone action is initiated by the binding of L-T₃ to non-histone chromatin receptors (Oppenheimer, 1979; Schwartz & Oppenheimer, 1978) within the nucleus. To date, no one has investigated the mechanism by which the hormones traverse the two membranes comprising the nuclear envelope on their way to the chromatin receptors. It is possible that T₃, a small and relatively lipophilic molecule, enters the nucleus via passive diffusion, perhaps via the nuclear-pore complexes. It is equally possible that T₃ entry into the nucleus is by a controlled passage across the nuclear envelope. Thyroid hormone action may be modulated by such a transport process. The first step toward identifying such a process is identification of a binding component for T₃ on nuclear envelopes from a T₃-target tissue. We have therefore isolated intact nuclear envelopes from male-rat liver and have characterized T₃ binding to the membranes.

The binding experiments involved incubation *in vitro* of the isolated nuclear-envelope fraction with labelled hormone and were not designed to distinguish between binding of the labelled T₃ to membrane sites previously occupied by endogenous hormone and binding to unoccupied sites. However, at 0°C we were unable to measure any specific binding, which, we conclude, shows either that the binding process requires energy or that dissociation of endogenous ligand is required. Silva *et al.* (1977) have shown that 40% of the measured binding sites in extracts from rat liver nuclei are occupied. Since the K_D of the T₃-binding site from the unthyroidectomized animals was not different, however, the binding process probably requires energy and is not a reflection of endogenously bound hormone.

Scatchard analysis of the binding of T₃ to nuclear envelopes from livers of intact rats revealed a class of binding sites having a K_D of 86×10^{-9} M. The dissociation constant of T₃ binding to isolated nuclei as well as to chromatin receptors is reported to be 0.1×10^{-10} M– 2×10^{-9} M (Schwartz & Oppenheimer, 1978; DeGroot & Torresani, 1975; Surks *et al.*, 1975). A cytoplasmic binding component has also been identified which in the rat liver has a K_D of approx. 10^{-7} M (Dillman *et al.*, 1974; Davis *et al.*, 1974; Visser *et al.*, 1976). We conclude therefore that the nuclear-envelope binding site characterized in the present studies differs in its affinity from both the nuclear and the cytosolic binding sites.

The maximum binding capacity of the nuclear envelope for T₃ is 223 pmol/mg of protein, or 24 ng of T₃ for nuclear envelopes from 1 g of liver. Reported values for the maximum binding capacity of nuclei are approx. 10-fold less (DeGroot &

Torresani, 1975). Although previous estimates of nuclear binding capacities have been obtained with nuclei which have been treated with non-ionic detergents, and such treatment removes the outer nuclear membrane (Aaronson & Blobel, 1975), perhaps a rich source of T₃-binding sites, the nuclear-envelope site is probably a higher-capacity lower-affinity site than are previously reported nuclear sites.

Specificity studies showed that the most effective competitors for binding of [¹²⁵I]T₃ to the nuclear envelopes were T₃ itself and rT₃. Tri-iodothyroacetic acid was a less effective competitor, and L-T₄ and D-T₄ did not compete at all. The order of potency of the T₃ analogues correlates with that demonstrated for the binding of T₃ to nuclei and chromatin receptors, except that rT₃ is a less effective competitor in nuclei and chromatin than is tri-iodothyroacetic acid (Schwartz & Oppenheimer, 1978; Smith *et al.*, 1980). Tri-iodothyroacetic acid is a more effective competitor of T₃ binding to nuclear envelopes than it is in the cytosol binding system (Smith *et al.*, 1980).

We have also identified a binding site with a K_D similar to that of nuclear envelopes on nuclear matrices, but with approx. 30% of the binding sites compared with the nuclear-envelope site. These matrices were prepared after high-salt extraction. Our studies of NaCl extraction of nuclear envelopes showed that NaCl extracted most of the binding sites, and perhaps this explains the decreased number of sites in the matrix. However, once T₃ has bound to the sites, they are protected from salt extraction and then even high concentrations of salt cannot remove the occupied site. Wilson *et al.* (1982) have described specific T₃-binding sites on rat liver nuclear matrices with a K_D of 1.3 nM. The T₃-binding site reported here is of lower affinity and appears to be present on both nuclear envelopes and nuclear matrices.

Oppenheimer *et al.* (1975) reported that the T₃-binding capacity of hepatic nuclei is the same in euthyroid and thyroidectomized animals. Our studies suggest that, although the affinity of the nuclear-envelope T₃-binding site in the thyroidectomized animal is similar, the number of binding sites is slightly decreased. On the other hand, nuclear matrices prepared from thyroidectomized animals did not display a lower binding capacity.

In summary, we have identified in the rat liver a nuclear-envelope binding site which differs from previously described nuclear and cytosolic binding sites in its affinity, binding capacity and specificity. The site is also present on nuclear matrices. The physiological role of this binding site remains to be elucidated.

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