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Regulation of activity of chromatin receptors for thyroid hormone: Possible involvement of histone-like proteins

(ligand-binding specificity)

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ABSTRACT **Thyroid hormone receptors lose their capability for high-affinity binding of the biologically active triiodothyronine after solubilization and separation from other chromatin proteins. The high-affinity triiodothyronine-binding capacity can be reconstituted by addition of a histone-containing extract of chromatin or purified core histones (H2A, H2B, H3, and H4); a number of other acidic or basic proteins tested were ineffective. The data support a model of the receptor in which a "core" receptor subunit that contains a thyroid hormone-binding site interacts with a regulatory subunit, which is possibly a histone or histone-like species. This interaction with the "core" subunit enables the resulting "holo" receptor to bind biologically active hormones. These data also suggest that histones or related proteins can modulate the activity of nonhistone chromosomal proteins that are involved in regulating the expression of specific genes.**

Numerous studies have focused upon chromosomal proteins and their functions in the eukaryotic genome (1–4). Histones are involved in the structural organization of DNA (1, 2), whereas nonhistone chromosomal proteins are thought to function in regulating gene expression (3, 4). The thyroid hormone receptor is an intrinsic nonhistone chromosomal protein (5–7) that regulates the expression of specific genes, including those for growth hormone in cultured pituitary cells (8, 9) and α_{2u}-globulin in liver (10).

Thyroid hormone receptors bind triiodothyronine (T_3), thyroxine (T_4), and other iodothyronine analogues with affinities that parallel the hormone's thyromimetic potencies (11, 12). Solubilized receptors maintain the same binding specificity as those in chromatin (13); however, we previously found that these preparations lose their high-affinity T_3, but not T_4, binding activity after further purification, acidification, or heating (13, 14). T_3 and T_4 bind to the same protein(s) in the initial extracts used for these studies (11, 13–15). For instance, in these extracts, the concentration of high affinity T_3- and T_4-binding sites is identical and [^{125}I]T_4 binding is inhibited more avidly by nonradioactive T_3 than by T_4. Thus, the data suggest that the receptor is being modified and that specific influences on the binding site are required for ligand recognition. The current studies focus on the reconstitution of the original receptor-binding activity in preparations that have lost high-affinity T_3-binding activity and on the possible involvement of histones or related proteins in the ligand-recognition mechanism of the receptor.

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MATERIALS AND METHODS

Reagents. 3,3′,5[3′-^{125}I]Triiodo-L-thyronine (900 Ci/g; 1 Ci = 3.7 × 10^{10} becquerels) and 3,3′,5,5′[3′,5′-^{125}I]tetraiodo-L-thyronine (1200 Ci/g) were from New England Nuclear. Pancreatic RNase, DNase I, trypsin, and pancreatic trypsin inhibitor were from Worthington. All other reagents were from Sigma. Buffer A is 10 mM Tris·HCl, pH 7.0/0.25 M sucrose/50 mM sodium bisulfite/0.24 mM spermine/1 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride; buffer B is 20 mM *N*-tris(hydroxymethyl)methylglycine, pH 7.6/2 mM $CaCl_2$/1 mM $MgCl_2$/0.5% Triton X-100; buffer C is 50 mM sodium bisulfite, pH 4.0/0.14 M NaCl/1 mM phenylmethylsulfonyl fluoride; buffer D is 50 mM sodium bisulfite, pH 4.0/1 mM phenylmethylsulfonyl fluoride.

Chromatin Extracts. Solubilized extracts were obtained from purified rat liver nuclei as described (13). Heated extracts were prepared by incubating 5 ml of the nuclear extract at 60°C for 10 min followed by centrifugation (27,000 × *g* for 10 min). The supernatant was stored in liquid nitrogen.

Sephadex G-100 and QAE-Sephadex Chromatography. Chromatography was performed as described (13). Included receptor fractions were obtained by chromatographing 10 ml of the nuclear extract on a 2.5 × 96 cm column of Sephadex G-100. Fractions from the included volumes of the column were pooled and stored in liquid nitrogen. Scatchard analysis of [^{125}I]T_3 binding by the included fractions indicated that the receptor was purified 60-fold compared to nuclei.

Binding Assay. These were performed as described (13). In assays that contained added proteins or heated chromatin extracts, control binding assays were performed to correct for hormone binding by these proteins. For Scatchard analyses, the free hormone concentration was determined by subtracting the total bound hormone from the total hormone so that nonspecifically bound hormone was not included as free hormone. Proteins were determined by the method of Bradford (16).

Preparation of Histones. Histones were prepared from fresh steer thymus by a modified procedure provided by R. D. Cole (University of California, Berkeley, CA). All procedures were performed at 4°C. Minced tissue (900 g) was washed with 1 liter of buffer A. Tissue was strained through cheesecloth, combined with 1.5 liters of buffer A, and homogenized for 1 min (Waring Blendor) and 2 min (Tekmar Polytron). The homogenate was strained successively through two, four, six, and eight layers of cheesecloth, the filtrate was centrifuged (700 × *g* for 10 min),

Abbreviations: T_3, triiodothyronine; T_4, thyroxine; TBPA, thyroid hormone-binding prealbumin.

and the supernatant was discarded. Nuclei were suspended in 1 liter of buffer B and centrifuged (700 × *g* for 10 min), the supernatant was discarded, and the procedure was repeated. The pellet was washed twice with 1 liter of buffer C (centrifugation at 700 × *g* for 10 min) and suspended in 120 ml of buffer D; 1/3 vol of 20% trichloroacetic acid was added with stirring. After 15 min, the suspension was centrifuged (8000 × *g* for 10 min) and the supernatant was saved (H1). The pellet was extracted with 200 ml of 5% trichloroacetic acid with stirring for 15 min. After centrifugation of the suspension (8000 × *g* for 10 min), the H1 supernatants were combined, filtered (s intered glass), and dialyzed exhaustively with distilled water. After extraction of H1, the pellet was extracted with 200 ml of 0.25 M HCl for 15 min with stirring and centrifuged (8000 × *g* for 10 min). The supernatant was saved and the pellet was extracted with 0.25 M HCl as above. The combined supernatants (core histones) were filtered (sintered glass) and dialyzed exhaustively with distilled water. The dialyzed H1 and core histone extracts were lyophilized and stored at −20°C. Eighty milligrams of each histone fraction (in 5 ml of 10 mM HCl) was chromatographed on Sephadex G-100 (2.5 × 96 cm) equilibrated in 10 mM HCl; 5-ml fractions were collected at a flow of 30 ml/hr and elution was monitored by absorbance at 230 nm. Pooled fractions were dialyzed against water and stored at −20°C. The concentrations of the individual histones were determined absorptiometrically (230 nm) with the following extinction coefficients: H1 = 1.85 (1 mg/ml) and core histones = 2.90 (1 mg/ml).

Enzyme Treatment. RNase (10 mg/ml in 0.1 M sodium acetate, pH 5) was heated at 90°C for 10 min. The heated extract was treated with RNase at 0.1 mg/ml (30 min at 25°C). DNase I was dissolved in 20 mM Tris·HCl, pH 7.4/3 mM $MgCl_2$ (2 mg/ml). Heated extracts were digested with DNase I at a concentration of 20 μg/ml (2 hr at 37°C). Trypsin (2 mg/ml in 1 mM HCl) digestions of the receptor-containing extracts were performed at a concentration of 0.1 mg/ml (2 hr at 37°C). Trypsin was inhibited with pancreatic trypsin inhibitor (0.01 mg/ml). A nuclear extract treated under these conditions lost 95% of its [^{125}I]T_3 binding activity in the absence of inhibitor and maintained 90% of its original binding activity in its presence.

RESULTS

Selective Loss of T_3 Binding after Dilution. Sephadex G-100 chromatography of receptor-containing extracts yields an included receptor fraction corresponding to a size of 50,500 daltons (13). Fig. 1 shows the dependence of T_3 and T_4 binding on the protein concentration in the included fractions. High-affinity binding site concentrations for [^{125}I]T_3 and [^{125}I]T_4 were determined at each protein concentration by Scatchard analyses (examples are shown in Figs. 3 and 5). The high-affinity T_4-binding site concentration is linearly related to the protein concentration; by contrast, the high-affinity T_3-binding site concentration varies nonlinearly with the protein concentration. At a protein concentration of 35 μg/ml, the ratio of the concentration of T_3 to T_4 binding sites is 1, but it decreases markedly with decreasing concentrations of the included fractions (Fig. 1 *inset*). Thus, dilution appears to affect specifically the high-affinity receptor binding of T_3 as compared with T_4.

Reconstitution of High-Affinity T_3 Binding Activity. Because the influences of dilution on the T_3-binding properties of the receptor (Fig. 1) might be explained by an association–dissociation reaction, we sought conditions to separate the essential components and to reconstitute high-affinity T_3 binding in a receptor fraction that had lost this activity. We found that T_3-binding activity could be stimulated by addition of an extract of chromatin (*Materials and Methods*) to the included receptor fractions that had lost high-affinity T_3-binding activity (Fig. 2). This extract had been heated at 60°C to destroy endogenous high-affinity T_3-binding activity. When portions of the heated extract were added to the fractions from the Sephadex G-100-filtered crude extract (Fig. 2*A*) or QAE-Sephadex-purified receptors (Fig. 2*B*), T_3-binding activity was stimulated in the included column fractions but not in the excluded fractions. These results could imply that the heated nuclear extract contains a component whose concentration in the included fractions was lowered by dilution or separation during the gel filtration.

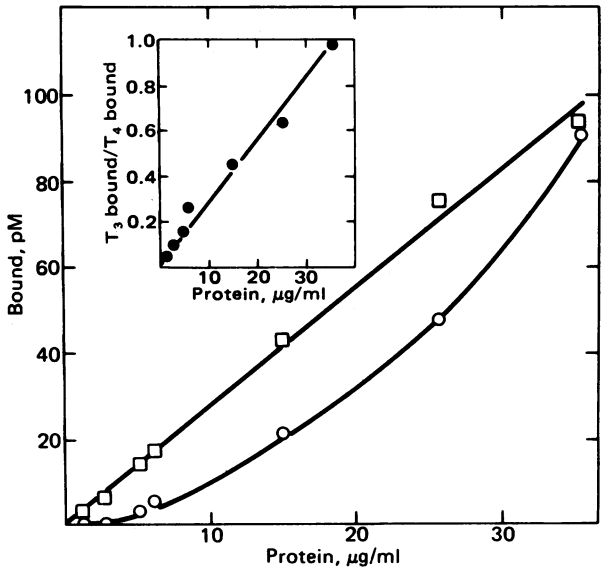

FIG. 1. Effect of dilution (indicated by the protein concentration) of the Sephadex G-100 included receptor fraction on [^{125}I]T_3 (○) and [^{125}I]T_4 (□) binding. At each protein concentration, the concentration of high-affinity [^{125}I]T_3- or [^{125}I]T_4-binding sites was estimated by Scatchard analysis (examples are shown in Figs. 3 and 5).

Shown in Fig. 3 is an examination of the reconstituted binding activity by Scatchard analysis. In this experiment, the concentration of high-affinity T_4-binding sites in the included fractions exceeded by 3-fold the concentration of high-affinity T_3 sites. Fig. 3 demonstrates that addition of the heated extract

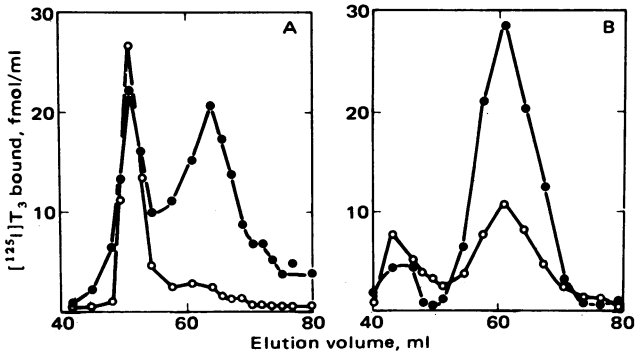

FIG. 2. [^{125}I]T_3 binding by the Sephadex G-100 receptor fractions from the initial nuclear extract (*A*) or an extract partially purified by QAE-Sephadex chromatography (*B*) in the presence (●) or absence (○) of a heated chromatin extract. One milliliter of the initial extract or QAE-Sephadex-purified extract was chromatographed on Sephadex G-100 in the absence of ligand (13). [^{125}I]T_3 binding was assayed in 200 μl of each column fraction in the presence or absence of 50 μl of the heated chromatin extract. The small amount of residual [^{125}I]T_3 binding in the heated extract (determined in separate assays) has been subtracted from each plotted point.

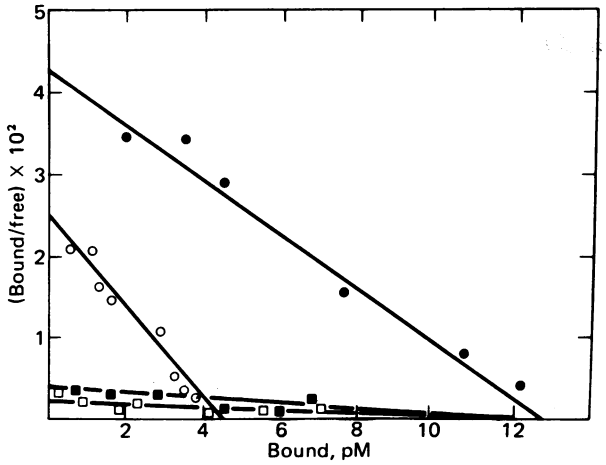

FIG. 3. Scatchard analysis of the binding of [^{125}I]T_3 (○,●) and [^{125}I]T_4 (□,■) by the Sephadex G-100-included receptor fraction in the presence (●,■) or absence (○,□) of the heated chromatin extract. Residual [^{125}I]T_3- and [^{125}I]T_4-binding activity in the heat-treated chromatin extract (50 μl) was monitored in separate assays and has been subtracted from the data.

to the included fractions increased the concentration of high-affinity T_3-binding sites to a level that equalled, but did not exceed, the concentration of T_4 sites. There was no significant influence on the number of T_4-binding sites.

Characterization of Stimulated T_3-Binding Activity. The nature of the components that stimulate T_3 binding was examined by adding various components to the included receptor fractions to determine their ability to stimulate T_3 binding and by testing the effect on the reconstitution of various experimental manipulations of either the included fractions or the heat-treated chromatin extract. Addition of DNA or RNA (each at 400 μg/ml) to the included fractions did not influence the binding of T_3 by the receptor. Likewise, treatment of the heated extract with DNase or RNase did not influence the stimulation of T_3-binding activity. Therefore, DNA or RNA is not required for reconstitution. Dialysis of the included fractions or the heated chromatin extract did not influence the stimulation of T_3 binding, indicating that micromolecular ligands are not required for reconstitution. When either the included fractions or the heated chromatin extract was treated with trypsin, there was a complete loss of T_3 stimulation which could be prevented by pancreatic trypsin inhibitor. Thus, protein–protein interactions may be responsible for the reconstitution. High concentrations of ovalbumin, poly(L-lysine), cytochrome *c*, or lysozyme (each at 200 μg/ml) did not affect the binding of T_3 by the included fractions, suggesting that stimulation is due to a specific protein component(s) in the heated nuclear extract rather than to nonspecific protein influences.

Histone Stimulation of T_3 Binding. The possible interaction of basic proteins with the receptor was suggested from isoelectric focusing studies. When the receptor was focused under nonequilibrium conditions, two forms with isoelectric points of 5.8 and 7.8 were observed (data not shown). By contrast, in equilibrium isoelectric focusing experiments most of the bound hormone focused at pH 5.8, with greatly reduced levels of the form with an isoelectric point of 7.8. These results suggest that the acidic form of the receptor may be initially associated with a more basic protein(s) that dissociates from it under equilibrium focusing conditions.

When histones were added to the included receptor fractions, there was a stimulation of T_3-binding activity. Fig. 4 shows the results of adding increasing concentrations of various histone fractions to the included fractions. Core histones (H2A, H2B, H3, and H4) devoid of detectable H1, as analyzed by gel electrophoresis (17, 18), produced maximal stimulation at 50 μg/ml. Histone fractions containing high concentrations of individual H1 subfractions also produced stimulation; however, greater concentrations of these were required and, as judged by gel electrophoresis (17, 18), these fractions contained small quantities of proteins indistinguishable from histones H4, H2B, and possibly H3. A mixture of H1 subfractions devoid of detectable core histones was much less effective than core histones in producing stimulation. Crude whole histones were the least effective. The basic proteins poly(L-lysine), lysozyme (Fig. 4), and cytochrome *c* did not stimulate T_3 binding. Thus, it does not appear that it is merely the basic nature of histones that produces stimulation. When core histones were fractionated by differential solvent extraction (19), three fractions, containing H4 and H2A, H3 and H2A, and H2B and H2A, were obtained. None of these fractions possessed any stimulatory activity; however, when these fractions were recombined and further purified by chromatography on Sephadex G-200, the fractions corresponding to a size of approximately 60,000 daltons possessed 90% of the stimulatory activity of the original core histones. Moreover, these reconstituted histone fractions exhibited maximal stimulation at the same protein concentration as the original core histones (data not shown). This result was obtained despite the loss of 80% of the original protein during the fractionation.

The nature of the influence of histone on the stimulation of T_3 binding by the included receptor fractions was examined by Scatchard analysis (Fig. 5). Addition of core histones (50 μg/ml) to the included receptor fractions increased the concentration of high-affinity T_3-binding sites to a level equalling but not exceeding the concentration of T_4-binding sites without significantly changing the concentration of high-affinity T_4-binding sites. In five experiments (as in Fig. 5) the concentrations of T_4-binding sites were within 10% in the presence or absence of histones, and the equilibrium dissociation constant

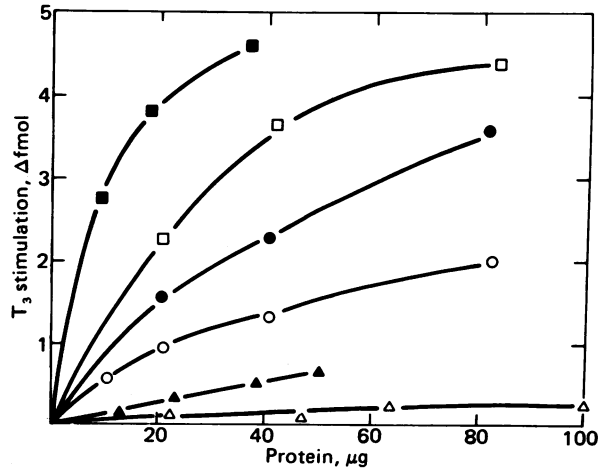

FIG. 4. Stimulation of [^{125}I]T_3 binding by the Sephadex G-100-included receptor fraction by core histones (H2A + H2B + H3 + H4, ■), H1 subfraction 2 (□), H1 subfraction 3 (●), unfractionated H1 (○), whole histone (▲), and lysozyme (△). Histones were prepared from fresh steer thymus. The abscissa represents the amount of proteins added to a 0.5-ml assay mixture; the ordinate represents the difference in the binding of [^{125}I]T_3 by the included receptor fractions in the presence and absence of the indicated protein fractions. All data are corrected for the small amount of [^{125}I]T_3-binding activity by the individual protein fractions, which was determined in separate assays.

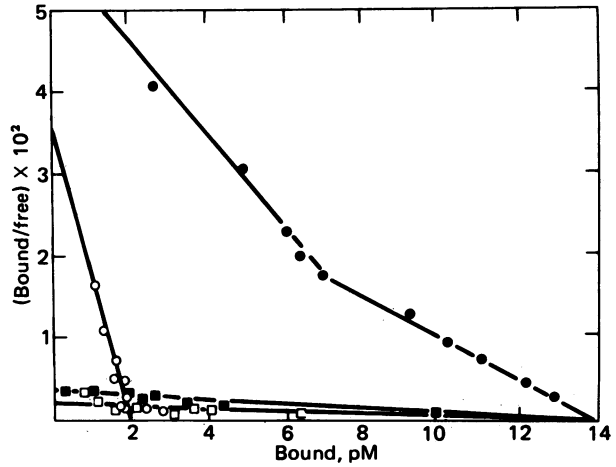

FIG. 5. Scatchard analysis of the binding of [^{125}I]T_3 (○,●) and [^{125}I]T_4 (□,■) by the included receptor fractions in the presence (●,■) or absence (○,□) of core histones. Assays were performed as described in *Materials and Methods* except that the concentration of [^{125}I]T_3 or [^{125}I]T_4 was varied (500–10,000 pM).

in the absence (2.38 nM ± 0.37; SD) or presence (1.83 nM ± 0.78; SD) of histones was nearly the same. In the plot shown in Fig. 5, there is a suggestion of two classes of binding; however, in four of five experiments the data were more consistent with a single straight line. The dissociation constant for T_3 obtained from the individual Scatchard plots (as in Fig. 5) was 0.21 nM ± 0.10 (SD, $n = 5$) in the absence or 0.19 ± 0.07 (SD, $n = 5$) in the presence of histone.

Analysis of the histone stimulation of T_3-binding activity as a function of protein concentration in the included receptor fraction is shown in Fig. 6. Each datum point represents the extrapolated concentration of high-affinity T_3- or T_4-binding sites from Scatchard analyses. The histone concentration was constant at 50 μg/ml, and the concentration of the included receptor fraction was varied. As discussed previously, the ratio

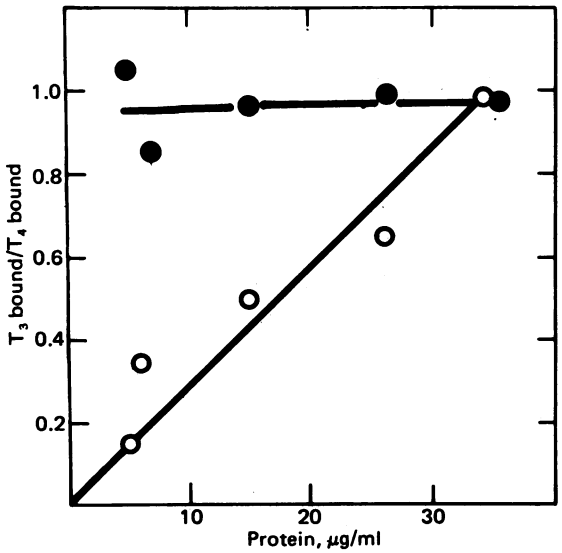

FIG. 6. Dependence of the ratio of [^{125}I]T_3 to [^{125}I]T_4 binding sites (ordinate) on the included receptor fraction concentrations (abscissa) in the absence (○) or presence (●) of core histones. T_3- and T_4-binding site concentrations were determined from individual Scatchard analyses.

of the concentration of high-affinity T_3 to T_4 binding sites in the absence of added histones decreased at lower protein concentrations (Fig. 1). Inclusion of core histones restored the concentration of high-affinity T_3-binding sites to a level equalling that of the high-affinity T_4-binding sites. At the highest protein concentration there was no loss of high-affinity T_3-binding capacity and no effect of histones on the binding of T_3.

The possibility was excluded that the histones are merely protecting the receptors from a proteolysis occurring during the binding assay that converts the receptors to the low-affinity T_3-binding form. To do this, included receptor fractions were incubated with T_3 with or without histones under the standard assay conditions at 22°C for 4 hr. Then histones were added to the mixture that had not received them and the incubations were continued for an additional 2 hr. Binding in the two incubations was identical. If the histones were merely protecting against receptor proteolysis, binding in the mixture first incubated in the absence of histones should have been less than that in the incubation that contained histones for the duration of the experiment.

DISCUSSION

The present studies focus on the factors that affect the ability of the thyroid hormone receptors to bind T_3, the major iodothyronine active *in vivo*. When receptor-containing nuclear extracts are partially purified (60-fold) by Sephadex G-100, the major form of the receptor elutes in included fractions of the column corresponding to a size of 50,500 daltons (13). If these fractions are kept concentrated, high-affinity T_3-binding activity is maintained. However, upon dilution, there is a loss of T_4 binding (Fig. 1). Because T_3 and T_4 are bound by the same protein(s) in the initial extracts used for these studies (11, 13–15), the loss of T_3-binding activity upon dilution is not due to a selective loss of a species that exclusively binds T_3 but not T_4. The data suggest that dilution of the receptor results in an alteration of the receptor that influences T_3- but not T_4-binding activity. These results are consistent with the possibility that dissociation of receptor subunits could be involved in the changes in ligand-binding specificity; they also further support our previous studies (14) which suggest that some fundamental unit of the receptor can exist that differs from the active form of the receptor and resembles thyroid hormone-binding prealbumin (TBPA) in its hormone-binding characteristics. A possible relationship between the receptor and TBPA has also been postulated from crystallographic data suggesting the presence of a putative DNA-binding site on TBPA (20); however, earlier studies (21) were unable to detect DNA binding of TBPA. In any event, other thyroid hormone-binding species such as TBPA may more closely resemble the receptors than has been previously thought.

Reconstitution of high-affinity T_3-binding activity in the included receptor fractions could be obtained by addition of a chromatin extract that was heated to destroy endogenous T_3-binding activity (Figs. 2 and 3). The stimulating component could be destroyed by trypsin but not DNase or RNase; it could not be replaced by DNA, RNA, ovalbumin, poly(L-lysine), lysozyme (Fig. 5), or cytochrome *c*. Thus, the stimulating component appears to be a specific protein present in the chromatin extract, providing additional support that subunit interactions may influence the receptor's ligand-binding specificity. We discovered that reconstitution of high-affinity T_3-binding activity could also be obtained by addition of core histones (H2A, H2B, H3, and H4) and, to a lesser extent, certain H1 subfractions that appeared to contain small amounts of H2B, H4, and possibly H3 (Fig. 4). Mixtures of H1 subfractions that contained

no detectable core histones possessed very little stimulatory activity (Fig. 4). In further studies, mixtures of H2A and H2B, H2A and H3, or H2A and H4 were found not to possess stimulatory activity. However, when these fractions were combined, resulting in the formation of a complex of approximately 60,000 daltons isolated by Sephadex G-200 chromatography, 90% of the stimulatory activity (despite loss of 80% of total protein) of the original core histones was recovered. The reconstitutions resulted in a full recovery of the high-affinity T_3-binding capacity to a level that equalled the high-affinity T_4-binding capacity of the extract, and there was no stimulation of T_4-binding activity (Figs. 3, 5, and 6). These observations suggest that the alteration in receptor-binding properties is a reversible process that can be regulated by a protein(s) extracted from chromatin or by histones. Further, some aggregate of the histones or histone-like protein present in the preparation appears to be responsible for stimulating the high-affinity T_3-binding activity.

These studies suggest the following model of the thyroid hormone receptor. A "holo" receptor complex exists which consists of subunits and which binds thyroid hormones in relation to their biological activities. The "holo" receptor complex consists of a "core" subunit which has a thyroid hormone binding site and a regulatory subunit, possibly a histone. The "core" subunit binds T_4 with about the same affinity as the "holo" receptor; however, it binds T_3 less avidly than T_4. Interaction of the "core" receptor with the regulatory subunit causes a change in the ligand-binding properties of the "core" receptor and generation of the "holo" receptor.

Because the current findings of reversible changes in specificity of receptor binding were obtained in cell-free extracts, it is not possible to make conclusions about the role of these influences on the receptor in the cell. However, the findings do suggest the following hypothesis, which can explain many known receptor characteristics. The receptor is initially synthesized as the "core" receptor. This could explain why cytosol proteins that bind iodothyronines proportional to their thyromimetic potencies have not been found (22, 23). The "core" receptors then interact in the nucleus with histones or histone-like species, forming "holo" receptors. The receptors may simultaneously interact with DNA, although from previous studies of the DNA–receptor interaction (24) it cannot be ascertained whether the observed DNA binding was due to interactions with DNA-binding proteins or whether there is a DNA-binding site located on the receptor itself. This model for thyroid hormone action contrasts with steroid hormone action; chromatin stimulates the thyroid hormone receptors' ability to bind the active ligand (T_3), whereas steroids stimulate the binding of receptors to chromatin.

The possibility that histones can affect ligand recognition by the thyroid hormone receptor also raises the possibility of a role for the histones in addition to their function in packaging of DNA. Histones may also function in the maintenance or modulation of the activity of nonhistone chromatin proteins involved in the regulation of specific genes. It is not yet possible to know how general such effects may be; chromatin proteins known to regulate the expression of specific genes other than the thyroid hormone receptor have not been identified. However, from other studies, there is evidence for interactions between nonhistone chromosomal proteins and histones. The nonhistone chromosomal proteins HMG-1 and HMG-2 can form specific complexes with various H1 subfractions (25). In isolated rat liver nuclei, exogenous H1 and H4 stimulate phosphorylation of several nonhistone proteins as well as inhibit the phosphorylation of a specific low molecular weight protein (26). However, the functions of the HMG proteins or the nonhistone chromosomal proteins whose phosphorylation is affected by the histones (26) are not known. These studies, in conjunction with our results, suggest that histone–nonhistone chromosomal protein interactions must be considered as participants in the regulation of genetic expression.

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