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Affinity labeling of rat liver thyroid hormone nuclear receptor

(*N*-bromoacetyl-3,3′,5-triiodo-L-thyronine/*N*-bromoacetyl-L-thyroxine/ligand–receptor covalent interaction)

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ABSTRACT The thyroid hormone receptor from rat liver nuclei has been covalently labeled with the *N*-bromoacetyl derivatives of L-thyroxine (T4) and 3,3′,5-triiodo-L-thyronine (T3). Displacement binding studies showed that, in the presence of 100-fold molar excess of unlabeled *N*-bromoacetyl-T3 or T4, binding of [125I]T3 or [125I]T4 was nearly totally inhibited. Heat inactivation of the receptor (55°C for 15 min) resulted in parallel losses in the binding of T3 (95%) and *N*-bromoacetyl-T3 (93%). These results indicated that T3 and T4 and their bromoacetyl derivatives compete for the same binding site. The nuclear receptor showed identical behavior in high-pressure liquid chromatography (HPLC) whether bound to T3 or T4 or covalently labeled with their bromoacetyl derivatives. HPLC provided a single-step 100-fold purification of the nuclear receptor. $NaDodSO_4$ gel electrophoresis of the nuclear receptor labeled with *N*-bromoacetyl derivatives of [125I]T3 or [125I]T4 showed one major radioactive component with a molecular weight of 56,000. Furthermore, in the absence of denaturant, the nuclear receptor either bound to [125I]T3 or covalently labeled with *N*-bromoacetyl-[125I]T3 showed identical mobility. These results suggested that the nuclear receptor is a single polypeptide chain and binds either T3 or T4. Nuclear receptors covalently linked with *N*-bromoacetyl derivatives of [125I]T3 or [125I]T4 may be useful as a marker for the preparative purification of receptor.

Thyroid hormones play a fundamental role in regulating tissue differentiation and development and in influencing numerous metabolic processes (1). Recently, several specific mRNAs have been shown to be under thyroid hormone control (2–4). A large body of evidence suggests that the initiation of thyroid hormone action is through the interaction of 3,3′,5-triiodo-L-thyronine (T3) with nonhistone proteins in the chromatin (5). These receptors can be solubilized from nuclei of target tissues (6–7) and have binding characteristics similar to those found in intact nuclei.

In order to characterize the thyroid hormone receptor, to understand the nature of its interaction with thyroid hormones and to study its functions, receptor in a reasonable quantity and purity is required. However, it has been reported that manipulations such as heating, pH changes, dilution, and partial purifications result in loss of T3 binding activity (7–9). Therefore, it became apparent that, if the receptor covalently labeled with radioactive thyroid hormone could be prepared, it would serve as a marker to follow preparative purification, and more intensive studies would no longer be limited to conditions consistent with normal, reversible, hormone-binding activity. In the present communication, we report the affinity labeling of rat liver thyroid hormone nuclear receptor with 125I-labeled *N*-bromoacetyl-T3 (BrAcT3) and *N*-bromoacetylthyroxine (BrAcT4).

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MATERIALS AND METHODS

Reagents. T4,T3,*N*-hydroxysuccinimide, and *N,N′*-dicyclohexylcarbodiimide were obtained from Sigma. [125I]T4 (900 μCi/μg; 1 Ci = 3.7 × 10^{10} becquerels) and [125I]T3 (1200 μCi/μg) were from New England Nuclear. Bromoacetic acid was obtained from Chem Service (Media, PA). Other chemicals were the highest purity available.

Synthesis of *N*-Hydroxysuccinimide Bromoacetate. To a solution of 0.695 g (5 mmol) of bromoacetic acid in 10 ml of dimethoxyethane was added 0.575 g (5 mmol) of *N*-hydroxysuccinimide (purified by recrystallization from ethyl acetate) and 1.13 g (5 mmol) of *N,N′*-dicyclohexylcarbodiimide (purified by distillation). The mixture was stirred for 3 hr, and the dicyclohexylurea was removed by filtration. The filtrate was evaporated to dryness under reduced pressure. The dry residue was purified by recrystallization from ethyl acetate/hexane, 5:1 (vol/vol), to give colorless crystals (0.57 g; yield, 48%), mp 114–116°C.

Synthesis of BrAc[125I]T4. To 167 ng (0.215 nmol; 855 μCi/μg) of [125I]T4 in 5 μl of dimethylformamide was added 30 μg (112 nmol) of *N*-hydroxysuccinimide bromoacetate in 20 μl of dimethylformamide. After the mixture has been at room temperature for 10 min, 5 μl of 10% triethylamine in dimethylformamide was added to it. After further standing at room temperature for 45 min, the resultant solution was applied to a prewashed silica gel plate (KF1, Whatman) with 10% acetic acid in ethyl acetate as developing solvent. Unlabeled BrAcT4, synthesized as described by Cheng (10), was used as a reference. The radioactive band with a R_F identical to that of BrAcT4 was scraped off and the BrAc[125I]T4 was eluted with 1 ml of 30% methanol in ethyl acetate. Purity of the product was confirmed by autoradiography of the thin-layer chromatogram.

Synthesis of Unlabeled and 125I-Labeled BrAcT3. To a solution of T3 sodium salt (50 mg; 74 μmol) in 0.5 ml of dimethylformamide was added *N*-hydroxysuccinimide bromoacetate (20 mg; 75 μmol). After the mixture had been stirred for 30 min at room temperature, the resultant solution was added to 10 ml of ice-cold 20% (wt/vol) citric acid. The white precipitate was collected by filtration and dried under reduced pressure. The product was purified by preparative thin-layer chromatography (PLK5F, Whatman) with 10% acetic acid in ethyl acetate as the developing solvent. The band with a R_F of approximately 0.58 was eluted with 10% methanol in ethyl acetate. Thin-layer chromatography of the eluted product showed one spot.

Abbreviations: T4, L-thyroxine; T3, 3,3′,5-triiodo-L-thyronine; BrAcT4, *N*-bromoacetyl-L-thyroxine; BrAcT3, *N*-bromoacetyl-3,3′,5-triiodo-L-thyronine; HPLC, high-pressure liquid chromatography; T3Ac-, L-thyroxyl-*N*-acetyl; T4Ac-, L-thyroxyl-*N*-acetyl.

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BrAc[^{125}I]T3 was synthesized similarly. To a solution of 0.12 μg (0.184 nmol; 1015 μCi/μg) of [^{125}I]T3 and 20 μg (74.6 nmol) of *N*-hydroxysuccinimide bromoacetate in 20 μl of dimethylformamide was added 5 μl of 10% triethylamine in dimethylformamide. After the mixture had been at room temperature for 50 min, BrAc[^{125}I]T3 was isolated and purified by thin-layer chromatography as described above. Autoradiography of the chromatogram showed one spot which comigrated with the unlabeled BrAcT3; no free T3 was detected.

Preparation of Nuclear Extract. All steps were carried out at 5°C. Nuclei from 10 g of rat liver were prepared as described (11). The nuclear pellets were washed twice with 0.02 M potassium phosphate, pH 7.2/0.32 M sucrose/3 mM magnesium chloride/0.5% Triton X-100 and centrifuged at 750 × *g* for 10 min. The nuclear pellets were suspended in 25 ml of the same buffer without Triton X-100 and centrifuged as above. The nuclei were then suspended in 7.5–10 ml of 0.02 M phosphate, pH 7.2/5% (wt/vol) glycerol/1 mM EDTA/0.4 M potassium chloride. The suspension was kept on ice for 45 min (with frequent mixing by pipetting) and the nuclear residue was pelleted by centrifugation at 14,500 × *g* for 10 min. The supernatant, which contained the nuclear receptor, was used for affinity labeling or binding studies. The protein concentration was 0.92–1.2 mg/ml, and the solution had a A_{260}/A_{280} ratio of approximately 1.4.

Affinity Labeling of Nuclear Receptor, Binding Assay, and Heat-Inactivation Studies. Fresh nuclear extract was used in all experiments. Nuclear extract (0.5 ml; 0.5 mg of protein) was incubated with 0.1–0.5 nM of ^{125}I-labeled T3, T4, BrAcT3, or BrAcT4 in polypropylene tubes at 5°C for 15–18 hr. Nonspecific binding was determined under identical conditions except in the presence of 100- to 1000-fold molar excess of T3, T4, or BrAcT3, respectively. For heat-inactivation experiments, the nuclear extract was first heated at 55°C for 15 min. In spite of the findings that the presence of sulfhydryl reagents enhances the binding of T3 or T4 to the nuclear receptor, it was necessary to exclude it from the incubation mixture because of its reactivity with *N*-bromoacetyl groups. To avoid losses up to 75% due to adsorption of the receptor protein to glass, polypropylene tubes and plastic pipettes were used in all operations.

Chromatography on Sephadex G-25. To determine nonspecific binding and to establish that T3, T4, BrAcT3, and BrAcT4 compete for the same binding site, binding studies were carried out using Sephadex G-25 (coarse) columns prepared from pasteur pipettes. The Sephadex G-25 column was equilibrated with the same buffer used for the extraction of nuclear proteins. The excluded peak, containing hormone-bound nuclear receptor, was collected and the amount of ^{125}I was determined with a Beckman Gamma 9000 spectrometer.

High-Pressure Liquid Chromatography (HPLC). Binding of T3, T4, and their BrAc derivatives was also measured by HPLC. Two hundred microliters of the nuclear extract labeled with BrAcT3 or -T4 or bound to T3 or T4 as described above was applied to dual protein column I-125 (Water Associates, Milford, MA), with the exclusion limit of 80,000 daltons. The hormone-bound receptor eluted at a retention time of 6.7 min with a flow rate of 2 ml/min in 0.02 M potassium phosphate, pH 7.2/0.4 M KCl at room temperature. The fractions corresponding to peaks of labeled material (fraction 19, 20, 21, and 22 in Fig. 1) were pooled, dialyzed overnight against 0.05 M sodium bicarbonate (pH 7.8), lyophilized, and used for gel electrophoresis.

Polyacrylamide Gel Electrophoresis. NaDodSO$_4$ gel electrophoresis was performed according to the method of Laemmli (12). Gel electrophoresis of native proteins was by method of Laemmli but without NaDodSO$_4$, and the running buffer system contained 0.02 M 2-mercaptoethanol. For 7.5% analytical gel electrophoresis, the incubation mixture of [^{125}I]T3 and nuclear protein was directly applied to the gel. Nuclear extracts labeled with BrAc[^{125}I]T3 were used after purification by HPLC followed by dialysis and lyophilization. NaDodSO$_4$ (0.1%) was required to solubilize the proteins. The gel electrophoresis was performed at a constant potential of 100 mV for 60 min and increased to 140 mV for 240 min at 5°C. Bovine serum albumin (M_r, 68,000; isoelectric point, 4.6) was used as a marker protein.

To locate the labeled nuclear receptor, the slab gel strips were sliced into 2-mm slices and the radioactivity was quantitated in a Beckman Gamma 9000 spectrometer. For NaDodSO$_4$ slab gel electrophoresis, 10% gel was used. The nuclear extracts covalently labeled with BrAc[^{125}I]T3 or -T4 were treated as described above. They were further boiled in 1% NaDodSO$_4$/10 mM 2-mercaptoethanol for 1 min. The reduced products were applied to the gel. Gel strips were cut and the radioactivity was measured in the same manner as described for the native gels. Methylated phosphorylase b (M_r, 92,500), bovine serum albumin (M_r, 68,000), ovalbumin (M_r, 43,000), carbonic anhydrase (M_r, 30,000), and cytochrome *c* (M_r, 12,300) were used as marker proteins.

RESULTS AND DISCUSSION

High-affinity, low-capacity binding sites in nuclei are measured by binding assays that are elaborate and time-consuming. Therefore, a receptor covalently labeled with derivatives of T3 and T4 would be useful. We approached this problem by using BrAc derivatives of T3 and T4. The specificity of these derivatives was established by competitive and heat-inactivation studies using HPLC and Sephadex G-25 chromatography. Finally, gel electrophoresis was used to compare labeled protein.

Sephadex G-25 Chromatography. In order to show the presence of low-capacity, high-affinity thyroid hormone binding sites in the nuclei, several experiments were performed.

(*i*) Freshly prepared nuclear extract was incubated with [^{125}I]T3, [^{125}I]T4, BrAc[^{125}I]T3, or BrAc[^{125}I]T4 and assayed. Thirty-two percent of total added [^{125}I]T3 or BrAc[^{125}I]T3 was bound to the nuclear proteins. On incubation of the nuclear extract with 0.1–0.5 nM [^{125}I]T3 in the presence of 100-fold molar excess of unlabeled T3, this binding was inhibited by 95% after 15–18 hr at 5°C as indicated by chromatography on Sephadex G-25 (Table 1). When the nuclear extract was incubated with [^{125}I]T3 in the presence of nonradioactive BrAcT3

Table 1. Competitive binding of T3 and BrAcT3 to rat liver nuclear receptors

Conditions	^{125}I-Labeled ligand (0.5 nM)	Unlabeled competitor (50 nM)	Inhibition, %*
Normal†	T3	T3	95
Normal	T3	BrAcT3	93
Preincubation‡	T3	BrAcT3	100
Heated extract§	T3	—	95
Heated extract	BrAcT3	—	93

* Normalized.

† The ^{125}I-labeled ligand was incubated with the nuclear receptors for 18 hr at 5°C; assay was by Sephadex G-25 chromatography.

‡ The nuclear receptor was preincubated with BrAcT3 (100 nM) for 18 hr at 5°C; then, the unbound ligand was removed by Sephadex G-25 chromatography and the nuclear extract was incubated with [^{125}I]T_3 for 3 hr at 30°C.

§ The nuclear extract was heated for 15 min at 55°C.

at 100-fold molar excess the binding was inhibited by 93%. These results clearly demonstrate that BrAcT3 competes for the same binding site on the nuclear receptor and is able to displace bound [^{125}I]T3 from a solubilized nuclear receptor protein. Similar results were obtained with [^{125}I]T4 but a decrease in specific binding was observed as noted by others (7, 13).

(ii) The nuclear extract was preincubated with 100 nM BrAcT3 at 5°C for 18 hr. The free BrAcT3 was removed by chromatography on Sephadex G-25 and the eluted nuclear extract was incubated with 0.5 nM [^{125}I]T3 for 3 hr at 30°C. No bound [^{125}I]T3 could be detected by Sephadex G-25 chromatography (Table 1). The control experiment without pretreatment of the nuclear receptor with BrAcT3 resulted in the binding of 12% of total added [^{125}I]T3. This is to be compared with 32% binding of [^{125}I]T3 to freshly prepared nuclear extract that was not stored at 5°C for 18 hr and was not chromatographed on Sephadex G-25. The loss of binding activity is probably due to dilution (7–9) of the nuclear extract after chromatography on Sephadex G-25 and instability of the receptor protein. Nonetheless, this result indicates that BrAcT3 covalently labeled the binding site of T3 and added [^{125}I]T3 could no longer be bound.

(iii) Eberhardt et al. (8) reported that heating of a crude extract from rat liver nuclei at 50° C for 10 min destroyed 90% of T3 binding activity. To determine if BrAcT3 binding was similarly affected by such treatment, 500 μl of freshly prepared rat liver nuclear extract was heated for 15 min at 55°C. Under these conditions, 51% of the total protein was precipitated as determined by absorbance at 280 nm. The extract was centrifuged, and the supernatant was incubated with 0.5 nM BrAc[^{125}I]T3 at 5°C for 18 hr. The control samples containing nuclear extract with or without heating were treated in a similar fashion. The binding of [^{125}I]T3 or its BrAc derivative was determined after chromatography on Sephadex G-25. The amount of the radioactivity was compared as cpm/A_{280} nm. As shown in Table 1, heat inactivation of the nuclear extract resulted in a parallel loss in binding to [^{125}I]T3 (95%) and BrAc[^{125}I]T3 (93%).

High-Pressure Liquid Chromatography. Further evidence that the protein that binds T3 or T4 is the same as that labeled with radioactive BrAcT3 or BrAcT4 was provided by HPLC: they showed identical elution profiles. This method also allowed a rapid and efficient separation of labeled nuclear receptor protein from other contaminating proteins, achieving a 100-fold purification. The products of incubations were directly applied to the column. The results of the binding experiments are illustrated in Fig. 1. The nuclear receptor bound to [^{125}I]T3 or [^{125}I]T4 was eluted in the same fractions with maximal radioactivity at fraction 19 (prealbumin, M_r 54,000, showed a peak at fraction 20). At 100-fold molar excess of unlabeled T3 or T4 the binding of [^{125}I]T3 or [^{125}I]T4 was inhibited. A similar result was obtained when [^{125}I]T3 was incubated with the nuclear extract in the presence of 100-fold excess of BrAcT3. Results of these experiments are consistent with those using Sephadex chromatography. The recovery of labeled receptor from HPLC was about 25% of that obtained when Sephadex G-25 columns were used. This lower recovery is probably due to adsorption of the labeled protein to the column. The nuclear receptor covalently labeled with BrAc[^{125}I]T3 or with BrAc[^{125}I]T4 eluted from HPLC in the same fractions, with the maximal activity in fraction 19 and with identical chromatographic properties as the nuclear receptor labeled with [^{125}I]T3 or [^{125}I]T4. Furthermore, the shapes of the activity peaks for the nuclear receptor bound to [^{125}I]T3 or covalently labeled with BrAc[^{125}I]T3 and BrAc[^{125}I]T4 were strikingly similar. All peaks containing labeled receptor show asymmetry towards lower molecular weight species.

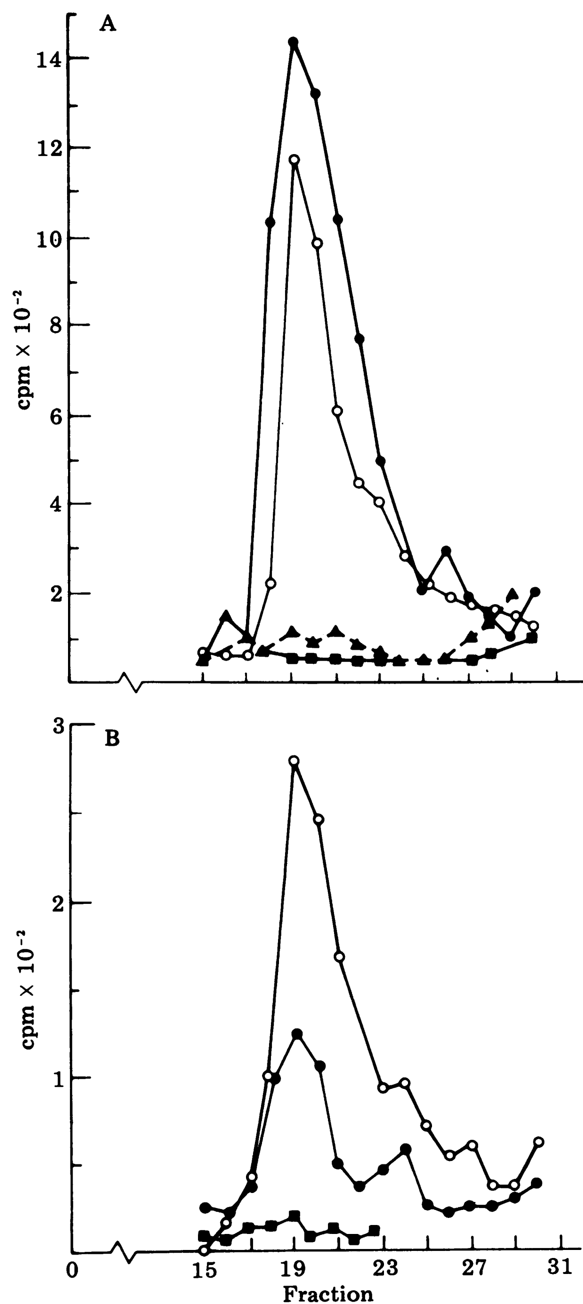

FIG. 1. HPLC. (A) Nuclear extract (≈0.5 mg of protein) was incubated in 0.5-ml aliquots with 0.5 nM [^{125}I]T_3 alone (●), with 0.5 nM [^{125}I]T3 plus unlabeled T3 at 100-fold molar excess (■), with 0.5 nM [^{125}I]T3 plus unlabeled BrAcT3 at 100-fold molar excess (▲), or with BrAc[^{125}I]T3 (○) for 15–18 hr at 5°C. (B) Incubations of nuclear extract with 0.5 nM [^{125}I]T4 alone (●) or plus unlabeled T4 (■) at 100-fold molar excess or with BrAc[^{125}I]T_4 (○), under the same conditions as above. The incubation mixture (200 μl) was directly applied to the column. The elution buffer was 0.02 M phosphate, pH 7.2/0.4 M KCl; 0.71-ml fractions (flow rate, 2 ml/min) were collected and the amount of ^{125}I was determined.

Analytical Gel Electrophoresis. It has been shown (14) that the nuclear receptor bound to T3 exhibits an anodic migration and has a broad peak with some T3-bound material not entering the gel. When gel electrophoresis under native conditions was performed using the nuclear extract bound to [^{125}I]T3 (Fig. 2A) or labeled with BrAc[^{125}I]T3 (Fig. 2B), a major portion of the radioactive materials did not enter the gel. However, a suffi-

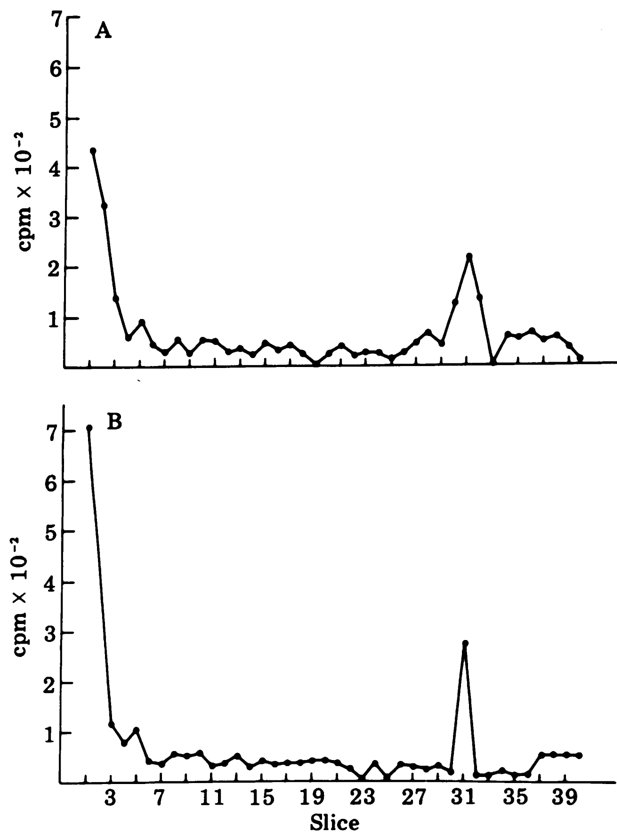

FIG. 2. Polyacrylamide gel electrophoresis of the [^{125}I]T3-bound (*A*) or BrAc[^{125}I]T3-labeled (*B*) nuclear extracts. Nuclear extract (0.5 ml; ≈0.5 mg of protein) was incubated with 0.5 nM [^{125}I]T3 for 15–18 hr at 5°C, and 50 μl of the sample was applied to 7.5% polyacrylamide gel (*A*). BrAc[^{125}I]T3-Labeled nuclear extract was fractionated by HPLC. Fractions containing labeled receptor were pooled, dialyzed, and lyophilized. The lyophilized material was dissolved and the sample was applied to 7.5% polyacrylamide gel (*B*). At the end of the run, gel strips were cut into 2-mm slices and ^{125}I was measured.

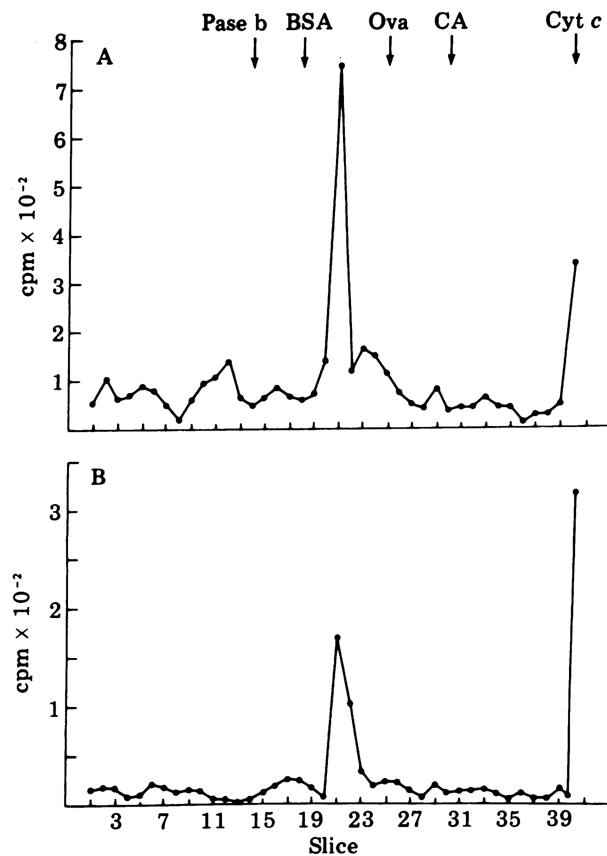

FIG. 3. NaDodSO$_4$ gel electrophoresis of the nuclear extract labeled with BrAc[^{125}I]T3 (*A*) or BrAc[^{125}I]T4 (*B*). Nuclear extract (0.5 ml; ≈0.5 mg of protein) was incubated with 0.1–0.5 nM BrAc[^{125}I]T3 (*A*) or BrAc[^{125}I]T4 (*B*) for 15–18 hr at 5°C. The product of incubation was fractionated by HPLC, dialyzed, and lyophilized. Ac[^{125}I]T3–nuclear receptor or Ac[^{125}I]T4–nuclear receptor was dissolved in buffer containing 1% NaDodSO$_4$ and 0.01 M 2-mercaptoethanol, boiled for 1 min, and analyzed in 10% gel. At the end of run, gel strips were sliced into 2-mm pieces, and ^{125}I was measured. Marker proteins were methylated phosphorylase b (Pase b; 96,500), bovine serum albumin (BSA; 68,000), ovalbumin (Ova; 43,000), carbonic anhydrase (CA; 30,000), and cytochrome *c* (Cyt c, 12,300).

cient amount of T3-bound or BrAcT3-labeled materials migrated toward the anode and was detected in the region of bovine serum albumin as a relatively sharp peak. The identity of electrophoretic mobilities provides further evidence that T3-bound and BrAcT3-labeled nuclear protein are the same molecule.

NaDodSO$_4$ Gel Electrophoresis. Electrophoresis on 10% NaDodSO$_4$ slab gels was used to determine if the nuclear receptor is covalently labeled with BrAc[^{125}I]T3 and BrAc[^{125}I]T4. The radioactive fractions after HPLC were pooled, dialyzed, lyophilized, boiled in 1% NaDodSO$_4$/10 mM 2-mercaptoethanol, and applied to the gel. The results of such an experiment are shown in Fig. 3. The fact that Ac[^{125}I]T3- was not dissociated from the nuclear receptor by boiling in 1% NaDodSO$_4$, compared to the complete release of T3 from [^{125}I]T3-bound nuclear receptor, was direct evidence that covalent interaction had occurred. Furthermore, the reaction of BrAc[^{125}I]T3 (Fig. 3*A*) or BrAc[^{125}I]T4 (Fig. 3*B*) with the nuclear receptor resulted in the covalent labeling of one major protein. The molecular weight of this protein was 56,000, which is consistent with that observed by HPLC with prealbumin as a marker and is in a good agreement with that determined by gel filtration as reported by others (7, 15). This suggests that the nuclear receptor for thyroid hormones is a single monomeric protein and binds either T3 or T4.

It is clear from the binding studied by Sephadex gel chromatography and HPLC that BrAc derivatives of T3 or T4 compete for the same binding site as thyroid hormones in the nuclear receptor from rat liver. The same chromatographic pattern after HPLC and identical electrophoretic mobilities in nondenaturing gels of [^{125}I]T3-bound and Ac[^{125}I]T3-labeled nuclear receptor showed that BrAc[^{125}I]T3 labeled the nuclear receptor. Furthermore, the inability of BrAc[^{125}I]T3 to label heat-treated nuclear receptor is additional proof of the specificity in labeling of the T3 binding sites. Gel electrophoresis of Ac[^{125}I]T3- or Ac[^{124}I]T4-labeled nuclear receptor in the presence of denaturating agent under reducing conditions indicated that a single protein species was covalently labeled with the BrAc derivatives. In the present study, the findings that Ac[^{125}I]T3- and Ac[^{125}I]T4-labeled nuclear receptor protein showed the same chromatographic pattern and identical electrophoretic mobilities demonstrated that T3 and T4 bind to the same protein, which has a molecular weight of 56,000.

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