A panel of anti-thyroid hormone receptor (TR) antisera were generated to allow direct assay of the concentrations of the α1 and β1 receptor isoforms in nuclear extracts from adult rat liver, kidney, brain and heart, and fetal brain. An antiserum, immunoglobulin G (IgG)-β1, raised against amino acid sequence 62–92 of the rat TR-β1 specifically precipitated only TR-β1 in vitro translation products. A second antiserum, IgG-α1/β, generated against a sequence that is identical in the ligand binding region of rat TR-α1 and TR-β isoforms immunoprecipitated both TR-α1 and -β1 translation products. These IgG preparations were used to specifically immunoprecipitate thyroid hormone receptor binding activity from nuclear extracts. IgG-β1 cleared almost 80%, and the IgG-α1/β immunoprecipitated nearly all binding from hepatic nuclear extracts. This distribution of TR protein, 80% β1 and 20% α1, is the same as previously reported for their respective mRNAs in liver. In heart, kidney, and brain IgG-β1 cleared 45%, 43%, and 28% of total binding, respectively, and IgG-α1/β cleared all T3 binding activity from these tissues. In agreement with an earlier study, marked variations in specific protein/mRNA ratios were noted among these tissues. Consistent with our earlier report of the presence of only very low levels of TR-β1 mRNA in fetal brain, IgG-β1 cleared just 5% of binding in this tissue. Studies using an antiserum (IgG-ch) generated against homologous segments of the hinge region in both TR-α1 and -β1 yielded results which contrasted sharply with those of IgG-α1/β. Whereas IgG-ch could also immunoprecipitate virtually all binding from hepatic extracts it cleared only 40–50% of binding from the other tissues, including fetal brain in which TR-α1 accounts for >90% of binding protein. The data suggest the presence of postranslational modification of the TR-α1 protein in the hinge region, consistent with the presence in this segment of potential phosphorylation sites.

Early studies from our laboratory as well as others suggested that the thyroid hormone nuclear receptor (TR) was a single protein in all species and tissues examined (1). However, since the initial reports of Sap et al. (2) and Weinberger et al. (3) it has become clear that the receptor is the product of at least two genes, the proto-oncogenes c-erbA α and β. Both these genes code for at least two proteins, the products of alternate splicing. One of the products of the c-erbA α gene, TR-α1, has been shown to bind T3, the other, TR-α2, does not, since it is missing a critical portion of the amino-terminal ligand binding region (4–6). In transfection studies, as expected, only the α1 protein can act to regulate gene expression. Both the protein products of the c-erbA β gene, TR-β1 and -β2, can bind thyroid hormone and both have been shown to induce target gene expression in transfection studies. The characterization of these receptors and variants has recently been reviewed (7). Although all three T3-binding receptor proteins have been shown to be trans-acting factors in transfection systems little is known as to their relative contributions to thyroid hormone action in the target tissues of the intact animal.

Initial attempts to determine the role of each of these receptor proteins described the tissue distribution of their respective mRNAs. These reports indicated a wide distribution of the α1, α2, and β1 mRNAs among target tissues (8–10), whereas the β2 mRNA is limited to the pituitary (11). Recently, we reported a study in which we compared the absolute concentration of the mRNAs for the hormone binding species, α1 and β1, to the T3 nuclear binding capacity measured by in vitro saturation analysis in several rat tissues (12). Our data clearly indicated that the T3 binding capacity in any tissue was not a simple function of the concentration of the mRNA. Our results emphasized the likely role of translational and/or posttranslational processes as determinants of the concentrations of the receptor proteins. This conclusion was further strengthened by the findings of Bigler and Eisenman (13) who reported that whereas during the maturation of embryonic chick erythroblasts the concentration of TR-α mRNA fell 19–20-fold, the TR-α protein levels rose 3–5-fold.

An initial approach toward elucidation of the relative contribution of the TR-α and -β receptor proteins to hormone action clearly requires assay of the individual protein levels in the various target tissues of the normal animal. We have for this purpose developed a panel of specific polyclonal antisera to thyroid hormone receptor and determined the percentage of the nuclear binding capacity which could be immunoprecipitated with each. One of these was generated against a unique peptide segment in the amino-terminal domain of the rat β1 protein. A second antiserum was developed against a sequence identical in the α1 and β1 T3-binding rat receptor isoforms that is located in the carboxyl-terminal ligand binding domain. We reasoned that if for a given tissue...
the nuclear binding capacity determined by displacement analysis represented the sum of the \( \alpha_1 \) and \( \beta_1 \) receptors, the antisera generated against this common sequence should immunoprecipitate all the binding capacity. Furthermore, the difference between the total binding capacity and that immunoprecipitated by the anti-\( \beta_1 \) antisera could be assumed to represent \( \alpha_1 \) receptor. The results of these maneuvers have provided the first estimation of the nuclear content of the \( \alpha_1 \) and \( \beta_1 \) receptor in rat liver, adult and fetal brain, kidney, and heart.

The availability of an antisera directed to a peptide sequence in the hinge region of the human \( \beta_1 \) receptor that bears significant homology to the corresponding region of the rat \( \alpha_1 \) and \( \beta_1 \) receptors prompted us to apply it to the immunoprecipitation of rat nuclear \( T_3 \) binding capacity. The results of these studies proved to be of interest in as much as they point to heterogeneity of structure among rat \( \alpha_1 \) receptors.

**MATERIALS AND METHODS**

**Animals**—Adult Sprague-Dawley rats, weighing 200-225 g, and late gestational females were purchased from BioLabs (St. Paul, MN). All rats had free access to food (Purina Chow) and water.

**Receptor Extraction**—Animals were killed under ether anesthesia by exsanguination from the abdominal aorta. Tissues were excised, immediately frozen in liquid nitrogen, and stored at -80°C until needed. Portions of tissue were thawed and homogenized in 0.32 M sucrose, 3 mM MgCl₂. A crude nuclear pellet was prepared by centrifugation at 1000 x g for 10 min. The pellet was resuspended in 2.4 M sucrose, 3 mM MgCl₂ and centrifuged at 50,000 x g for 45 min in an SW-41 rotor. The nuclear pellet was washed by resuspension in 0.14 M NaCl, 3 mM MgCl₂ and centrifuged at 1000 x g for 10 min. The receptor protein was extracted in ice-cold TEM buffer (20 mM Tris-HCl, pH 8.0 (at 22°C), 1 mM dithiothreitol, 5 mM MgCl₂, 2.0 mM EDTA, 10% glycerol) containing 0.4 M NaCl with vortexing for 15 s every 5 min for 45 min (14). All procedures were carried out at 4°C.

**Synthesis of Peptides**—To prepare an antisera specifically targeted against the \( \varepsilon \)-RBA receptor protein an amino acid sequence comprising amino acids 62-92, TWASSIFHLDPDDVNDQSVSS-AQTPQTEKKK, was chosen from the deduced amino acid sequence of the rat \( \beta_1 \) receptor protein reported by Koenig et al. (8). This sequence, in the amino-terminal portion of the protein, is unique to the \( \beta_1 \) receptor form. A second peptide, PTETFPLPLEVFEFD, represents the carboxy-terminal 14 amino acids (amino acids 447-461) in the ligand binding region of the rat \( \beta_1 \) TR (8) and is identical to amino acids 393-407 in the rat \( \alpha_1 \) receptor (10). A third antisera was initially generated against the hinge region of the human TR \( \beta_1 \) receptor (amino acids 186-214, LIQQNRQGRRRGGLQKSIGHKPQPTD-QGW) (3). Since this sequence has a strong homology to the rat \( \alpha_1 \) and \( \beta_1 \) receptors, 45 and 70%, respectively (Fig. 1), our original intent was to generate an antisera which would react with all known receptor species. The positions of each of the peptides within the receptor sequences is shown in Fig. 2. The peptides were synthesized by solid-phase methodology on \( s \)-methylbenzhydrolamine resin as previously described (15). The peptides had the correct amino acid composition by amino acid analysis. 20 mg of each peptide were conjugated to keyhole limpet hemocyanin (Sigma) using 4% gluteraldehyde. Male New Zealand White rabbits were immunized by subcutaneous injection of 100 \( \mu \)g of the conjugate mixed with complete Freund’s adjuvant. Booster injections of 100 \( \mu \)g in incomplete Freund’s adjuvant were given every 2 weeks. Blood was collected for screening for antipeptide activity after 8 weeks and at 2-week inter-

**FIG. 2. Structure of the \( T_3 \) receptor \( \beta_1 \) and \( \alpha_1 \) isoforms.** The positions of the peptide sequences used for raising antisera are indicated. Identity of sequences within the receptors is indicated by same design. Numbers above sequences refer to amino acid position at the domain junctions. Numbers within boxes indicate degree of homology with \( \beta_1 \) protein.

**RESULTS**

**Specificity of Antibodies**—Fig. 3 presents the results of the immunoprecipitation of the \( \alpha_1 \) or \( \beta_1 \) translation products by each of the IgG preparations. For both the \( \alpha_1 \) and \( \beta_1 \) translations the pattern of products is consistent with those previously published (5, 6, 9, 17). In each case there appears a major product with an expected molecular mass in the order

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*Quantitation of Rat Tissue \( T_3 \) Receptor Isoforms*
FIG. 3. Specific immunoprecipitation of translation products by the prepared IgG fractions. Five μg of each IgG preparation was used throughout these studies. A, aliquots of rat TR-β1 translation products (lane 1) incubated with: lane 2, IgG-PIS (preimmune serum); lane 3, IgG-β1; lane 4, IgG-β1 plus 2 μg of peptide 62-92; lane 5, IgG-β1 plus 2 μg of peptide 186-214; lane 6, IgG-ch; lane 7, IgG-ch plus 2 μg of peptide 186-214; lane 8, IgG-ch plus 2 μg of peptide 62-92. B, aliquots of rat TR-α1 translation products (lane 1) incubated with: lane 2, IgG-PIS; lane 3, IgG-β1; lane 4, IgG-β1 plus 2 μg of peptide 62-92; lane 5, IgG-β1 plus 2 μg of peptide 186-214; lane 6, IgG-ch; lane 7, IgG-ch plus 2 μg of peptide 62-92. The precipitation of a lesser fraction of TR-α1 than of TR-β1 (compare Fig. 3A, lane 6 and 3B, lane 6) is attributed to a lower affinity for the TR-α1 since addition of greater amounts of IgG-ch results in equal precipitation of both products. C, rat TR-β1 translation products (lane 1) incubated with: lane 2, IgG-PIS; lane 3, IgG-α1/β; lane 4, IgG α1/β plus 2 μg peptide 447-461; lane 5, IgG-α1/β plus 2 μg peptide 186-214. D, rat TR-α1 translation products (lane 1) incubated with: lane 2, IgG-PIS; lane 3, IgG-α1/β; lane 4, IgG-α1/β plus 2 μg peptide 447-461; lane 5, IgG-α1/β plus 2 μg peptide 186-214.

of 50,000–60,000 daltons as well as several bands with smaller molecular weight. The specificity of each IgG for the relevant product is demonstrated by the immunoprecipitation of only the specific product(s) and the finding that immunoprecipitation is blocked only by the appropriate peptide. Of interest is the finding that, whereas all three IgG preparations immunoprecipitate the α1 or β1 translation products with molecular mass greater than 43,000 daltons, only the IgG-ch and IgG-α1/β also recognize the bands of M, 30,000–40,000. This is consistent with the position of the epitopes within the sequences of the TRs (Fig. 2) and suggests the smaller products result from the in vitro initiation of translation from alternate internal start sites rather than being truncated translation products.

Quantitation of the Nuclear Binding Capacity by Immunoprecipitation with IgG-β1 and IgG-α1/β—These IgG preparations were used to quantitate the contribution of the α1 and β1 receptor isoforms to the total binding capacity in nuclear extracts from adult liver, heart, kidney, cerebrum, and cerebellum and fetal total brain (Tables I and II). Fig. 4 illustrates one of eight studies of nuclear extracts from rat liver. Incubation of receptor extract with preimmune IgG (IgG-PIS) caused no consistent change in the binding capacity in the hepatic or other tissue extracts. However, the binding capacity following incubation with the IgG-β1 was reduced by an average of 78 ± 6% (mean ± S.D.) (Table I). For each hepatic extract studied, incubation with IgG-α1/β decreased the binding capacity more than did IgG-β1. Although in the example displayed in Fig. 4 approximately 15% of the binding capacity remained after incubation with IgG-α1/β, in all other hepatic extracts nearly all binding (97 ± 3%) was immunoprecipitated by this antibody. Since IgG-α1/β immunoprecipitated virtually all nuclear binding capacity, the difference between the total binding capacity and TR-β1 capacity was assumed to represent the contribution of TR-α1. Furthermore, only mRNAs for the α1 and β1 T3-binding TRs are known to be present in liver (11). Thus, the data suggest that the β1 form accounts for about 80% and the α1 isoform the remaining 20% of the total binding activity.

In the extracts from adult cerebrum (Fig. 4, Table I), 28 ± 7% of the total binding could be assigned to the β1 receptor. Identical results (28 ± 4%) were observed with cerebellar extracts (Table I). In extracts from heart and kidney (Fig. 4) 45 ± 4% and 43 ± 7%, respectively, of the total binding could
be cleared with the IgG-β1. Essentially all binding could be cleared by IgG-α1/β from nuclear extracts of these tissues. In all these studies no consistent difference was observed between the affinity of binding of T3 by the total extract and by the residual receptors, presumably consisting of TR-α1, following treatment with IgG-β1 (Fig. 4). Whereas IgG-α1/β can clear fetal brain extracts of all binding (Fig. 5) incubation with the IgG-β1 caused only a very small, 5 ± 3%, reduction in binding capacity (Table I). This implies that the binding protein in the brain at this stage of development consists almost entirely of the α1 species. This would be expected from our previous report (12) that in the fetal brain at least 90% of the mRNA coding for T3-binding TR consists of the α1 mRNA. The finding that the β1 protein is present in the adult brain but not easily detectable in the fetal brain is also consistent with the earlier study (12) in which we demonstrated that the mRNA for the β1-TR rises from very low levels in the fetus approximately 40-fold during the first 2 weeks of life. These data now allow us to estimate the absolute concentration of each receptor isoform in these tissues by multiplying the total binding capacity previously measured (12) by the fraction of β1 receptor activity immunoprecipitated with IgG-β1 (Table II).

**Immunoprecipitation with IgG-ch**—The homology of the IgG generated against the hinge region of the human TR-β receptor (IgG-ch) to rat TR-α and TR-β had led us to expect that this antibody would also immunoprecipitate the binding activity of nuclear extracts due both to α and β receptors in a manner analogous to the behavior of IgG-α1/β. The likelihood of such a result was strengthened by the studies showing that this IgG preparation could specifically interact with the α1 and β1 translation products (Fig. 3). Although IgG-ch immunoprecipitated virtually the same fraction of total activity in hepatic extracts as did IgG-α1/β, IgG-ch immunoprecipitated a substantially lower fraction of receptor activity in other tissues. Thus, whereas IgG-α1/β cleared essentially all binding from the nuclear extracts of adult cerebrum, cerebellum, kidney and heart, and fetal brain, only 40–50% of receptor binding was immunoprecipitated by IgG-ch (Table I).

These findings raised questions as to the nature of the nonprecipitable portion of the receptor proteins. One potential explanation of the incomplete immunoprecipitation of nuclear binding capacity in heart, kidney, and brain by IgG-ch was that insufficient quantities of antisera were used. To test this possibility, we incubated aliquots of cerebral nuclear extract as described with 150 or 600 μg of IgG. Binding capacity was reduced only 45 and 48%, respectively. In a parallel experiment, after clearing cerebral nuclear extracts with 150 μg of IgG-ch as described, we added a second aliquot of the same IgG to the cleared supernatant. The second treatment of the extract did not result in further reduction in binding capacity (42 and 41%, respectively). We could, therefore, conclude from both these experiments that the incomplete immunoprecipitation of binding capacity by IgG-ch did not result from the addition of a submaximal amount of IgG.

A second potential explanation for our results was considered. Bigler and Eisenman (13) have reported that multiple shortened forms of the α receptor arise from alternate initiation sites in the chicken erythroblasts. Such forms were also apparent in the in vitro translations which were immunoprecipitable by IgG-ch and IgG-α1/β (Fig. 3). Several potential alternate start sites exist within the mRNA sequence which could lead to products less than 30,000 daltons lacking the possibility of such a result was strengthened by the studies showing that these IgG preparations could specifically interact with the α1 and β1 translation products (Fig. 3). Although IgG-ch immunoprecipitated virtually the same fraction of total activity in hepatic extracts as did IgG-α1/β, IgG-ch immunoprecipitated a substantially lower fraction of receptor activity in other tissues. Thus, whereas IgG-α1/β cleared essentially all binding from the nuclear extracts of adult cerebrum, cerebellum, kidney and heart, and fetal brain, only 40–50% of receptor binding was immunoprecipitated by IgG-ch (Table I).

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**TABLE I**

| IgG          | Receptor region | Liver | Cerebrum | Cerebellum | Heart | Kidney | Fetal brain |
|--------------|----------------|-------|----------|------------|-------|--------|-------------|
|              |                | %     |          |            |       |        |             |
| Anti-β1      | Amino terminus | 78 ± 6 | 28 ± 7   | 28 ± 4     | 45 ± 4 | 43 ± 7  | 5 ± 3       |
|              |                | (8)   | (6)      | (5)        | (4)   | (4)    | (5)         |
| Anti-α1/β    | Carboxyl terminus | 95 ± 6 | 89-90    | 94-98      | 97-97 | 97-97   | 96-98       |
|              |                | (4)   | (2)      | (2)        | (2)   | (2)    | (2)         |
| Anti-ch      | Hinge region   | 92 ± 6 | 48 ± 5   | 48 ± 5     | 44 ± 4 | 46 ± 5  | 40 ± 8      |
|              |                | (6)   | (6)      | (5)        | (4)   | (4)    | (5)         |
| Unaccounted  |                | 52    | 52       | 56         | 54    | 60     |             |

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![Graphs showing binding capacity precipitated from various tissues](image-url)
Quantitation of Rat Tissue T$_3$ Receptor Isoforms

Our findings, therefore, suggest that the incomplete immunoprecipitation of binding activity from nuclear extracts by IgG-ch may be due to modification or sequestration of a critical epitope in one or more of the TR isoforms. As will be discussed below, a likely mechanism for achieving such heterogeneity is posttranslational modification, such as phosphorylation. Since the T$_3$ binding activity of fetal brain nuclear extracts is due almost exclusively to the TR-a1 isoform, and since IgG-ch only incompletely immunoprecipitates binding activity from these extracts (Fig. 5), we infer that such modification must at the very least involve the a1 receptor.

**DISCUSSION**

These studies demonstrate the usefulness of specific antisera for the measurement of the various isoforms of the thyroid hormone receptor. Together with our previous report (12), these studies represent the first efforts to quantitate the concentrations of the individual T$_3$-binding receptor isoforms. We have previously commented on the marked variation among tissues in the ratio of total nuclear binding capacity and the sum of the mass of mRNA coding for T$_3$ receptors (12). The present findings in which we have directly related specific receptor protein to the coding mRNA further emphasize the importance of translational or posttranslational factors in regulating receptor content in tissues. Whereas the concentration ratios for protein to mRNA for the a1 and b1 isoforms are more or less equal in liver, they differ by 7-fold in kidney. And, consistent with our earlier finding, the protein/mRNA ratio varies by 18-fold for the TR-a1 and 17-fold for the TR-b1 among the tissues analyzed.

In line with previous reports (1, 21), there appear to be no consistent differences in the affinity constant for T$_3$ among the nuclear extracts derived from the diverse tissues studied. Moreover, the immunoprecipitation of b1 receptor by IgG-b1 did not result in a change in the affinity of the residual a1 receptor. These findings are of interest in connection with a previous study from our laboratory in which the in vitro translation products of the TR-b1 isoform showed an affinity 9-fold higher than the TR-a1 product (17). The basis of this difference remains unclear. The multiple protein bands generated in the in vitro translation may have differential effects on the binding of T$_3$ by the receptor. Binding could be differentially affected by receptor milieu in reticulocyte lysate and extraction buffer. Lastly, posttranslational modification of protein structure may alter the affinity of binding.

The use of IgG-ch for differential immunoprecipitation of receptor isoforms raised the strong possibility of further heterogeneity among receptor species. This antibody was directed to the hinge region of the human b receptor which shows homology with both the rat TR b1 and TR a1. We therefore anticipated that this antibody would serve to immunoprecipitate essentially all T$_3$-binding receptor activity similar to the behavior of IgG-a1/b. As expected, rat TR-a1 and b1 translation products were specifically immunoprecipitated by the IgG-ch. Contrary to our expectations, however, IgG-ch immunoprecipitated only approximately one-half of the total binding capacity of brain, kidney, and heart. As detailed under "Results," failure to immunoprecipitate the total binding capacity could not be related to inadequate amounts of antibody.

| Tissue | Binding capacity pmol/mg DNA | mRNA fmol/mgDNA | Protein/mRNA |
|--------|-----------------------------|-----------------|--------------|
| Liver  | 1.01                        | 0.23            | 870          |
| Cerebrum | 0.66                      | 0.49            | 144           |
| Cerebellum | 0.18                     | 0.49            | 144           |
| Kidney | 0.25                        | 0.13            | 250           |
| Heart  | 0.58                        | 0.27            | 482           |
nor to the existence of short T3 binding receptors generated as a result of the use of internal start sites of translation.

We believe that the most likely explanation of the failure of immunoprecipitation of T3 binding activity by IgG-ch is posttranslational modification of T3 receptors in the hinge region which effectively blocks access of the IgG-ch to this epitope. Phosphorylation would appear to be a possible mechanism. There is ample precedence for the phosphorylation of other hormone receptors acting at a nuclear level (22–25). Goldberg et al. (26) and Glineur et al. (27) have, in fact, reported phosphorylation of two sites in the chicken TR-α1, identified as serine residues in the amino-terminal portion of the molecule. Moreover, potential sites of phosphorylation do exist within the hinge sequence of the TR-α1 at the serine and threonine positions (Fig. 1). The serine (Ser-153), with its neighboring arginine and leucine residues as well as the triplet of arginines in positions 144–146, is a strong candidate for phosphorylation (28).

Our analysis has assumed that IgG-β1 immunoprecipitates all TR-β1 and that the difference between the fraction precipitated by IgG-α1/β and IgG-β1 is due to the α1 receptor. Although there are no apparent consensus sites for posttranslational modification in the sequence 62–92 of TR-P1 (Fig. 1), we cannot rule out the possibility that modifications elsewhere in the molecule may act to limit access of the IgG-β1 to this epitope. However, immunoprecipitation of the overwhelming proportion of receptor activity in hepatic extracts by IgG-β1 supports our assumption.

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