Isolation and Characterization of Rat cDNA Clones for Two Distinct Thyroid Hormone Receptors*

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Two distinct v-erbA-related cDNA clones representing the products of different genes were isolated from a rat liver cDNA library. The first, rc-erbA-α, was 82% identical to v-erbA and encoded a polypeptide with a calculated molecular mass of 45,000 daltons. This cDNA clone arises from the same gene product as a v-erbA-related cDNA isolated from rat brain by Thompson et al. (Thompson, C. C., Weinberger, C., Lebo, R., and Evans, R. (1987) Science 237, 1610–1614). The second cDNA clone, rc-erbA-β, was 76% identical to v-erbA and encoded a polypeptide with a calculated molecular mass of 52,000 daltons. Both rc-erbA-α and rc-erbA-β translational products bound 3,5,3'-triiodo-L-thyronine (T3) localized in the nuclear compartment of target tissues were first described by Oppenheimer and colleagues (1) in 1972 based on in vivo displacement techniques. A reasonable body of evidence now exists which supports this nuclear T3 binding activity on tissues and may thus represent the “classical” nuclear thyroid hormone receptor, whereas rc-erbA-α may encode an isoreceptor species with differing functional properties.

High affinity, limited capacity binding sites for 3,5,3'-triiodo-L-thyronine (T3) localized in the nuclear compartment of target tissues were first described by Oppenheimer and colleagues (1) in 1972 based on in vivo displacement techniques. A reasonable body of evidence now exists which supports this nuclear T3 binding activity on tissues and may thus represent the “classical” nuclear thyroid hormone receptor, whereas rc-erbA-α may encode an isoreceptor species with differing functional properties.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J03333.

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The abbreviations used are: T3, 3,5,3'-triiodo-L-thyronine; bp, base pairs; SDS, sodium dodecyl sulfate.
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EXPERIMENTAL PROCEDURES

cDNA Cloning and Sequencing—A rat liver cDNA library was constructed in the Agt11 vector from total hepatic poly(A)-containing RNA of a normal male adult Sprague-Dawley rat (23). First strand cDNA synthesis was carried out in two separate reactions using oligo(dT) and a random mixture of octanucleotides as primers for reverse transcriptase. Equal portions of the two reactions were mixed and used for second strand synthesis by the method of Gubler and Hoffman (24). Following methylation of internal EcoRI sites and addition of EcoRI linkers, double-stranded cDNA was size-selected on a Sephacryl CL-4B column to enrich for molecules greater than 1000 bp in length. A library of 1.3 X 10^6 independent recombinant plasmid phages was obtained.

The cDNA library was initially screened with a DNA fragment corresponding to nucleotides 277-1140 of the chicken v-erbA oncogene (numbering according to Deloubie et al. (25)). This fragment was radiolabeled by the method of Feinberg and Vogelstein (26) and used to screen 1 X 10^6 recombinant phage. Hybridization was performed in 50% formamide, 0.76 M NaCl at 42 °C and washing at 50 °C in 0.015 M NaCl, 0.1% SDS. A single positive plasmid was isolated with an insert size of 1290 bp. This fragment was subcloned into the EcoRI site of pTZ18R (27) and used for rescanning 5 X 10^6 additional plaques. A second positive plasmid was purified which carried an insert of approximately 2300 bp. This fragment was likewise subcloned into pTZ18R and designated rc-erbA-a. The library was rescanned with this inserts were excised by digestion with exonuclease III (29).

Translation of RNA was performed in the micrococcal nuclease-technique Inc.) was added to 0.5 mM to yield capped RNA molecules for protein labeling experiments. DNA sequencing was performed by the dideoxy chain termination method of Sanger (28) using [35S]dATP. For rc-erbA-β, a series of deletion clones from either end was generated by unidirectional digestion with exonuclease III as described by Henikoff (29). All portions of the sequence were determined independently in both directions from overlapping clones except the first 128 bases of the 5'-untranslated region, which was sequenced only in one direction.

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RESULTS

Isolation of Two erbA-related Rat Liver cDNA Clones—Liver is a major target organ for thyroid hormone, containing approximately 4500 receptors/cell in the rat (38). In addition, the expression of several genes in liver, such as S4 and malic enzyme, are known to be responsive to circulating thyroid hormone levels (for review, see Ref. 39). We therefore decided to screen a rat liver cDNA library for cDNA clones which cross-hybridized with erbA sequences. Using chicken v-erbA DNA and a human placental c-erbA cDNA as probes, we have isolated two distinct cDNA clones representing the products of different genes (Fig. 1A). The first was a cDNA with an insert size of 2300 bp which was about 82% identical to the chicken v-erbA oncogene at the nucleotide level. The restriction enzyme map of this cDNA was identical to that recently reported by Thompson et al. (40) for a rat erbA-homologous cDNA isolated from brain. Sequencing of three segments of this clone, representing 5'-flanking, coding, and 3'-flanking regions of the mRNA, revealed that it was identical to the sequence of the brain cDNA (data not shown). Thus, we conclude that this cDNA arises from the same gene product as the rat brain cDNA and have designated this clone, rc-erbA-α, consistent with the nomenclature suggested by Weinberger et al. (14).

The second rat liver cDNA clone had an insert size of 2450 bp and a restriction map which was distinct from rc-erbA-α (Fig. 1A). To demonstrate that this cDNA arose from a different gene, the two cDNA clones were used as probes for Southern hybridization of rat genomic DNA cleaved with a variety of restriction enzymes (Fig. 1B). The probes used in this experiment were generated from the 3'-portion of the respective cDNA clones in order to avoid the highly conserved DNA-binding motif. The pattern of hybridizing bands observed under these moderate stringency conditions was unique for each probe. For several restriction enzymes, only a single band was detected with either probe. Thus, the two erbA-related cDNA clones arise from two different genes in rat.

The nucleotide sequence of the second cDNA clone was determined (Fig. 2). This cDNA clone was only about 76% identical at the nucleotide level to the chicken v-erbA, a value similar to that reported for a human placental c-erbA isolated by Weinberger et al. (14) and designated as the β class. Thus, we designated this clone as rc-erbA-β. The nucleotide sequence contained a single long open reading frame. Two possible initiation codons are present at nucleotides 124 and 136. Based on analogy to the human placental cDNA in which only the initiation codon at position 136 is found and the somewhat weaker "context" of the AUG codon at position
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A

rc-erbA-α

rc-erbA-β

S

Pv X P

Pv X P

Pu X p

and from residues 411 to the C-terminal end of β, the two polypeptides are identical for 46 consecutive residues. In the 68-amino acid, cysteine-rich domain of c-erbA which is most related to the DNA-binding domains of the steroid hormone receptors, the two sequences are common at 59 out of 68 residues (Fig. 3B). The differences are clustered in the second half of the domain at positions which show a high degree of variability between different classes of steroid hormone receptors.

Thyroid Hormone Binding Activity of c-erbA Polypeptides—In order to confirm that both erbA-related cDNA species encode polypeptides capable of binding to T₃, hormone binding experiments were carried out. The two cDNA inserts were subcloned into the pTZ18R vector in proper orientation behind the T7 RNA polymerase promoter sequence. The encoded polypeptides were then expressed by in vitro transcription followed by translation in a rabbit reticulocyte lysate. To achieve significant levels of expression of the rc-erbA-α cDNA, it was necessary to remove a large segment of the 5'-untranslated region. Following this deletion, a major polypeptide product with an estimated molecular mass of 50 kDa was obtained (Fig. 4). The rc-erbA-β cDNA was expressed without removal of its 5'-untranslated region and resulted in a major polypeptide product of 57 kDa. In both cases, a number of lower molecular weight bands of lesser intensity could be seen. These products could result from proteolytic cleavage of the initial product, initiation at internal AUG codons or incomplete translation. The difference in size observed for the major translational products of the α and β cDNA clones is consistent with that predicted from the nucleotide sequences (i.e. 45,000 daltons for predicted α polypeptide versus 52,000 daltons for β).

Both α and β c-erbA clones encode polypeptides which bind T₃ with high affinity. The dissociation constants estimated for T₃ binding were 0.4 and 0.49 nM, respectively (Fig. 5). These values are in reasonable agreement with that reported for the nuclear T₃ receptor extracted from rat liver (43). Competition for binding of [³²P]T₃ to the c-erbA translational products by several thyroid hormone analogs was also very similar (Fig. 6). For both forms, competition with l-thyroxine required about eight times as much hormone as that observed for T₃, whereas 3,3',5'-triiodothyronine was less than 100 times as effective. 3,5,3'-triiodothyroacetic acid, an analog which binds with a 1.6-fold higher affinity to the extracted liver receptor than T₃ (2), was slightly more effective as a competitor than T₃ itself. Again, both α- and β-derived polypeptides were indistinguishable with respect to their 3,5,3'-triiodothyroacetic acid binding properties. Thus, the hormone binding properties of the two erbA-related polypeptides appear to be highly conserved.

Distribution of α and β c-erbA Transcripts—Major target organs for thyroid hormone in the adult rat, such as anterior pituitary, liver, kidney, heart, and brain, contain between 2000 and 6000 nuclear T₃ binding sites/cell (38). On the other hand, spleen and testis, two tissues generally considered as nonresponsive to T₃, have few, if any, nuclear T₃ binding sites. We compared the relative amounts of transcripts corresponding to the two forms of rc-erbA in these tissues. Poly(A)-containing RNA was extracted from each of the tissues and analyzed by electrophoresis in denaturing agarose gels. After transfer, filters were hybridized under conditions in which the two rc-erbA cDNA clones do not cross-hybridize (Fig. 7). In all tissues, three distinct RNA species with sizes of approximately 2600, 5500, and 6600 nucleotides hybridized to the rc-erbA-α probe. Under the stringency conditions used, the rc-erbA-α probe only hybridizes to a single gene in the rat

B

PROBE: rc-erbA-α rc-erbA-β

Fig. 1. rc-erbA-α and rc-erbA-β cDNA clones are derived from different genes in the rat genome. A, restriction maps of rc-erbA-α and rc-erbA-β cDNA clones. Restriction enzymes used were: B, BamHI; H, HincII; P, PstI; Pv, PvuII; S, SmaI; X, XbaI. Restriction enzymes which did not digest either cDNA were EcoRI, HindIII, SalI, and SphI. B, restriction enzyme analysis of genomic DNA. Rat genomic DNA (10 μg) was digested with BamHI (lane 1), EcoRI (lane 2), HincII (lane 3), HindIII (lane 4), SstI (lane 5), or a combination of BamHI and EcoRI (lane 6), and products were separated on a 1% agarose gel. Fragments were transferred to Zeta-Probe membranes and hybridized to 1 x 10⁶ cpm/ml of ³²P-labeled rc-erbA-α probe (left) or rc-erbA-β probe (right). The rc-erbA-α probe used was a fragment from the HindII site to the 3’-end of the cDNA, and the rc-erbA-β probe was a fragment from the PvuII site to the 3’-end of the cDNA. Filters were washed at 60 °C in 0.015 M NaCl, 0.1% SDS and subjected to autoradiography for 3 days.

124 (41, 42), we have designated the initiation codon at 136. This suggestion will need to be confirmed in future studies. The open reading frame using this AUG encodes a polypeptide of 456 amino acid residues and a calculated molecular mass of 52,100 daltons. It is preceded by a 5’ untranslated region. Following this deletion, a major polypeptide product with an estimated molecular mass of 50 kDa was obtained (Fig. 4). The rc-erbA-β cDNA was expressed without removal of its 5’-untranslated region and resulted in a major polypeptide product of 57 kDa. In both cases, a number of lower molecular weight bands of lesser intensity could be seen. These products could result from proteolytic cleavage of the initial product, initiation at internal AUG codons or incomplete translation. The difference in size observed for the major translational products of the α and β cDNA clones is consistent with that predicted from the nucleotide sequences (i.e. 45,000 daltons for predicted α polypeptide versus 52,000 daltons for β).

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The complete nucleotide sequence of the cDNA clone rc-erbA-β is shown. Numbers to the right refer to the nucleotide locations with the putative initiation codon designated as +1. The predicted amino acid sequence of the largest open reading frame is indicated underlined. The position of all upstream AUG codons is indicated by ***. The putative DNA binding region of the polypeptide is shown.

**FIG. 2. Nucleotide and predicted amino acid sequence of rc-erbA-β.** The complete nucleotide sequence of the cDNA clone rc-erbA-β is shown. Numbers to the right refer to the nucleotide locations with the putative initiation codon designated as +1. The predicted amino acid sequence of the largest open reading frame is shown with numbers below indicating amino acid location. The position of all upstream AUG codons is underlined and the putative DNA binding region of the polypeptide is indicated by the arrows.
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Fig. 3. Comparison of predicted amino acid sequences encoded by rc-erbA-a and rc-erbA-β. A, the amino acid sequences of the predicted polypeptides have been schematically drawn from the N terminus to the C terminus and aligned to yield maximal sequence identity. The percentage of identical amino acid residues in the N terminus to the amino acid positions of the respective polypeptide chain. DNA indicates putative DNA binding region. B, the amino acid sequences in the putative DNA binding domain of c-erbA polypeptides are aligned. Amino acid differences between each pair are indicated by a colon or asterisk. hc-erbA-β, human placental c-erbA of Weinberger et al. (14); rc-erbA-β, rat liver c-erbA from Fig. 2; rc-erbA-α, rat brain c-erbA from Thompson et al. (40); hc-erbA-α, human testis c-erbA from Benbrook and Pfaller (44); cc-erbA-α, chicken embryonic c-erbA from Sap et al. (13).

Hybridization of RNA from various tissues to the rc-erbA-β probe revealed a distinct pattern. A single RNA species with a size of approximately 6500 nucleotides was detected. The relative concentration of this species was high in pituitary, liver, kidney, heart, and brain, all tissues which contain relatively high levels of nuclear T₃ binding sites. On the other hand, transcripts capable of hybridizing to rc-erbA-β were undetectable in spleen and testis, even on longer exposures of this filter. Thus, this form of erbA-related transcript showed a reasonable correspondence to the previously reported nuclear T₃ binding sites in these various tissues.

We have also analyzed the relative levels of erbA transcripts in several cell lines which contain different levels of nuclear T₃ binding sites. For this purpose, we used the mouse 3T3-L1 preadipocyte cell line, which possesses approximately 1200 nuclear T₃ binding sites/cell, and a series of rat hepatoma cell lines containing varying numbers of sites. These hepatoma cell lines included a clonal descendant of the cell line FAO, which had about 350 nuclear T₃ sites/cell, and three additional cell lines formed by somatic cell fusion of the FA0 hepatoma cell line with mouse fibroblasts. These latter cell lines contained 7500, 24000, and 70000 sites/cell. RNA was extracted from these cell lines and hybridized to the rc-erbA-α probe (Fig. 8). Again, three transcripts were detected, but the relative abundance of these transcripts was inconsistent with results from T₃ binding experiments. The 3T3-L1 cells contained a relatively low number of T₃ receptors and the highest level of RNA, whereas the fusion cell with the highest content of T₃ binding sites contained one of the lowest levels of α transcripts. No consistent pattern of hybridization and T₃ binding sites could be discerned.

By contrast, a reasonably good correlation between levels of T₃ binding sites and RNA capable of hybridizing to rc-
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Fig. 6. Competition of thyroid hormone analogs for binding of [125I]T3 to rc-erbA-α and rc-erbA-β translational products. Varying concentrations of each indicated thyroid hormone analog were incubated together with 1 nM [125I]T3 and either rc-erbA-α translational product (A) or rc-erbA-β translational product (B) for 18 h at 4°C. The amount of [125I]T3 bound was determined by the nitrocellulose filter binding assay and normalized to the binding observed in the absence of added analog. TRIAC, 3,5,3′-triiodothyronic acid; T4, l-thyroxine; rT3, 3,3′,5′-triiodo-l-thyronine.

Fig. 7. Tissue distribution of rc-erbA-α and β transcripts. Poly(A)+ RNA (25 μg) isolated from brain (B), pituitary (P), liver (L), kidney (K), heart (H), spleen (S), and testis (T) of adult male rats was loaded onto a 1.5% agarose gel containing 2.2 M formaldehyde. Hybridization was carried out using rc-erbA-α or β cDNA inserts as described under “Experimental Procedures” using 2 × 10⁶ cpm/ml. Autoradiography was carried out for 15 h.

Fig. 8. Distribution of α and β transcripts in various cell lines. Total cellular RNA (25 μg) was isolated from the following cell lines: lane 1, 3T3-L1 preadipocyte cells prior to differentiation; lane 2, 3T3-L1 cells following differentiation; lane 3, FAO hepatoma cells; lane 4, NZ-2 hepatocyte-hepatoma fusion cells; lane 5, NZ-21 fusion cells; lane 6, NZ-12 fusion cells. The latter cell lines have 750, 2400, and 7000 nuclear T3 receptors/cell, respectively. Autoradiograms were exposed for 9 h for rc-erbA-α probe and 46 h for rc-erbA-β.

erbA-β was found in the rat hepatoma cell lines. In this case, transcripts homologous to the β probe were found at increasing levels in each of the hepatoma cell lines with successively higher contents of T3 binding sites. However, 3T3-L1 cells contained no detectable level of β transcript and yet displayed 1200 sites/cell. We surmise that these sites must be formed from the α transcripts. Note that in this case the autoradiogram for the α probe was exposed for only one-fifth the time of that for the β probe, despite the fact that the probes were labeled to identical specific activities. It thus appears that the α transcripts are far less effectively used to form sites detectable in the nuclear T3 binding assay than the β transcript.

DISCUSSION

We have isolated two different v-erbA-related cDNA clones from rat liver which encode thyroid hormone binding proteins. Comparison of the predicted amino acid sequences of the α and β forms of c-erbA between different species reveals that these two classes of potential thyroid hormone receptors have been conserved evolutionarily. The rat c-erbA-α is more closely related to the chicken embryonic cDNA reported by Weinberger et al. (14) than to the human c-erbA-β. Likewise, the rat c-erbA-β is more closely related to the human placental cDNA reported by Benbrook and Pfahl (44) than it is to the rat c-erbA-α. This trend is particularly noticeable in the DNA binding domains of the various forms of c-erbA (Fig. 3B). These data imply that the duplication event that gave rise to the α and β gene families occurred prior to the evolutionary separation leading to present day avian and mammalian species. We have not been able to detect further v-erbA-related genes in the rat genome by Southern hybridization at low stringency with any of the clones. However, in humans, there are at least two additional erbA-related genes (14, 21, 22). Whether these additional genes form functional polypeptides has not been determined.

Comparison of the amino acid sequences between α and β polypeptides reveals two regions in the C-terminal half which are particularly highly conserved. These regions are nearly identical in c-erbA polypeptides from chicken and human. Thus, the sequences in these regions have remained identical for a reasonably long period of evolutionary time.
Based on the regions of the steroid hormone receptors which have been shown to be involved in hormone binding, it is likely that these two regions may form essential elements in the thyroid hormone binding domain. Alterations in the gag-v-erbA hybrid polypeptide (relative to c-erbA) which result in the thyroid hormone binding domain. Thus, it is likely that both polypeptides function in vivo as thyroid hormone binding proteins. Based on these binding properties, it is reasonable to suggest that both proteins may be receptors for thyroid hormone. Direct verification of this hypothesis awaits further experiments for both forms of c-erbA.

The levels of rc-erbA-β mRNA in various tissues and cell lines correlate reasonably well with measurements of nuclear T3 binding sites. A large amount of correlative data suggests that the nuclear T3 binding sites in liver and pituitary tumor cells are true receptors for thyroid hormone (for review see Ref. 2). This leads us to hypothesize that rc-erbA-β represents the classical T3 receptor, that is, the receptor whose activity is measured in nuclear T3 binding assays and has been correlated with bioactivity. If rc-erbA-β encodes the classical T3 receptor, then what does rc-erbA-α encode? One possibility is that rc-erbA-α encodes a second form of nuclear T3 receptor. However, there is little or no correlation between α mRNA levels and nuclear T3 binding activity. There are several possible explanations which could account for this observation. The c-erbA-α polypeptide may have a short half-life in vivo and thus not accumulate to levels consistent with its mRNA content. The α mRNA may be inefficiently translated in vivo under normal circumstances and require certain physiological conditions for effective utilization. Finally, it is possible that only a fraction of the α transcripts may encode full-length receptor. c-erbA-α cDNA clones have been isolated from rat brain which lack T3 binding activity, but whose sizes are indistinguishable from full length mRNA by Northern analysis. Sequence analysis of these clones revealed that they differed by small amino acid substitutions in the hormone binding domain. Thus, it is possible that a sizable fraction of the rc-erbA-α encoded polypeptides do not correspond to thyroid hormone hormone-binding forms. The possible physiological roles of these alternate forms is not clear.

If a portion of the rc-erbA-α gene product encodes a second nuclear T3 receptor, then what are the functions of the α and β forms? The amino acid differences in the DNA binding domains of the two receptors may indicate that each recognizes a different set of target genes. This region has been postulated to form two zinc binding “fingers” involved in interaction with receptor with DNA. Comparison of the differences between α and β polypeptides in this region indicates that the first putative zinc finger is completely conserved. All of the nine amino acid changes between the two forms occur in the second finger or the region between the two fingers. If these differences are sufficient to alter the DNA binding specificity of the receptors, the two forms may be responsible for regulating the expression of different sets of genes in a tissue-specific or developmentally controlled pattern. On the other hand, the two c-erbA polypeptides may interact with the same DNA sequences, but have somewhat different properties relative to the activation process. For example, the two forms may interact differently with transcriptional factors in activating or inhibiting gene expression. Distinguishing between these possibilities will require further work on the DNA binding properties of the various forms of c-erbA and the functional significance of this interaction.

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