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IN VIVO EXPRESSION OF RAT LIVER c-*erbA* β THYROID HORMONE RECEPTOR IN YEAST (*Saccharomyces cerevisiae*)

Chao LU, Yong-Fan YANG, Hiroyuki OHASHI, and Paul G. WALFISH*

Thyroid Research Laboratory, Samuel Lunenfeld Research Institute of Mt. Sinai Hospital, University of Toronto, 600 University Avenue, Toronto Ontario, Canada M5G 1X5

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SUMMARY: To study thyroid hormone receptor (TR), we developed an *in vivo* expression system in yeast by using a copper-responsive yeast metallothionein promoter and ubiquitin-fusion protein technology. The cDNA encoding full-length rat liver TRβ was expressed under the control of copper. The [^{125}I]T3 binding activities to yeast extracts were significantly correlated with the added copper sulfate into the medium. Partially purified TR from the transformed yeast had a high hormone binding affinity (Kd=0.34) for T3 and could bind thyroid hormone response element in gel retardation analysis.

L-triiodothyronine (T3) has a profound effect on the growth, development and metabolism *via* the interaction with its nuclear receptor (TR) (1). The cDNA sequences of several isoforms of TR have been recently isolated and characterized to be the proto-oncogene c-*erbA*, which showed that TR is one of the members in steroid superfamily (2-4). Like other members in the superfamily, TR is also a *trans*-activating factor, and can bind to some specific DNA sequences, thyroid hormone response element (TRE), to regulate target gene expression (4). The yeast *S. cerevisiae* has recently been used as a model system for study of eukaryotic biology (5) and the development of yeast genetics has provided us with a successful and powerful tool to study the characteristics of the steroid superfamily of related genes in eukaryotes. By using ubiquitin-fusion protein technology, in which fusion protein can be cleaved automatically by endogenous enzymes (6), vitamin D and progesterone receptors have been successfully expressed and studied in yeast (7,8). To have a suitable model for the study of TR, we employed the similar technology to express in yeast rat liver TRβ which had high hormone and DNA binding activities.

* To whom reprint requests should be addressed.

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MATERIALS AND METHODS

Materials: rat c-*erbA* β cDNA (9) in pTZ19R vector was kindly from Dr. H.C. Towle (MN, USA). A polylinker plasmid Pnmhub-poly5253, the ubiquitin expression plasmid YEp46 (6) and yeast *S. cerevisiae* strain F762 (6) were kindly from Dr. T.R. Butt (SmithKline & French Lab., PA, USA). Restriction enzymes, Nuclease S1, Nuclease *Bal 31*, T4 DNA ligase and *Hind III* and *Nco I* linkers were purchased from Promega Biotec and Boehringer Mannheim Biochemicals. T4 polynucleotide kinase and poly(dI-dC) were purchase from Pharmacia. [^{125}I]T3 (2200 Ci/mmol) was purchased from Du Pont-New England Nuclear. Filter membrane (HAWP02500) was purchased from Millipore Co. (MA, USA). Glass beads (425-600 microns), uracil and Phenylmethyl sulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co.

Construction of Expression Vector: rc-*erbA* β plasmid was cleaved by *Hind III* and then digested by *Bal 31* to position -14 (start from the putative initiation codon (9)) to cut the stop codon at -21, followed by ligation with *Hind III* linker and T4 DNA ligase. Sequence was confirmed by DNA sequencing. After digestion, the *Hind III-Eco RI* fragment was inserted into the same sites of Pnmhub-poly5253 to have an in-frame fusion of ubiquitin to rc-*erbA* β protein, called Pnmhub TR-β. After the conversion of *Kpn I* site to *Nco I* site in YEp46 plasmid, the *Afl II-Nco I* fragment of Pnmhub TR-β was excised and subcloned into the corresponding sites of YEp46. The resulting plasmid containing the rat c-*erbA* β cDNA encoding full-length TRβ was referred to as YEp TR-β (Fig. 1). This plasmid was amplified, purified and used to transform *S. cerevisiae* (10,11), and transformants were selected by tryptophan auxotrophy.

Production and Partial Purification of Rat c-*erbA* β TR: After grown at 30°C overnight in a medium containing 0.67% yeast nitrogen base, 2% glucose and 0.0025% uracil, yeast cells transformed by YEp TR-β expression plasmid were diluted and then grown to an optical density at 600nm of 0.5. Induction was initiated by the addition of copper sulfate (final 100 μM) into the medium (7). After grown for another 2 hours, cells were harvested by centrifugation and washed twice with ice-cold PBS. The following was conducted at 4°C unless otherwise stated. The pellet was suspended in 20 mM HEPES buffer containing 1mM $MgCl_2$, 5mM EDTA, 300mM KCl, 1% Triton X-100, 10% glycerol, pH 7.8 and freshly added PMSF (0.5mM) and vortexed with glass beads three times for 1 min. After centrifugation, the pellet was resuspended, vortexed and centrifuged. This step was repeated twice. Yeast extract from the pooled supernatant was loaded onto Sephadex G-100 column (2.5x92 cm). Fractions from Sephadex G-100 chromatography in 20mM HEPES buffer containing 1mM $MgCl_2$, 2mM EDTA, 50 mM NaCl, 10% glycerol and 0.1% Triton X-100, pH 8.0 were collected. Fractions with high T3 binding activities were pooled and loaded onto DEAE-Sephacel column (1.5x9.0 cm) in the same buffer. Gradient elution

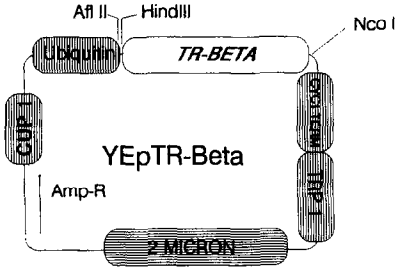


FIG.1: Yeast Expression Vector, YEp TR-β. The cDNA encoding full-length rat liver c-*erbA* β protein was inserted so as to produce a fusion protein with ubiquitin. *CUP 1* is the yeast metallothionein promoter. *TRP 1* is the tryptophan selective marker. TR-β is rat c-*erbA* β.

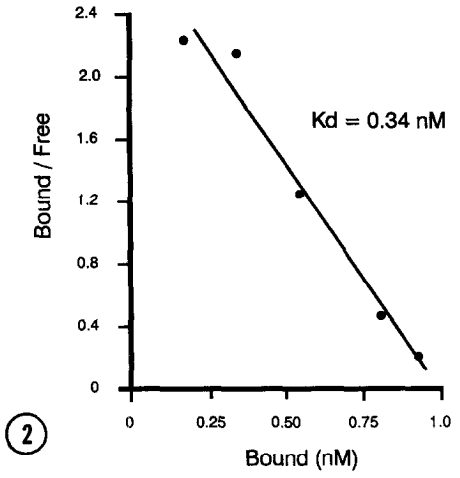
of 50 to 500 mM KCl in the buffer was begun after the column was washed in 50 ml of the loading buffer. Fractions with high T3 binding activities were aliquoted and stored at -70°C. Protein concentration was measured by Lowry's method (12) using bovine serum albumin as standard.

Hormone Binding Assays: [^{125}I]T3 binding assay was performed in 20 mM HEPES buffer containing 1mM $MgCl_2$, 50 mM NaCl, 200 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, 10% glycerol, pH 7.8 and 0.1mM PMSF. To test T3 binding activity of yeast extract and fractions from chromatography, yeast extracts were incubated at 22°C for 90 min followed by separation of free and bound [^{125}I]T3 using Dowex resin 1-X8, 200-400 mesh, Cl^-. In the Scatchard analysis, partially purified TRβ protein was incubated with 7.5-150 fmol [^{125}I]T3 at 4°C for 18 hours in a total volume of 30 μl followed by separation of free and bound [^{125}I]T3 using filter membrane method (13). Non-specific binding was determined in the presence of 1μM unlabelled T3 (Sigma) and subtracted from total binding to calculate the specific binding of [^{125}I]T3 (14).

Gel Retardation DNA Binding Assay: A synthesized palindromic oligonucleotide of TRE (5'-AATTCTCAGGTCA.TGACCTGAG-3') (15) was annealed and end-labelled with ^{32}P and T4 polynucleotide kinase. TRβ was incubated with ^{32}P-oligonucleotide and poly(dI-dC) (200 μg/ml) in 25mM phosphate buffer, pH 7.6 containing 1mM $MgCl_2$, 40mM NaCl and 5% glycerol at 22°C for 20 min. Electrophoresis was performed in 6% polyacrylamide gel in a buffer containing 6.7 mM Tris, 1 mM EDTA and 3.3 mM sodium acetate (16).

RESULTS AND DISCUSSION

In the high copy number expression vector YEp TRβ, the yeast metallothionein *CUP 1* promoter is regulated by copper ions (7). The ubiquitin-fusion protein method was shown to result in a higher yield of the expressed protein and the fusion protein could be cleaved by yeast endogenous enzymes (6-8,17). To assess the production of TRβ, copper sulfate was added into the media to final 0, 10, 50 and 100 μM. Results of [^{125}I]T3 binding to the yeast extract showed that there was a basal level of TR expression in noninduced yeast cells (0 μM $CuSO_4$), and a significant linear correlation between the concentrations of copper induction and specific [^{125}I]T3 binding activities. However, there was no specific binding of [^{125}I]T3 in the control transfected by YEp46 plasmid (data not shown). The noninduced expression was recently observed in such system of a truncated chicken oviduct progesterone receptor, but not the full length progesterone receptor, nor vitamin D receptor (7,8). For further studies of TRβ, partial purification was performed. The [^{125}I]T3 binding peak was obtained at 220-270 mM of KCl gradient in DEAE-Sephacel column. By using the pooled fractions of TRβ from DEAE-Sephacel chromatography, Scatchard analysis revealed a high binding affinity for [^{125}I]T3 to TRβ (Kd=0.34nM, Fig.2), which was higher than that obtained by using *in vitro* translational product with rabbit reticulocyte lysate (Kd=0.49nM) (9) and compatible to that using highly purified human TRβ from *in vivo* expression in *E. coli* (Kd=0.36 nM) (18), indicating a normal hormone binding activity of the TR expressed in yeast. The [^{125}I]T3 binding capacity was 6.0-8.0 pmol per mg protein after DEAE-Sephacel chromatography. In our previous study, we have observed a differential effect of zinc chloride on [^{125}I]T3 binding to rat c-*erbA* α and β proteins obtained by *in vitro*



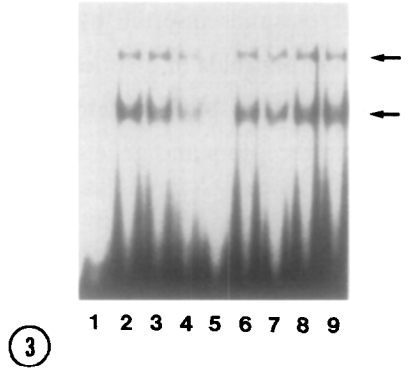


FIG.2: Scatchard Analysis of [^{125}I]T3 Binding to Rat TRβ Obtained by *in vivo* Expression in Yeast. Rat liver TRβ (5.0μg protein) was incubated with 7.5-150 fmol of [^{125}I]T3 in the presence or absence of 1.0μM unlabelled T3 at 4°C for 18 hours, followed by separation of free and bound [^{125}I]T3. Experiment was conducted by triplicate determinations.

FIG. 3: DNA Binding by Rat c-*erbA* β Protein. ^{32}P-labelled TRE oligonucleotide (≈0.5 ng, 28,000 cpm) was incubated without (lane 1) or with the rat c-*erbA* β protein in the presence of 0, 10, 50 and 250 ng of unlabelled TRE (lane 2, 3, 4, and 5, respectively) or in the presence of 0, 50, 250 and 500 ng of linearized pUC 19 plasmid DNA (lane 6, 7, 8, and 9, respectively) as described in *Materials and Methods*.

transcription and translation (14). By using the TRβ synthesized in yeast, an identical and inhibitory effect of $ZnCl_2$ on T3 binding to rat TRβ was found with approximately 50μM of $ZnCl_2$ causing 50% inhibition of T3 binding (data not shown).

In comparison to other steroid hormone receptors, the binding of TR to the TRE *in vitro* does not require the induction of ligand binding (18). In rat growth hormone (rGH) gene, multiple site of TRE have been identified (19,20). A synthetic variant of rGH TRE consisting of a palindromic motif was shown to have a higher binding affinity and transcriptional activation for TR (15). Assessed by gel retardation analysis, the *in vivo* expressed rat TRβ could specifically bind TRE of rGH promoter (*BagII-BamHI* fragment, -237-+11, start from CAP site, (21)) (data not shown). As shown in Fig. 3, the binding of ^{32}P-labelled palindromic TRE oligonucleotide to rat TRβ could be displaced by the unlabelled TRE, but not by the linearized nonspecific pUC 18 plasmid DNA. The amount of competitive oligonucleotide required for complete inhibition (lane 2-5, Fig.3) was higher than expected, which was likely because of the low efficiency in annealing of the single-stranded oligonucleotide.

In summary, we have utilized the advantage of ubiquitin-fusion protein technology to express rat liver TR *in vivo* in yeast. This system may provide a very useful model system for the study of these *trans*-activating factors. The expression of chicken progesterone

receptor (8), vitamin D receptor (7), glucocorticoid receptor (22) and human estrogen receptor (23) in yeast has been recently reported. Studies are in progress to cotransfect the reporter plasmids inserted by different TRE fragments into a pC2 vector (7) with the expression plasmids of a series of receptor derivatives bearing a deletion of the hormone binding domain. Such system could assist in enhancing our understanding of thyroid hormone receptors and gene regulation.

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