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c-erb-A mRNA content and triiodothyronine nuclear receptor binding capacity in rat liver according to vitamin A status

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Vitamin A is required for normal growth and development and synergistically acts with thyroid hormones in these processes. Effects of retinol dietary intake (0, 5, 50 U retinol/g food) on the triiodothyronine (T3) nuclear receptor were examined in rat liver. Properties of this receptor have been investigated by an in vitro binding method. The amount of c-erb-A mRNA has been deduced by reverse transcription and amplification methods (PCR). Results show that both retinol deficiency and excess leads to a reduced amount of c-erb-A mRNA and to a decreased T3 binding capacity, suggesting that retinol, or retinoic acid which is its natural metabolite, contributes to the regulation of the T3 nuclear receptor properties.

Retinoid; Vitamin A; Thyroid hormone; c-erb-A; T3-nuclear receptor

1. INTRODUCTION

New roles are emerging for vitamin A (retinol) and for its natural metabolite: retinoic acid. Recent studies have shown that the mechanism of action of retinoic acid was closely similar to that of the steroid and thyroid hormones, involving the activation of the expression of specific genes, thus placing retinoids in the category of hormones regulating growth, differentiation and embryonic development [1–5]. Structural homologies among the receptors of these factors had led to consider them as belonging to a 'superfamily' of DNA binding proteins [3,6]. Relationships have been observed between members of this 'superfamily'. For example, retinoic acid acts synergistically with thyroid hormone and glucocorticoids to control growth hormone gene expression [7] and we have also shown recently in our laboratory that a short-term administration of retinoids modifies the binding characteristics of T3 receptors [8]. The aim of this work was the study of the long-term effect of vitamin A on the properties of the triiodothyronine (T3) nuclear receptor in rat liver. In order to study this effect, experiments have been performed in which diets administered to animals contained various amounts of retinol. The properties of the receptor have been investigated using an in vitro binding method and then a Scatchard analysis. Data resulting from this analysis have been related to the results of c-erb-A mRNA measurement. Indeed it was known that the c-erb-A protein is a high-affinity receptor for thyroid hormone [9] and that in the liver c-erb-A mRNA content is related to tissue T3 nuclear binding capacity [10].

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2. MATERIALS AND METHODS

2.1. *Animals and diets*

Livers were obtained from male Wistar rats given diets for a period of 8 weeks which were either a control diet (5 U retinol/g food), or a vitamin A-deficient diet (without retinol), or a vitamin A-overloaded diet (50 U retinol/g food).

2.2. *Properties of T3 nuclear receptor*

Liver was homogenized in 0.32 M sucrose plus 1 mM $MgCl_2$ (0.32 SM). After centrifugation the crude pellet was washed and then centrifuged through a layer of sucrose (2.2 M sucrose plus 1 mM $MgCl_2$) [11]. The nuclei were resuspended in 0.32 SM plus 0.25% Triton X-100, centrifuged, washed and then resuspended in TKEM (20 mM Tris-HCl, 0.4 M KCl, 2 mM EDTA and 1 mM $MgCl_2$). After frequent pipetting of the suspension, the supernatant containing nuclear proteins was obtained by centrifugation [12]. Proteins were incubated for 3 h at 20°C in TKEM containing 0.006–0.12 μmol [^{125}I]T3. The binding reaction was stopped by addition of an ice-cold Dowex IX8-400 resin suspension in TKEM. After mixing the resin was sedimented and protein-bound T3 estimated in the supernatant. Non-specific binding was determined by incubation in the presence of 1000-fold excess of unlabelled T3. Scatchard analysis was then performed.

2.3. *Quantification of c-erb-A mRNA*

The absolute value of c-erb-A mRNA cannot be determined directly but a proportion can be deducted by comparing with the β-actin internal standard simultaneously reverse-transcribed and amplified in the same test tube.

2.3.1 *Extraction of RNA.* Total RNA was extracted from rat liver in 10 ml of extraction buffer (10 mM Tris-HCl, pH 7.4; 100 mM EDTA; 100 mM LiCl; 0.1% SDS and 100 μg of proteinase K), with an equal volume of phenol/chloroform/isoamylalcohol (49:49:2) [13]. Poly(A)$^+$RNA was purified as described by Maniatis et al. [14].

Table I

Synthesized primers used for PCR

Primers		Sequence 5'3'	Complementary site
β-actin*	A1	AGGATGCAGAAGGAGATTACTGCC	2814 to 2837
	A2	GTAAAACGCAGCTCAGTAACAGTCC	3159 to 3135
erb-A**	E1	TCCTGATGAAGGTGACGGACCTGC	1247 to 1270
	E2	TCAAAGACTTCCAAGAAGAGAGGC	1364 to 1341

*from rat c-erb-A cDNA according to the sequence of Murray et al. [15].
**from rat cytoplasmic β-actin gene according to the sequence of Nudel et al. [16].
Primers A1 and A2 being chosen in two different exons, the size of the PCR products allowed us to ensure that there was no genomic DNA contamination.

2.3.2. *Oligonucleotide primer.* Primers used for PCR were synthetized using an Applied Biosystem Model 381A DNA synthesizer. The position and sequence of the different primers are summarized in Table I.

The positions of the primers were chosen to hybridize with α 1 and β c-erb-A that code for T3 binding protein, but not with α 2 c-erb-A that codes for protein (called 'receptor variant') which does not bind T3 [10]. These positions were chosen according to Mitsuhashi et al. [17] who compared amino acid sequence of rat α 1 and α 2 c-erb-A.

2.3.3. *Preparation of cDNA.* 1 μg of poly(A)$^+$RNA, 200 ng of primer A2 and 200 ng of primer E2 were used for the reverse transcription in the presence of 2.5 μl of reaction buffer (1.25 M Tris-HCl, pH 8.3, 1.875 M KCl, 75 mM $MgCl_2$, 250 mM DTT), 80 U of Moloney murine leukemia virus reverse transcriptase, 25 U of RNase inhibitor and 0.4 mM of each dNTP in a total volume of 25 μl. Synthesized cDNA was then amplified by the polymerase chain reaction (PCR) technique using Taq polymerase [18]. Absence of genomic DNA contamination has been verified using, during the reverse transcription, control tubes containing 10 μg of RNase.

2.3.4. *PCR analysis.* 10 μl of cDNA was then used for amplification performed in a Perkin-Elmer/Cetus thermocycler. The reaction mixture (100 μl) contained 600 ng of each primer (A1, A2, E1, E2), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dGTP, 0.2 mM dATP, 0.2 mM dTTP, 0.02 mM dCTP, 50 μCi [α-^{32}P]deoxycytosine triphosphate (sp. act., 370 MBq/ml) and 1 U Taq polymerase. The reaction was carried out for a total of 31 cycles. The cycles times were as follows: denaturation, 1 min at 95°C; annealing, 1 min at 60°C; primer extension, 2 min at 72°C.

2.3.5. *Quantitative analysis of PCR products.* 10 μl of the PCR reaction were sampled after 10, 13, 16, 19, 22, 25, 28, 31 amplification cycles [19] and the co-amplified fragments were separated by electrophoresis on a 10% acrylamide gel. The incorporated radioactivity was visualized by autoradiography, the bands were excised from the gels and quantified by scintillation counting.

3. RESULTS

3.1. *Binding characteristics of T3 nuclear receptor*

Fig. 1A represents binding, after 3 h of incubation at 20°C, of increasing amounts of [^{125}I]T3 with solubilized nuclear receptors isolated from the liver of rats fed different diets. The binding of T3 with nuclear receptors was less in vitamin A-deficient rats than in control rats. Such a result confirms previous data of our laboratory [20]. Vitamin A overloaded diet also led to a similar decreased binding. A Scatchard analysis of the binding values (Fig. 1B) allowed the determination of the main properties of receptors, i.e. capacity and affinity. The slope of the straight line gave the affinity constant and the intercept of the slope with the abscissa represented

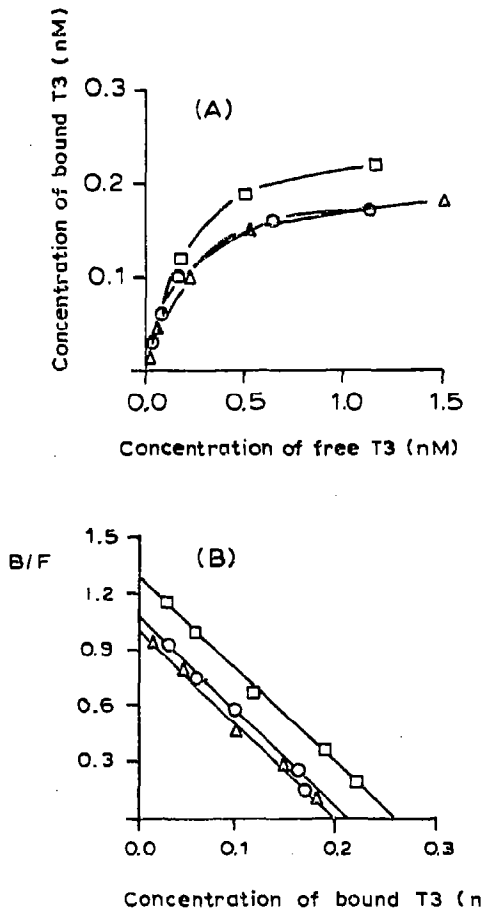

Fig. 1. Properties of triiodothyronine (T3) nuclear receptor. Formation of receptor-T3 complexes at increasing concentrations of T3 in the incubation medium (A). Scatchard analysis of T3 binding to the nuclear proteins: results are plotted as the ratios of bound to free hormone (B/F) vs bound hormone concentrations (B). Hepatic nuclear fractions were obtained from control (□), vitamin A-deficient (△), and vitamin A-overloaded (○) rats.

Table II

Capacity of T3 nuclear receptor and proportion of c-erb-A mRNA to β-actin mRNA.

	Control	Vitamin A-deficiency	Vitamin A-overloading
Capacity[a] (pmol/mg prot)	1.03 ± 0.08	0.79* ± 0.07	0.84* ± 0.08
$A_{erb\text{-}A}/A_{act}$[b]	0.215	0.138	0.068

[a]Each value is the mean ± SEM of 8 animals; *$P<0.05$ compared with control (Student's t-test).
[b]Each value represents the mean of two separate measurements; $A_{erb\text{-}A}$ and A_{act} are absolute values of c-erb-A mRNA and β-actin mRNA.

the maximum binding capacity, i.e. the binding site concentration. The results of such an analysis showed that in vitamin A deficiency or vitamin A overloading, the affinity of receptors was not affected while the binding capacity was significantly reduced near to 20% (Table II).

3.2. *Quantitative analysis of PCR products*

Our purpose was to determine the proportion of c-erb-A mRNA in vitamin A-deficient rats or vitamin A-overloaded rats to c-erb-A mRNA in control rats. The proportion of c-erb-A mRNA was estimated against an internal standard known to be unmodified by thyroidal [21] and nutritional conditions [22]: the β-actin mRNA. After a first step of reverse transcription allowing the production of cDNA from the different nutritional groups of rats, co-amplification of β-actin cDNA and c-erb-A cDNA were performed using primers A1 and A2 for the first one, E1 and E2 for the second one. PCR reactions were stopped after different amplification cycles and the reaction products were separated by electrophoresis on acrylamide gels: 222 bp for the β-actin fragment and 117 bp for the c-erb-A fragment (Fig. 2A). Logarithms of radioactivity incorporated during these different amplification cycles were plotted against the number of PCR cycle. Straight lines with different slopes were obtained as shown in Fig. 2B,C,D.

The proportion of c-erb-A mRNA to β-actin mRNA

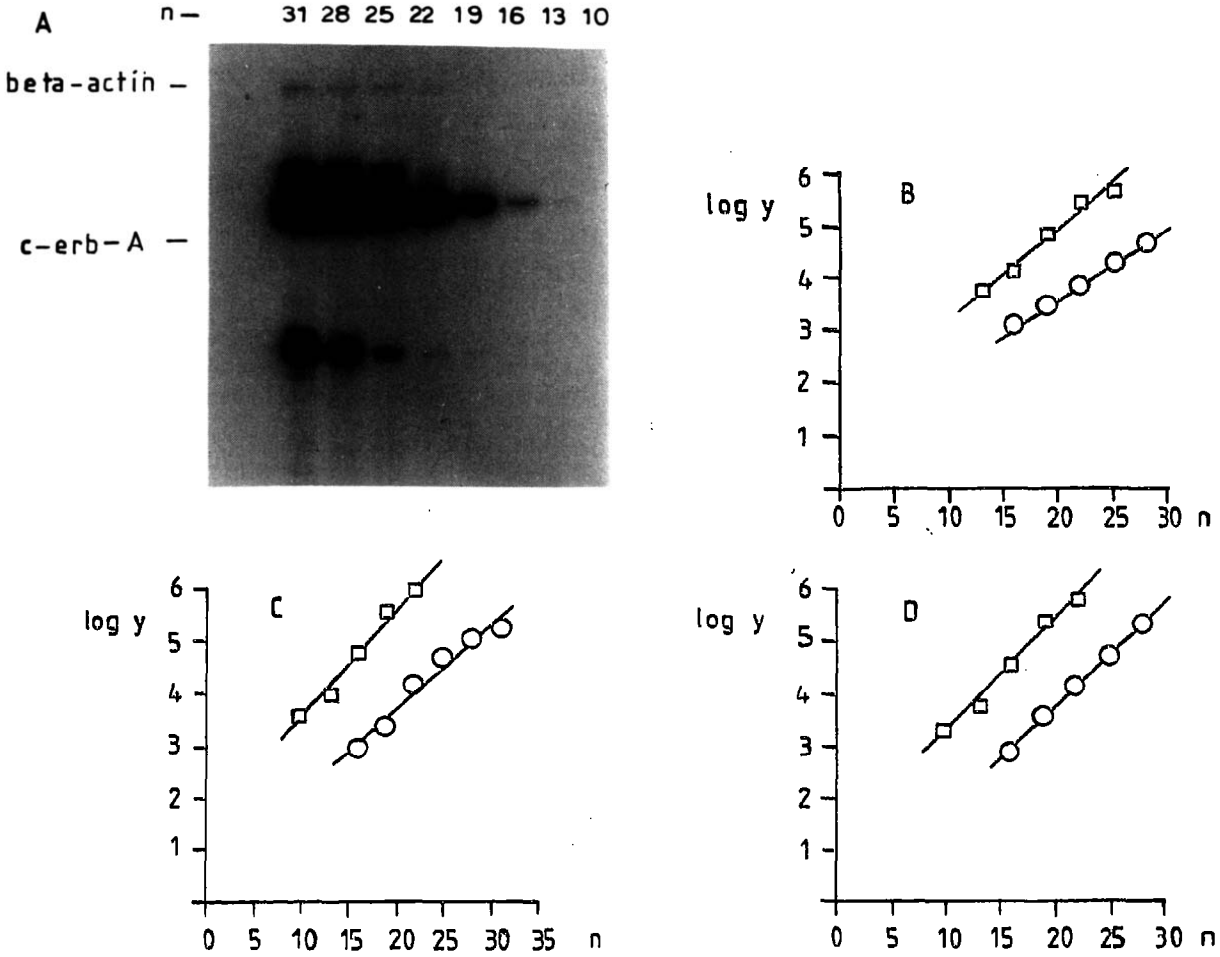

Fig. 2. Kinetic analyses of PCR products from β-actin and c-erb-A mRNAs. Autoradiograms of acrylamide gel performed on PCR co-amplified products of rat transcripts of β-actin (222 bp) and erb-A (117 bp) gene (A). Semi-logarithmic representation of the relative extent of amplification in control rat (B) measured by counting the amount of ^{32}P incorporated into the fragments visualized in (a); (C) and (D), same plot as in (B) but from deficient rat and overloaded rat respectively; n is the number of PCR cycles; Y is the incorporated radioactivity (cpm); (□) β-actin; (○) erb-A.

was calculated using the formula: $A_{erb-A}/A_{act} = Y_{erb-A}(1+R_{act})^n / Y_{act}(1+R_{erb-A})^n$ according to Chelly et al. [23], where A is the initial amount of material, R the efficiency, n the number of cycles and Y the radioactivity incorporated; R can be deduced from the slope of the semi-log plot: $\log(1+R)$. The proportion of c-erb-A mRNA to β-actin mRNA is decreased by 42% (Fig. 2C) and 78% (Fig. 2D) in vitamin A-deficient rats and vitamin A-overloaded rats, respectively (Table II).

4. DISCUSSION

The present investigation demonstrates that in the liver of rats fed for two months on either a vitamin A-deficient or a vitamin A-overloaded diet there was a decreased binding capacity of the T3 nuclear receptor and a decreased amount of c-erb-A mRNA. The level of dietary intake of retinol influences the amount of efficient T3 receptors. The mechanisms implied must be complex and still remain to precise. Therefore, it appears that retinol, or probably retinoic acid which is its natural metabolite, contributes to the regulation of T3 nuclear receptor properties. Such a result can be related to other recent data showing interrelations between retinoids and triiodothyronine: (i) the administration of a single dose of retinol or retinoic acid induced a few hours later an increase in protein kinase C activity and a subsequent increase in the affinity of the T3 nuclear receptor [8] while the capacity was not affected; (ii) T3 receptor and retinoic acid receptor could be associated in the nucleas as an heterodimer resulting in positive or negative regulation of gene transcription [24]; (iii) retinoic acid increased mouse mammary tumor virus expression but only when T3 was also present [25]. In addition, interrelations between retinoids and receptors of other hormones implied in growth, development, and differentiation have also been described. For instance it was observed that retinol modulates the glucocorticoid receptor in normal human cultured T-cells [26] and that retinoic acid stimulates 1,25-Dihydroxyvitamin D3 binding in rat osteosarcoma cells [27].

So the multiplicity of the interactions between either an exogenous factor (retinol) or its metabolite (retinoic acid) and endogenous elements (as hormone receptors) could greatly increase the spectrum of cellular phenomena implied in the regulation of growth, development, and differentiation.

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