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Functional properties of human thyroid hormone receptor β1 overexpressed using baculovirus

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We have overexpressed the human β1 thyroid hormone receptor in insect cells using a recombinant baculovirus to a level of 5–10% of total cellular protein The recombinant protein migrates as a 50 kDa band by SDS-PAGE and Western blot analysis The expressed receptor binds to L-T3 with a K_d of 1 3±0 4 × 10^{-10} M and to thyroid hormone analogues with an affinity hierarchy of TRIAC>L-T3>L-T4>rT3 Gel retardation assays show highly specific receptor binding to a TRE which is modified by the presence of ligand and avidin–biotin complex DNA analysis shows a K_d of 6 2±2 0 × 10^{-10} M for this interaction These results indicate high level expression of *h*TRβ with authentic hormone and DNA binding properties

Thyroid hormone receptor, Baculovirus, Gene expression

1 INTRODUCTION

The effects of thyroid hormone are now known to be mediated via a nuclear receptor protein which is part of a superfamily of ligand-inducible transcription factors that include steroid hormone, retinoic acid and vitamin D receptors [1] The receptor protein interacts with specific DNA sequences called thyroid response elements (TREs) usually located in the promoter region of target genes Both positive and negative regulation of gene transcription by ligand activated receptor has been demonstrated [2,3], but the precise nature of the interaction between the receptor and the transcription initiation complex is poorly defined

The relatively low (~2000–10 000 molecules/cell) levels of endogenous receptor in mammalian tissues [4] demonstrate the need for expression systems which generate considerably higher levels of the protein Other groups have successfully overexpressed functional receptor protein by introducing expression vectors containing the thyroid hormone receptor cDNA into bacterial or yeast cell culture systems [5,6] A potential disadvantage with such expression systems is that the host cells are incapable of modifying the protein post-translationally by processes such as phosphorylation or glycosylation There is evidence to suggest that the thyroid hormone receptor is phosphorylated [7] and studies using its oncogenic counterpart *v-erb*A have shown that its capacity to transform cells is phosphorylation-dependent [8]

The rat glucocorticoid receptor is phosphorylated when expressed in insect cells using a recombinant baculovirus [9] and oestrogen receptor expressed in this system is transcriptionally active [10] In this paper we describe the overexpression of functional human thyroid hormone receptor in insect cells using a recombinant baculovirus

Abbreviations L-T3, 3,3′,5-triiodo-L-thyronine, TRIAC, triiodothyroacetic acid, L-T4, thyroxine, rT3, 3,3′,5′-triiodo-L-thyronine, hTRβ, human β thyroid hormone receptor, TRE, thyroid response element, ABCD, avidin–biotin complex DNA

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2. MATERIALS AND METHODS

2 1 *Cells and viruses*

Spodoptera frugiperda (*Sf*) cells (from R Possee, Oxford, UK) were grown at 27°C in TC-100 medium (Flow, Rickmansworth, UK) containing 10% foetal bovine serum Wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV) was obtained from Dr Max Summers (Texas A & M University) Procedures for cell culture, and viral infection were as described previously [11]

2 2 *Production of recombinant baculovirus*

A 1 4 kilobase *Eco*RI fragment containing hTRβ1 cDNA [12] was isolated, filled-in with Klenow and then ligated to *Bam*HI linkers This fragment was then introduced into the *Bam*HI site of a transfer vector p36C [13], downstream of the polyhedrin promoter in the correct orientation to generate p36-TRβ This recombinant vector DNA was co-transfected with wild type AcNPV genomic DNA into *Sf* cells Recombinant polyhedrin-negative virus was identified, plaque-purified and the TRβ cDNA insert verified by hybridisation [11]

2 3 *Cell extracts*

Sf cells were infected with either wild type or recombinant virus at a multiplicity of infection of 5 and harvested 48–72 h later The cells were washed, lysed in hypotonic buffer and then high salt extracts of nuclear proteins were prepared according to a previously described procedure [14]

2 4 *SDS-PAGE and Western blot analysis*

SDS-polyacrylamide gel electrophoresis (10% T, 2 7% C) was carried out under reducing conditions, with 20 μg protein per lane. After electrophoresis the protein was either stained with Coomassie blue or electro-transferred to a nitrocellulose membrane and probed with a polyclonal rabbit antiserum to a *lacZ/v-erb*A fusion protein [15] and then developed using a goat anti-rabbit IgG alkaline phosphatase conjugate (Promega, Southampton, UK)

2 5 *Ligand binding assays*

For hormone binding assays, aliquots of cell extracts were incubated with 0 01 nM ^{125}I-L T3 and increasing amounts of either unlabelled L-T3 or various thyroid hormone analogues Bound and free hormone were separated using a filter binding technique [16]

2 6 *DNA binding assays*

Gel retardation studies were performed using two ^{32}P-labelled oligonucleotide duplexes Pal and Ncon Pal contains a palindromic oligonucleotide sequence (5'-TCAGGTCATGACCTGA-3') which corresponds to a consensus thyroid response element known to bind thyroid hormone receptor [17], whereas Ncon contains a control DNA sequence from he human glycoprotein hormone α gene promoter (−113 to −97 bp) (5'-TAATTACACCAAGTAC-3') that does not bind receptor Wild type and recombinant cell extracts (3 μg total protein) were incubated for 20 min in buffer (20 mM HEPES, pH 7 4, 50 mM KCl, 10% glycerol, 1 mM dithiothreitol) in the presence of 5 mg ml^{-1} BSA, 0 1 mg ml^{-1} poly(dI-dC) Labelled probe (400 fmol) was then added followed by a further 30 min incubation prior to analysis on a non-denaturing polyacrylamide gel (6% T, 3 3% C) in 50 mM HEPES, pH 7 4 Where specified, excess unlabelled competitor oligonucleotide or 10 μM T3 was added

To measure DNA binding affinity, aliquots of recombinant cell extract were labelled with ^{125}I-L-T3 and then incubated with increasing amounts of biotinylated oligonucleotide containing either the TRE (BPal) or control (BCon) DNA sequences shown above Receptor–DNA complexes were quantitated after precipitation with streptavidin magnetic beads (Dynal Wirral, UK) as described previously [17] Receptor binding to control DNA was subtracted from the amounts bound to TRE DNA to calculate specific binding

3 RESULTS

Electrophoresis of wild type (Wt) and recombinant (Rec) virus-infected *Sf* cell extracts reveals a prominent unique band in the latter with a M_r of approximately 50 kDa (Fig 1a) Western blot analysis also demonstrates a novel band at ~50 kDa in the recombinant extract (Fig 1b) suggesting that this corresponds to expressed receptor protein It is estimated that this protein hTRβ, comprises around 5–10% of the total cell protein The majority of this expressed protein is associated with the nuclear fraction of cell extracts (data not shown)

Fig 2 shows a representative displacement curve and Scatchard analysis of L-T3 binding to recombinant cell extracts From this and similar experiments (n=5), the baculovirus-expressed hTRβ binds L-T3 with a mean K_d = 1 3±0 4 × 10^{-10} M. The relative affinity of various thyroid hormone analogues for the expressed receptor was assessed by measuring their ability to displace ^{125}I-L-T3 in a competitive binding assay (Fig. 3). When ranked in order of their relative affinities, these analogues show a hierarchy of TRIAC>L-T3>L-T4>r-T3

Receptor interaction with DNA was measured by the ability of expressed proteins to retard the mobility of

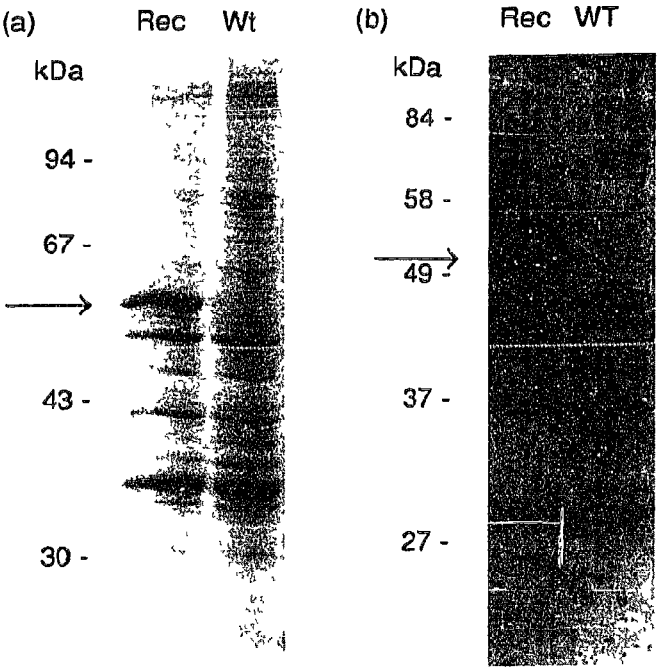

Fig 1 Electrophoretic (a) and Western blot (b) analysis of wild type (Wt) and recombinant (Rec) virus-infected *Sf* cell extracts Relative molecular masses of marker proteins in kDa and the position of hTRβ are indicated on the left

labelled oligonucleotides in gel shift assays (Fig 4) Both wild type (Wt) and recombinant (Rec) cell extracts contain protein which binds control DNA sequence (Ncon) to produce a complex with retarded mobility (lanes 1 and 2). However, wild type extract contains negligible binding activity when tested with the TRE sequence (Pal) (lane 3), whereas recombinant cell extract results in the formation of several bands (lane 4). Interestingly, the inclusion of ligand (10 μM T3) in the reaction alters the mobility and intensity of these retarded complexes (lane 5) The formation of these complexes is progressively inhibited by the addition of

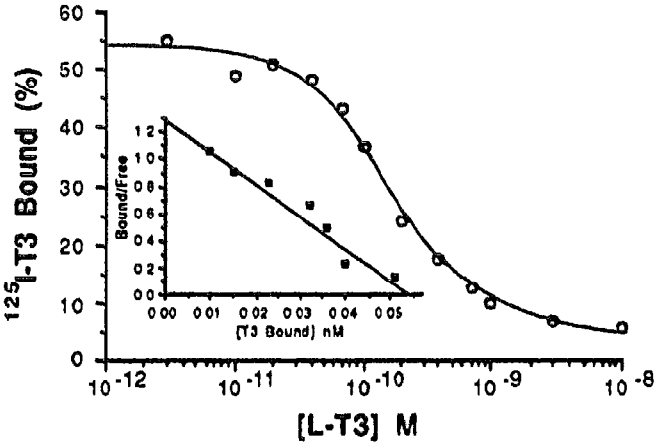

Fig 2 Displacement of ^{125}I-L-T3 binding to recombinant virus-infected cell extracts by unlabelled L-T3 and Scatchard analysis (inset).

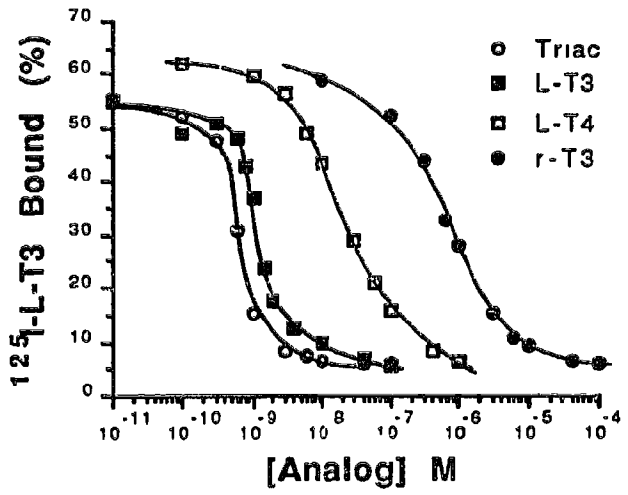

Fig 3 Competition of ^{125}I-L-T3 binding to recombinant virus-infected cell extract with unlabelled thyroid hormone analogues

increasing amounts of homologous non-labelled Pal DNA (lanes 6–9) However, the addition of similar amounts of unlabelled NCon DNA has no discernible effect (lane 10)

hTRβ containing recombinant cell extracts exhibits specific saturable binding to a biotinylated TRE sequence (Fig 5). Scatchard analyses from this and similar experiments (n=3) demonstrate that recombinant receptor protein binds the thyroid response element with a mean K_d=6.3±2.0×10^{-10} M

4 DISCUSSION

The properties of thyroid hormone receptor extracted from a variety of mammalian cell lines and tissues are well known. The protein is found in the nucleus, has a molecular weight of ~50 kDa and exhibits high affinity binding to T3 (K_d=10^{-10}–10^{-11} M) [4] The properties of our recombinant receptor protein overexpressed in insect cells are fully consistent with these observations. The majority of expressed receptor protein is associated with the nuclear fraction and binds L-T3 with the expected affinity Furthermore, the hierarchy of relative affinities observed with various thyroid hormone analogues of TRIAC>L-T3>L-T4>r-T3 is characteristic of authentic receptor

Gel retardation assays confirm that expressed hTRβ binds specificaly to a palindromic TRE. This consensus sequence represents an optimised high-affinity DNA binding site for thyroid hormone receptor and is capable of mediating receptor-induced activation of

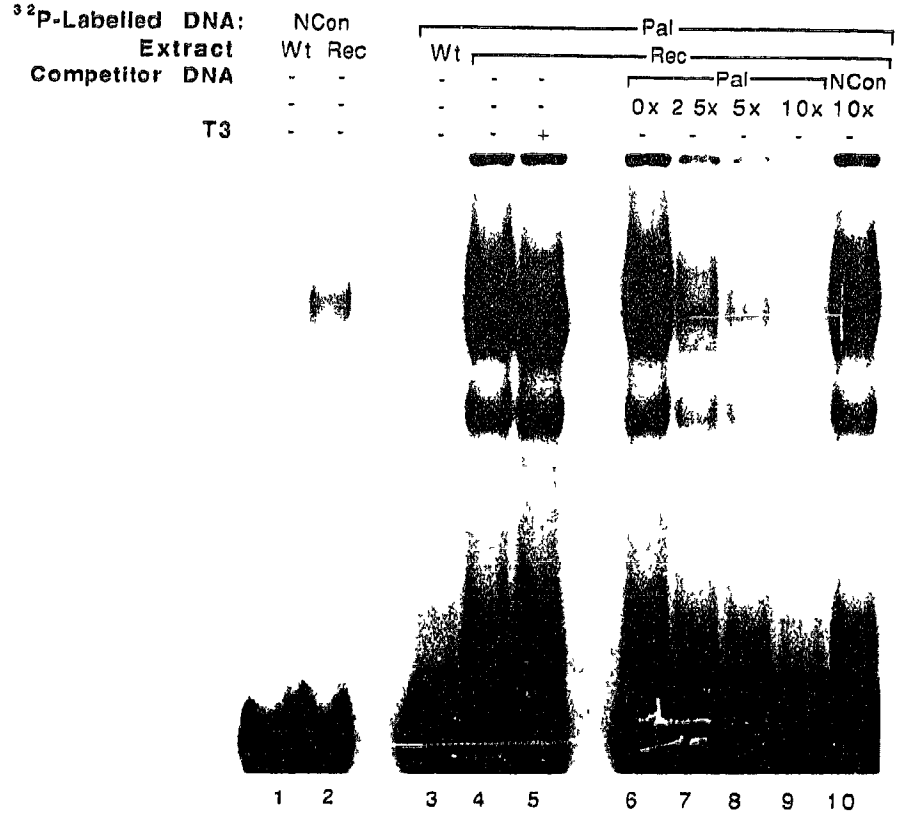

Fig 4 Gel retardation of ^{32}P-labelled palindromic TRE (Pal) or a control DNA sequence (NCon) by wild type (Wt) or recombinant (Rec) virus infected cell extracts The ratios of non-labelled competitor oligonucleotide shown are relative to the level of ^{32}P-labelled oligonucleotide present.

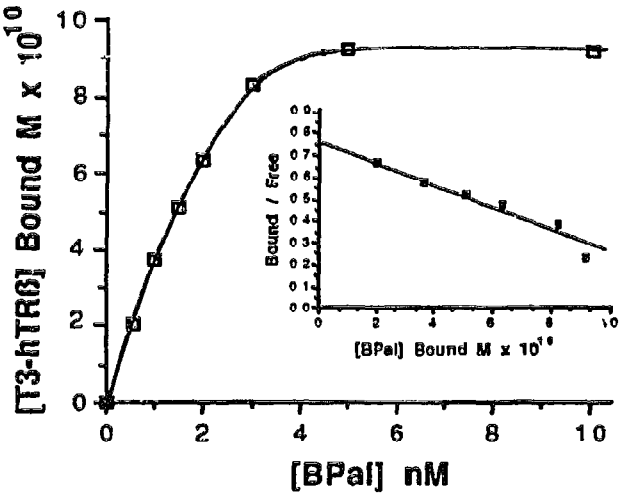

Fig 5 Avidin–biotin complex DNA (ABCD) binding assay of ^{125}I-L-T3 labelled recombinant cell extract to TRE and Scatchard analysis (inset)

target gene transcription [2] Binding studies confirm that the recombinant receptor binds with high affinity to this sequence with a K_d ($6.3 \pm 2.0 \times 10^{-10}$ M) that is also comparable with the previously reported value of 1.3×10^{-9} M [17]

Our data also indicate that incubation of the recombinant receptor with labelled TRE generates several complexes of differing mobilities in the gel retardation assays One explanation is that the expressed receptor has undergone proteolytic degradation to generate smaller molecular weight products which retain the ability to bind DNA – although SDS-PAGE and Western blot analyses do not support this Alternatively, these complexes may represent monomeric and homodimeric receptor – DNA interactions. The glucocorticoid and oestrogen receptors are known to bind as homodimers to related palindromic DNA response elements [18] A third intriguing possibility is that some of these complexes may represent heterodimer formation between hTRβ and other cellular proteins There is evidence to suggest that such thyroid receptor auxiliary protein (TRAP) activity is found in a variety of cell types and may interact with receptor–DNA complexes [19]

It is also interesting to note that the inclusion of T3 in gel retardation assays produces a retarded complex with a slightly increased mobility. The addition of ligand has been shown to have a similar effect on the binding of oestrogen receptor to DNA [20] It is tempting to speculate that this altered mobility reflects a ligand-induced conformational change in the receptor protein which may be related to the hormone-induced receptor activation observed in vivo

The high levels of functional thyroid hormone receptor obtained using the baculovirus expression system will facilitate studies using in vitro transcription assays to explore the interaction between the receptor and the transcription initiation complex We also plan to use the unique ligand and DNA binding properties of the expressed receptor to purify the protein using affinity chromatography

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