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HIGH LEVEL EXPRESSION OF FUNCTIONAL FULL LENGTH HUMAN THYROID HORMONE RECEPTOR β1 IN INSECT CELLS USING A RECOMBINANT BACULOVIRUS

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Summary—We have cloned the human thyroid hormone receptor β1 (hThR β) from the human breast cancer cell line T47D using the PCR technique. A recombinant baculovirus transfer vector pVL1392/hThR β was constructed and the full length receptor was expressed in the insect cell line *Spodoptera frugiperda* (Sf9). Approx. 10–15 × 10^6 receptors are expressed/cell which implies a production level of 2.5–4.0 mg hThR β/l of cell culture. The expressed hThR β displayed a single class of binding sites for T_3 with high affinity. Western blot analysis using a polyclonal antibody indicated that the molecular weight of the baculovirus expressed receptor is approx. 50 kDa. Crude nuclear extract of hThR β labeled with [^{125}I]T_3 sedimented as a 4 S peak on a glycerol gradient. No receptor could be detected in the cytoplasm indicating its proper translocation to the nuclear compartment. An oligonucleotide containing a palindromic thyroid hormone response element is specifically recognized and retarded in a gel-mobility-shift assay in the presence of nuclear extract of Sf9 cells expressing hThR β. These data suggest that hThR β expressed in Sf9 cells is functional and displays characteristics virtually indistinguishable from those of the thyroid hormone receptor (ThR) extracted from mammalian cells. Furthermore, the data indicate that the baculovirus expression system is adequate for large-scale production of receptor for detailed structural and functional studies.

INTRODUCTION

The thyroid hormone receptor (ThR) belongs to a family of soluble, intracellular receptors involved in development, differentiation and regulation of metabolic processes. ThR is a DNA binding protein that functions as a transcriptional regulator of gene expression in a hormone-dependent manner. The transcriptional control is mediated by a *cis*-active sequence specific thyroid hormone response element (TRE) which is present in a single or multiple copies upstream of or within target genes. However, the mechanism by which the receptor induces or represses the transcriptional response of target genes to thyroid hormone is poorly understood.

All members of the steroid/ThR family have a similar structural organization composed of distinct domains. Biochemical and mutational analysis of these hormone receptors identified domains responsible for hormone binding, DNA binding and *trans*-activation of gene expression, respectively [1–4]. The central region of 65–68 amino acids, which is highly conserved in the receptor superfamily, forms two so-called "zinc fingers" [5–7] or, rather, zinc-binding structures [8] that mediate the sequence specific interaction of the receptor with DNA. The less well characterized N-terminal domain is probably involved in modulation of *trans*-activation while the C-terminal region is functionally important for homone binding and dimer formation [4, 9, 10].

Synthetic hormones acting as agonists or antagonists have been described for the mineralocorticoid, glucocorticoid, estrogen, androgen and progesterone receptors [11, and references therein]. However, no antagonist that interacts with the ThR has yet been identified. In order to design potential ThR antagonists in the treatment of hypertension and cardiac arrhythmia we have initiated a program to determine the three-dimensional (3D) structure of the human

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thyroid hormone receptor $\beta 1$ (hThR β) and by 3D modelling identify and synthesize ThR antagonists.

Determination of the 3D structure of the ThR is also of importance for a more detailed understanding of the structure of the receptor in relation to its interaction with DNA and hormone as well as its interaction with other receptors or components of the transcriptional machinery.

Detailed structural studies of the ThR, including X-ray crystallography, have been prevented by the very small amount of receptor protein that can be purified from tissues or isolates of established cell lines expressing the receptor. In order to express large amounts of the human ThR β it was therefore necessary to take advantage of recombinant DNA techniques and a suitable expression system. In this report we describe high level expression of the full length hThR β in *Spodoptera frugiperda* (Sf9) cells using the baculovirus expression system.

EXPERIMENTAL

Methods

cDNA synthesis. Cytoplasmic mRNA was extracted from approx. 1×10^7 T47D cells in a buffer containing (10 mM Tris pH 7.9; 150 mM NaCl; 1.5 mM $MgCl_2$ and 0.65% NP40). After 10 min incubation on ice the lysate was transferred to an Eppendorf tube and the nuclei were pelleted. The supernatant was extracted twice with phenol–chloroform–isoamyl alcohol (1:1:24, v/v/v) and then precipitated with 1 vol of isopropanol. The RNA pellet was dissolved in diethylpyrocarbonate treated distilled water and further purified on oligo(dT)cellulose as described [12]. First and second strand cDNA was synthesized with a cDNA kit (Amersham) according to the recommendations of the supplier. As primer for the first strand synthesis we used a hThR β specific oligonucleotide, complementary to the 3′-end of the coding region of c-*erb*A [13], tailed with the recognition sequence for the restriction of endonuclease *Cla*I (5′ AGT*ATCGAT*CTAATCCTCGAAC-ACTTCCAG 3′).

Amplification by the polymerase chain reaction (PCR). In vitro amplification of the cDNA was performed in two steps. The 5′ half of the cDNA was amplified using the oligonucleotides Thr1 (5′-AGT*GGTACCA* TGGCAGAAAA TGG-CCTTACA-3′) and Thr2 (5′-CTTGGGC GTT GGTCGCCACA T-3′) as primers and for amplification of the 3′-end the primers Thr3 (5′-CAAAACTGTC ACCGAAGCC-3′) and Thr4 (5′-AGT*ATCGATC* TAATCCTCGA A-3′) were used. The sequence of the primers Thr2 and Thr3 were chosen in such a way that they flanked the recognition sequence for the unique restriction endonuclease *Bgl*I, which was used for reconstruction of the complete coding sequence for hThR β. Furthermore, to simplify cloning of the full length cDNA, the primers Thr1 and Thr4 were extended at their respective 5′-ends with the recognition sequence for the restriction endonucleases *Kpn*I and *Cla*I, respectively [italic sequences of Thr1 and Thr4 (see above)]. Additionally, extension of the Thr1 primer with the recognition sequence for *Kpn*I, immediately upstream of the initiation codon (ATG) and a change of the first nucleotide after the ATG, from an A to a G (bold letter in Thr1, see above) places the initiation codon close to a consensus "Kozak" sequence which should enhance the efficiency of translation [14].

PCR was performed with a GeneAmp reagent kit and Taq polymerase (Perkin–Elmer Cetus) according to the recommendations of the supplier. In each reaction 5 ng of cDNA and 1 μM of the respective primers were used. Annealing of primers was performed at 52°C and each PCR was run for 30 cycles.

Vector constructions. The *in vitro* amplified ThR β fragments were isolated from a 1% agarose gel and digested with *Bgl*I, ligated by T4 DNA ligase and then recut with *Cla*I and *Kpn*I. Full length cDNA was then cloned between the *Kpn*I and *Cla*I sites in pT7T3 19U (Pharmacia/LKB) generating the plasmid pT7-hThR β.

The recombinant transfer vector pVL1392/hThR β was constructed by insertion of a 1.4 kb fragment encoding the hThR β. Briefly, the plasmid pT7-ThR β was digested with *Cla*I, blunt-ended with the large fragment of DNA polymerase I in the presence of deoxynucleoside triphosphates and then digested with *Bam*HI. The *Cla*I and *Bam*HI cDNA fragment was purified from a 1% low-melting agarose gel and ligated between the *Bgl*II and *Sma*I sites of pVL1392 (kindly provided by Dr M. D. Summers). The structure of the resultant transfer vector pVL1392/hThR β was verified by restriction endonuclease cleavage.

In vitro transcription and translation. Capped mRNA transcripts were generated from the T7 promoter in the plasmid pT7-hThR β with a Transprobe T kit (Pharmacia/LKB). *In vitro* translation was performed with a rabbit reticulo-

cyte lysate (Promega). A typical translation assay included 1 μg of *in vitro* synthesized mRNA, 5 μCi [^{35}S]methionine (Amersham) and 35 μl of lysate in a total volume of 50 μl. The reaction was run for 60 min at 30°C. Subsequent steps were as recommended by the supplier. The translation products were analyzed on a 15% SDS–PAGE under denaturing conditions and exposed to film overnight.

Cell culture and viral infection. Cells of Sf9 were cultured at 27°C in TC 100 medium (Nordvac) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL). Sf9 cells, grown in monolayer cultures, were infected with wild type (wt) *Autographa californica* nuclear polyhedrosis virus (AcNPV) or recombinant AcNPV at a multiplicity of 10. Infected cells were harvested 48–72 h post infection.

Generation and isolation of recombinant virus. Plasmid pVL1392/Thr β and wt AcNPV DNA were used to transfect semiconfluent Sf9 cells by the calcium phosphate coprecipitation method [15]. Virus-containing medium was collected 96 h post transfection and used for plaque assays. After several rounds of plaque purification occlusion negative virus plaques were isolated. A number of plaque isolates were selected to infect Sf9 cells seeded in a 96 well plate. Forty-eight h post infection DNA was extracted from the cells as described [16] and analyzed by dot-blot hybridization [12]. A purified 1.4 kb ^{32}P-labeled hThR β cDNA fragment was used as a probe. Extracts from wt virus and uninfected Sf9 cells were used as controls. A single recombinant virus plaque was selected and amplified in Sf9 cells as described [15] for further characterization.

SDS–PAGE and Western blot. Forty-eight h post infection cells were lysed in 25 mM Tris, pH 7.6, 10 mM EDTA and 0.7% SDS. Lysates from approx. 50×10^3 cells were run on a 10% SDS–PAGE and either stained with a mixture containing 40% MeOH, 10% acetic acid and 0.1% Coomassie brilliant blue (R250) or electroblotted to a nitrocellulose membrane. The membrane was blocked with 10 mM Tris, pH 8.0, 150 mM NaCl and 0.1% Tween20 and incubated with a 1:500 dilution of rabbit anti-Thr β antiserum for 1 h at room temperature. The membrane was washed with TBS (10 mM Tris, pH 8.0, 150 mM NaCl and 0.05% Tween20) and then incubated with a 1:7500 dilution of goat anti-rabbit antibody conjugated with alkaline phosphatase (Promega). After extensive washing in TBS, bands were developed with an alkaline phosphatase substrate solution. Equal amounts of lysates from wt infected and uninfected Sf9 cells were used as controls.

Preparation of nuclei and crude extracts. Cells were scraped off the plate using a rubber policeman and suspended in cold buffer containing 20 mM Tris, pH 7.5, 2.5 mM $MgCl_2$ and 250 mM sucrose. After centrifugation at 300 *g* for 10 min the cells were taken up in the same buffer supplemented with 0.65% NP40 and incubated on ice for 10 min. The nuclei were pelleted by centrifugation at 1000 *g* and washed three times in buffer B (20 mM Tris, pH 7.5, 50 mM NaCl, 2 mM EDTA, 1 mM DTT and 10% glycerol) (nuclear fraction). High salt extraction of receptor from nuclei was carried out as described [17].

Ligand binding assays. Saturation dose assays were done by incubating the nuclear fraction with 10–2000 pM [^{125}I]T_3 in 200 μl buffer B in the absence (total binding) or presence (nonspecific binding) of a 500-fold excess of nonradioactive T_3 for 22 h at 4°C. The incubation was stopped by pelleting the nuclei at 2000 *g* for 6 min followed by two consecutive washes in PBS. The radioactivity was determined using a γ-counter [1277 gamma master (Pharmacia-Wallac)]. Specific binding of [^{125}I]T_3 was calculated by subtracting nonspecific from total binding.

The dissociation constant of the receptor for T_3 was determined by incubating aliquots of solubilized nuclear receptor or *in vitro* translated receptor in E400 buffer [20 mM KPO_4, pH 7.9, 400 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 8% glycerol and core histones (50 μg/ml)] with varying concentrations (10–2000 pM) of [^{125}I]T_3 $\pm$ a 500-fold excess of nonradioactive T3. The reaction mixture was incubated for 20–24 h at 4°C. Free ligand was removed by chromatography on 2 ml Sephadex G25 columns [17] (Pharmacia/LKB) and radioactivity was determined using a γ-counter. Specific binding of [^{125}I]T_3 was calculated by subtracting nonspecific from total binding. K_d was determined by plotting specifically bound hormone/free hormone vs specifically bound hormone.

Protein concentration was determined as previously described [18].

Glycerol density gradient centrifugation. Nuclear extract from Sf9 cells infected with recombinant virus was incubated with 1 nM [^{125}I]T_3 in the presence or absence of a 100-fold excess of nonradioactive T_3 at 4°C for 4 h. Free ligand was removed by chromatography on 2 ml Sephadex G25 columns and the eluate was layered on a 16–30% glycerol gradient in 20 mM Tris, pH 7.8, 1 mM EDTA, 150 mM NaCl,

1 mM DTT and insulin (100 μg/ml). Gradients were run at 4°C in an SW60Ti rotor in a Beckman L-8 55 M centrifuge at 55,000 rpm to a preset cumulative centrifugal effect (w^2t) of 1.80×10^{12} rad^2/s. Fractions of approx. 150 μl were collected from the bottom of the tube by gravity flow. Following fractionation, the bottom of the tube was cut off and assayed for radioactivity. ^{14}C-labeled bovine serum albumin (4.4S) was used as a sedimentation marker.

Gel-mobility shift assay. A synthetic, double stranded, palindromic thyroid hormone response element TRE-PAL [19] (5′-CGATTCAGGT CATGACCTGA GAGCT-3′) was end-labeled with [α-^{32}P]dATP using the large fragment of DNA polymerase I to a specific activity of 1×10^7 cpm/pmol. Aliquots (containing approx. 45 fmol of specific [^{125}I]T_3 binding sites) of solubilized nuclear receptor from Sf9 cells infected with recombinant baculovirus or of *in vitro* translated hThR β (unlabeled) were incubated for 20 min at room temperature in buffer containing 20 mM HEPES, pH 7.5, 80 mM KCl, 1 mM spermidine, 1 mM DTT, 4% Ficoll, 0.01% NP40, 1.6 μg poly(*d*I-C) and 15 fmol of ^{32}P-labeled TRE-PAL in a final volume of 25 μl. A 30-fold molar excess of nonradioactive TRE-PAL or unrelated oligonucleotide (5′-TCGAGGGCAC AGCCCAGAGG GTAGAGACAG TGCTGGAGGC TGGCGCTGCA-3′) was added in parallel reactions together with the ^{32}P-labeled TRE-PAL as specific and nonspecific competitors, respectively. Protein–DNA complexes were separated at 4°C on 4% PAGE in 22 mM Tris, 22 mM boric acid, 0.5 mM EDTA and 0.01% NP40 at 250 V. In control experiments, aliquots of nuclear extract from wt virus infected or uninfected Sf9 cells or unprogrammed reticulocyte lysate were used at the same protein concentrations as the extracts from

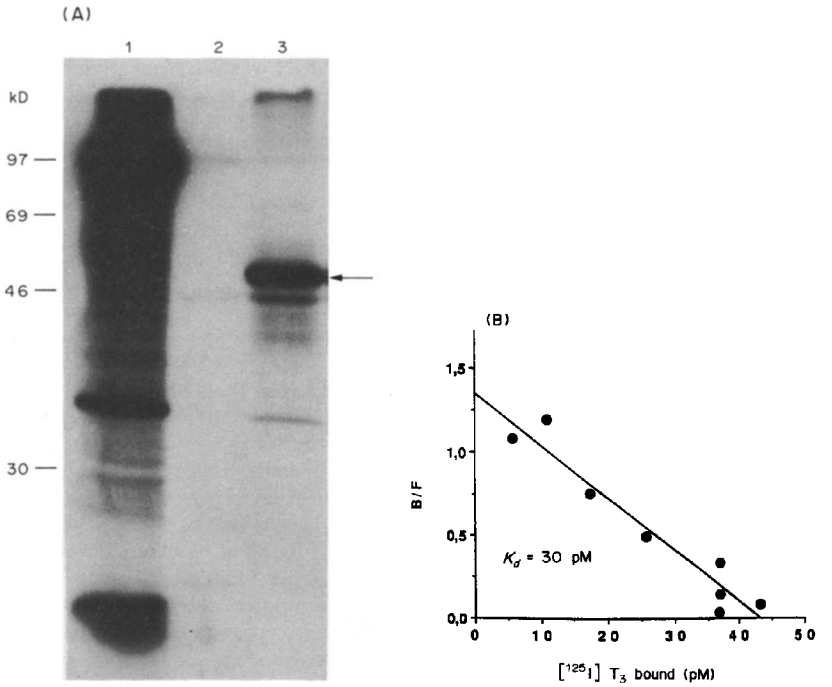

Fig. 1. *In vitro* synthesis of hThR β and Scatchard analysis of [^{125}I]T_3 binding. (A) *In vitro* capped and synthesized mRNA was translated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine as described in Experimental. [^{35}S]methionine labeled proteins were subjected to SDS–PAGE (15%) under reducing conditions and bands were visualized with autoradiography. Rainbow protein M_w markers were used as standards, Lane 1, positive control for *in vitro* translation (supplemented by the kit); lane 2, unprimed reticulocyte lysate as a control for unspecified translation; lane 3, reticulocyte lysate primed with RNA synthesized *in vitro* from the plasmid pT7/hThR β by T7 RNA polymerase. The position of hThR β is indicated by an arrow. (B) *In vitro* synthesized hThR β was incubated with varying concentrations of [^{125}I]T_3 in the presence or absence of an excess of nonradioactive T_3 as described in Experimental. K_d was determined by Scatchard analysis of the saturation binding data. B, specifically bound hormone; F, free hormone. Each point is the mean of 4 determinations.

recombinant virus infected cells or programmed reticulocyte lysate, respectively.

RESULTS

Cloning of hThR β

hThR β was cloned from mRNA isolated from the human breast cancer cell line T47D. The PCR technique was used to amplify the cDNA obtained after the 1st and 2nd strand cDNA synthesis. Amplified DNA was isolated and cloned into the transcription vector pT7T3 19U generating the plasmid pT7-hThR β. The structure and identification of the cDNA insert, verified by restriction enzyme cleavage and DNA sequencing, demonstrated perfect homology to the published sequence of the human c-*erb*A β [13]. Furthermore, the open reading frame of the cloned cDNA, deduced from the DNA sequence, comprised 456 amino acids which is in agreement with the ThR classified as β1 [20, 21]. Capped RNA transcripts, generated by T7 polymerase *in vitro*, were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. As shown in Fig. 1A the RNA directed the synthesis of a protein with $M_w \sim 50$ kDa, which is in close agreement with the reported size of hThR β (formerly referred to as c-*erb*A β) [13, 21]. In addition, hormone binding assays were performed to determine the affinity of the *in vitro* translated receptor for T_3. Scatchard analysis of the binding data indicated an apparent K_d of 30 pM (Fig. 1B) which is virtually identical to previously reported data [13].

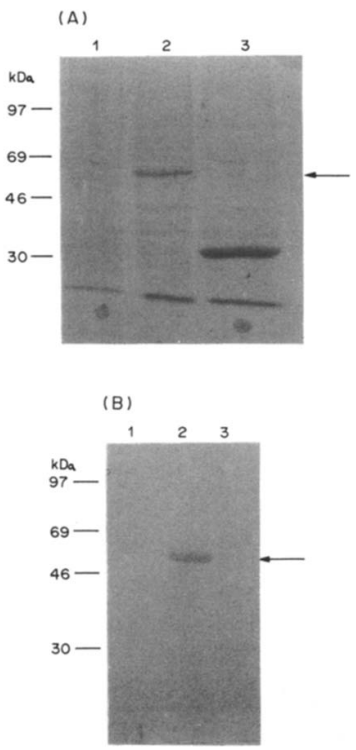

Fig. 2. Lysate of Sf9 cells subjected to SDS–PAGE and Western blot. Approx. 50×10^3 cells were lysed and analyzed on SDS–PAGE (10%) under reducing conditions and either stained with Coomassie brilliant blue (A) or electroblotted to nitrocellulose membrane and immunostained for hThR β (B). (A and B) lane 1, extract of uninfected Sf9 cells; lane 2, extract of cells infected with recombinant virus; lane 3, extract of wt virus infected cells. The arrow indicates the position of hThR β.

Expression of hThR β in Sf9 cells

High level expression of foreign proteins can be obtained in insect cells by inserting the desired cDNA gene under the control of the baculovirus polyhedrin promoter [15, 22, 23]. A 1.4 kb fragment from pT7-hThR β, containing the complete coding sequence of hThR β, was inserted into the vector pVL1392 resulting in the transfer vector pVL1392/hThR β. Recombinant virus, containing the hThR β cDNA in place of the viral polyhedrin gene, was generated by homologous recombination after cotransfection of Sf9 cells with wt viral DNA and the transfer vector pVL1392/hThR β. A single recombinant virus was isolated after several rounds of plaque purification. The presence of the hThR β cDNA sequence was verified by dot-blot hybridization. Infection of Sf9 cells with recombinant virus resulted in high level expression of the ThR 48–72 h post infection (Fig. 2A, B). The total number of T_3-specific binding sites, calculated from saturation dose assays on the nuclear pellet, was 80–110 pmol/mg protein (data not shown), representing 0.4–0.6% of total protein or approx. $10–15 \times 10^6$ receptors/cell. No expression of receptor could be detected in wt infected or uninfected Sf9 cells.

Ligand affinity

Ligand binding assays and Scatchard plots were carried out to determine the T_3 affinity of hThR β expressed in Sf9 cells. Scatchard analysis of T_3 binding assays, performed on crude high salt preparations of solubilized nuclei, point to a single class of binding sites for [^{125}I]T_3 with an apparent K_d of 105 pM (Fig. 3) which is a slightly higher K_d than that obtained for the *in vitro* translated receptor (Fig. 1B). No specific binding

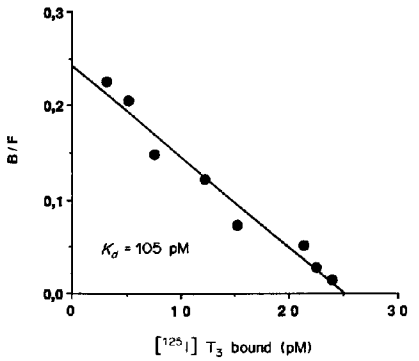

Fig. 3. Representative Scatchard analysis of baculovirus expressed hThR β. Preparation of crude extract and determination of the dissociation constant of the receptor for T_3 was done as described in Experimental. Each point is the mean of 4 determinations.

of [^{125}I]T_3 was detected in nuclear extracts from wt virus or uninfected Sf9 cells (data not shown).

Glycerol density gradient centrifugation

Unlike most steroid hormone receptors, the 1,25-dihydroxy-vitamin D_3, retinoic acid and ThRs are tightly bound to nuclear components in the absence of hormone [21, 24, 25]. The baculovirus expressed hThR β was also shown to be associated with nuclear components in Sf9 cells with undetectable receptor levels in the cytosolic compartment as determined by Western blot (data not shown). The [^{125}I]T_3 labeled extract from nuclei of Sf9 cells, infected with recombinant virus, sedimented in the 4S region of a 16–30% glycerol gradient (Fig. 4). This demonstrates that both the intracellular localization as well as the sedimentation coefficient of the baculovirus expressed hThR β are identical to those of mammalian ThR [26].

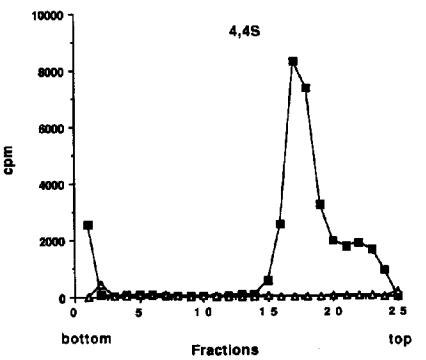

Fig. 4. Sedimentation profile of hThR β expressed in Sf9 cells. Crude nuclear extract of Sf9 cells infected with virus expressing hThR β was labeled with [^{125}I]T_3 in the presence (△) or absence (■) of a 100-fold excess of nonradioactive T_3. After removal of excess ligand the extract was layered on a 16–30% glycerol gradient and centrifuged at 55,000 rpm to a preset cumulative centrifugal effect of 1.80 rad^2/s. Fractions were collected from the bottom of the tubes and assayed for radioactivity. ^{14}C-labeled bovine serum albumin (4.4S) was used as an internal marker.

DNA binding assay

Nuclear extracts of Sf9 cells expressing hThR β were incubated with the ^{32}P-labeled TRE-PAL [19], followed by electrophoresis in a nondenaturing gel. As shown in Fig. 5A (lane 3), two protein–DNA complexes migrating more slowly than the free DNA were observed. These complexes were not detected when an identical amount of nuclear extract from uninfected or wt virus infected Sf9 cells was incubated with the TRE-PAL (Fig. 5A, lanes 1 and 2, respectively). This indicates that the appearance of the observed complexes was dependent on the presence of hThR β in the nuclear extract of Sf9 cells infected with the recombinant virus. When similar amounts of *in vitro* translated hThR β (as assessed by ligand binding) were used in the

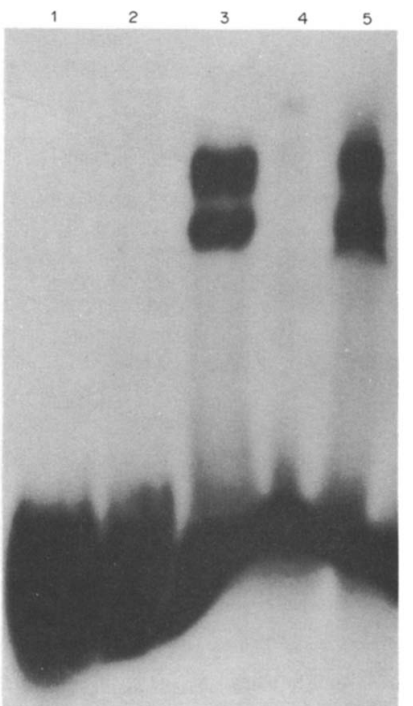

Fig. 5 (A)—*legend opposite.*

gel-mobility shift assay, formation of protein–DNA complexes with the same mobility as those formed in the presence of baculovirus expressed receptor was observed (Fig. 5A, lane 5). These protein–DNA complexes were not observed with an unprogrammed reticulocyte lysate (Fig. 5A, lane 4). Thus, the formation of both protein–DNA complexes seems to be dependent on the presence of expressed hThR β. Furthermore, there are no major differences in the apparent receptor–DNA affinity or mobility of the retarded protein–DNA complexes, whether the receptor was expressed in insect cells or in reticulocyte lysate. At the concentration of hThR β used (45 fmol) the two protein–DNA complexes formed seem to represent approximately equal amounts of labeled TRE-PAL. Titration with decreasing amounts of receptor demonstrated that at lower hThR β concentration, the formation of the slower migrating protein–DNA complex represents less amounts of shifted probe as compared to the faster migrating protein–DNA complex (data not shown). This suggests that complex formation may involve the binding of multiple ThR molecules to the response element. In competition experiments with unlabeled oligonucleotides, the formation of both complexes could be inhibited by an excess of TRE-PAL but not by an excess of an unrelated oligonucleotide (Fig. 5B, lanes 3 and 4, respectively), indicating the sequence specific nature of the binding of the expressed hThR β to TRE-PAL.

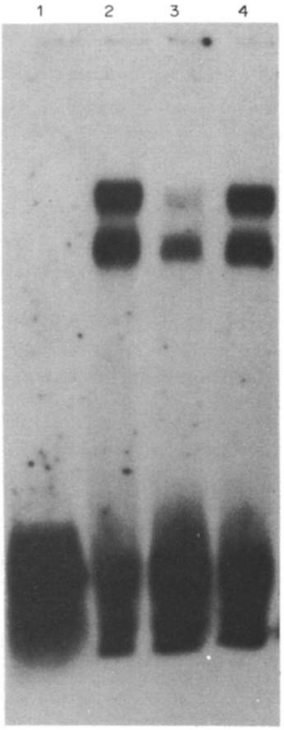

Fig. 5 (B)

Fig. 5. Formation of hThR β dependent protein–DNA complexes and specific and nonspecific competition with unlabeled oligonucleotides. (A) Aliquots (15–30 μg of total protein) of extract from nuclei of uninfected or wt virus infected Sf9 cells or from cells infected with virus encoding hThR β or from RNA primed or unprimed reticulocyte lysate, respectively, was incubated with 15 fmol of ^{32}P-labeled TRE-PAL as described in Experimental. Lane 1, uninfected Sf9 cells; lane 2, wt virus infected Sf9 cells; lane 3, hThR β expressed in Sf9 cells; lane 4, unprimed reticulocyte lysate; lane 5, hThR β synthesized *in vitro*. (B) Specific and nonspecific competition of the interaction of baculovirus expressed hThR β with TRE-PAL was analyzed by incubating the receptor (45 fmol) with ^{32}P-labeled TRE-PAL (15 fmol) in the presence or absence of a 30-fold molar excess of unlabeled TRE-PAL (specific competition) or unlabeled unrelated oligonucleotide (nonspecific competition). Lane 1, free probe (no receptor added); lane 2, ^{32}P-labeled TRE-PAL only; lane 3, competition with a 30-fold molar excess of unlabeled TRE-PAL; lane 4, competition with a 30-fold molar excess of unrelated oligonucleotide.

DISCUSSION

The repertoire of expression systems available for production of foreign gene products is large. There are several examples of bacterial, mammalian and yeast systems [27–29] that have been successfully used in order to express recombinant proteins at very high levels. The best choice of expression system for a particular gene product is therefore sometimes difficult to make. However, there are certain criteria that have an influence on the choice of system such as: (i) the purpose of expressing the gene product at a high level; (ii) the level of product generated and the percent biological activity of produced material; and (iii) the relative cost and easiness to go from small scale to production scale.

The baculovirus expression system has turned out to be a very powerful system for production of large quantities, ranging from 1 to 600 mg/l, of biologically active proteins and has been demonstrated to be capable of proper posttranslational modifications [23, 30, 31]. Furthermore, the system is easy to scale up as suspension culture under serum free conditions [32] with short production runs, 2–3 days. Additionally, the baculovirus system has recently been shown to be a proper system for production of biologically active human glucocorticoid receptor

[33]. For reasons stated above the baculovirus expression system seemed to be the system of choice for high level expression of hThR β.

We have cloned hThR β from the human breast cancer cell line T47D. The entire sequence of the cDNA was determined and showed 100% homology to the published sequence of the human c-*erb*A β [13], classified as β1 [20, 21]. The receptor expressed in Sf9 cells displayed characteristics virtually indistinguishable from those of ThR extracted from mammalian cells. No receptor could be detected in the cytosol indicating its proper translocation to the nuclear compartment and the receptor migrated as a monomeric 4S peak on a glycerol gradient. The receptor appeared largely intact with no shorter, degraded, fragments. The M_w of the baculovirus expressed receptor was similar to that of ThR from rat liver [17, 34, 35] and was in agreement with the M_w predicted from the translational open reading frame of the hThR β cDNA.

The apparent dissociation constant for T_3 of the *in vitro* translated receptor was somewhat lower than that of the baculovirus expressed receptor. This discrepancy could possibly be due to posttranslational modification(s) of receptor expressed in Sf9 cells as well as interference by other nuclear factor(s) co-extracted with the thyroid receptor from Sf9 cells. Similar differences in ligand binging characteristics between *in vitro* translated receptor and receptor extracted from cells have been reported [36]. However, a K_d of 105 pM for ThR in a crude nuclear extract is in good agreement with literature data on ThR β extracted from rat liver [11, 17].

In the DNA binding assays we detected two retarded protein–DNA complexes both when *in vitro* generated as well as when baculovirus expressed receptor were used. This is in disagreement with the data presented by Murray and Towle [37] who did not detect any thyroid receptor–DNA complex formation using *in vitro* synthesized receptor unless nuclear extracts from liver were added. One possible explanation for this discrepancy might be that we used a perfect palindromic TRE sequence which has been shown to have an increased affinity for the receptor as compared to the wt TRE found in the rat growth hormone gene [19]. Another possibility is of course that there are qualitative differences between extracts used for *in vitro* translation and that the reticulocyte lysate we have used contains accessory factors that stabilize the DNA–receptor complexes. In any case, we have shown that both the faster and the slower migrating gel-shifted bands were dependent on the presence of hThR β and the simplest and most straightforward interpretation we can make for the present, based on the data we have, is that the lower, faster migrating band is a receptor–DNA complex consisting of one receptor interacting with the target sequence and that the upper band contains two receptors.

The high affinity of the receptor for T_3 and its sequence specific binding to the TRE containing oligonucleotide suggest that the Sf9 cells express a functional hThR β and that the baculovirus expression system is adequate for large-scale production of receptor for detailed structural and functional studies. Extrapolation of receptor expression levels obtained in Petri dishes to large scale fermentation of suspension cultures of Sf9 cells, which can easily be grown to a density of 2–3 $\times$ 10^6 cells/ml, would imply production of 2.5–3.8 mg hThR β per litre. This level of production makes it possible to obtain sufficient amounts of purified receptor for X-ray crystallography and for more detailed functional studies regarding its interaction with DNA, other receptors and components of the transcriptional machinery.

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