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ORIGINAL ARTICLE

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Altered response to thyroid hormones by prostate and breast cancer cells

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Abstract Transferrin, an abundant bone marrow constituent, has been shown to be a potent mitogen in vitro in the prostate cancer cell line PC3. T4 (L-thyroxine) and T3 (3′,3,5-tri-iodo-L-thyronine) are regulators of cell metabolism. In this study, the effects of nonphysiological concentrations (about two orders of magnitude higher) of T4, T3, T2 (3,5-di-iodo-L-thyronine), RT3 (reverse T3, 3′,5′,3-tri-iodo-L-thyronine) and transferrin (about three orders of magnitude lower) were tested on the prostate cancer cell lines PC3, DU145 and LNCaP, and the breast cancer cell line MCF-7. In PC3 cells, increased proliferation by transferrin could be reversed by the addition of T3 or T4. T4 decreased proliferation in all cell lines tested, while transferrin increased proliferation in PC3 cells only. T3 decreased proliferation in PC3, LNCaP and MCF-7 cells but had no effect on DU145 cells. T4 and T3 gave two-state behavior in LNCaP cells. These results were combined to determine the essential iodines which produced the observed proliferative effects. Cell lines responded differently to T4, T3, T2, RT3 and transferrin suggesting a specific interaction among the compounds tested and the different cell lines. Finally, regulation of gene expression was demonstrated using DU145 cells. Upregulation of c-*fos* mRNA was observed in cultures at early time-points in the presence of T4, transferrin or both. Decreased expression was observed at later time-points with no expression at 4 h. An explanation for these results may be a change in thyroid hormone receptor/ligand affinity. Thus, the interactions between thyroid hormones and cancer cells may be different from those between thyroid hormones and normal cells.

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Introduction

Prostate cancer is the second leading cause of cancer-related deaths in men [3]. A unique property of prostate cancer cells is that metastasis usually occurs to bone [1, 17] and results in blastic lesions with poor bone integrity but dense bone formation. Transferrin is an abundant iron-carrying protein in bone marrow. Transferrin increases proliferation of prostate cancer PC3 and DU145 cells [10, 29] and MCF-7 cells with the addition of high-density lipoproteins [14]. Inhibition of proliferation in the presence of suramin, an anticancer drug, in prostate cancer cell lines PC3 and DU145 is reversed by the addition of transferrin [10].

Prostate cancer cell lines differ in several respects including their response to androgens, vitamin A and vitamin D, all of which require receptor binding followed by DNA transcription. Vitamin D_3 has been investigated in prostate cancer cells for receptor content and antiproliferative effects [5, 13, 15, 24, 35, 36, 47]. 1,25-Dihydroxyvitamin D_3 has an antiproliferative effect on LNCaP and PC3 cells but no effect on DU145 cells [35]. Vitamin A (retinoic acid) receptors are known to form dimers with vitamin D_3 receptors [47]. A biphasic proliferative response (stimulation and inhibition) has been observed following the addition of vitamin A to LNCaP cells [11] and in normal, benign hyperplasia and malignant prostatic epithelial cells [23]. Androgens have distinct effects on prostate cancer cells. PC3 and DU145 cells proliferate in the absence of androgen while LNCaP cells require androgen for proliferation [18, 37, 39].

Thyroid hormones (T4, T3, and RT3) differ in the position of their iodines which interact with receptor proteins and the iodine moiety confers specificity. It has been demonstrated that the difference in the position of the iodines between T4 and T3 is essential for receptor binding and that iodine at the 5′ position (T4) results in

2% of the affinity to receptor T3 [41]. Thyronine compounds with differing iodine substitutions may bind different proteins. An early study concluded that the iodines on thyroid hormones are necessary only for transport and that a methylated thyronine elicits the same responses as T3 [7]. Investigations of the electrostatic interactions between T4 and prealbumin have shown that binding is a function of interactions between the negativeness of iodine and positive charges in the binding pocket [43].

The c-*erbA* protein is a thyroid hormone receptor which is a member of the steroid hormone receptor family [31, 42, 48]. The v-*erbA* protein encodes a viral thyroid hormone receptor which acts as a constitutive repressor of thyroid hormone-responsive genes [8]. The v- and c-*erb* proteins bind response elements in the absence of hormone and suppress transcription [8]. The addition of T3 to cells containing c-*erbA* results in transcription of responsive genes while the addition of T3 to cells with v-*erbA* and c-*erbA* proteins results in continued suppression showing that v-*erbA* is dominant [8]. Mutated thyroid hormone receptors (v-*erbA*) are well characterized, cause erythroleukemia in birds [12, 26] and have been shown to influence actions of EGF (epidermal growth factor) receptors (v-*erbB*) [12].

Thyroid hormones are involved in gene regulation for growth and development [22, 30] and exert their actions by thyroid receptor interaction with DNA. In prostate cells, investigations of c-*fos* and c-*jun* have indicated that these proto-oncogenes can be regulated by androgens. TPA (12-*o*-tetradecanoylphorbol 13-acetate), an activator of protein kinase C (PKC), causes increased c-*fos* and c-*jun* (AP-1, activated protein) mRNA which counters androgen-induced increased levels of prostate specific antigen (PSA) mRNA in LNCaP cells [32]. Similarly, c-*fos* and c-*jun* repress androgen receptor-mediated gene induction in LNCaP cells [45]. The prostate cancer cell line PC82 implanted into nude mice shows decreased c-*fos* expression after androgen withdrawal [27].

Interactions among thyroid hormone receptor and proto-oncogenes have been demonstrated in other systems. For example, c-*erbA* receptors influence c-*jun* and c-*fos* activity [9, 34, 44, 46]. In osteoblasts, T3 decreases proliferation, and decreases EGF-enhanced c-*fos*/c-*jun* mRNA expression [40]. Similarly, thyroid hormone receptors and retinoic acid receptors repress c-*fos* and c-*jun* expression in pituitary GH1 cells [25]. Thyroid hormones may or may not compete with AP-1 (c-*fos* and c-*jun* proteins) for binding to DNA response elements [2, 33].

The relationships between thyroid hormone receptor and cancer cells have been investigated and binding constants (no difference) and receptor numbers have been compared for breast cancer, normal breast tissue, central nervous system tumors, epitheliomas, and sarcomas [19].

In this study, we demonstrated that the addition of thyroid hormones at nonphysiological concentrations (approximately two orders of magnitude higher) can alter proliferation. Using the observed effects we were able to determine the essential iodines which elicit the observed cellular event. These iodines may be involved in interactions with thyroid hormone receptor protein. We further demonstrated that, although thyroid hormone concentrations are high, the hormone exerts its effect through an expected pathway involving regulation of RNA synthesis. Thus, thyroid hormones may interact differently with these cancer cells than with normal cells due to decreased binding of ligand to receptor. This interaction may be integral to the cancerous state.

Materials and methods

Chemicals

L-thyroxine, 3′,3,5-tri-iodo-L-thyronine, 3′,5′,3-tri-iodo-L-thyronine, 3,5-di-iodo-L-thyronine, transferrin, RPMI 1640, Ham's F12 nutrient mixture, DME, M199 and D-glucose were purchased from Sigma Chemical Company (St. Louis, Mo.). Sodium pyruvate, penicillin-streptomycin, trypsin and a RadPrime labeling system were purchased from Gibco BRL (Grand Island, N.Y.). Bradford reagent was purchased from Bio-Rad Laboratories (Hercules, Calif.). ^{3}H-Thymidine and α^{32}P dCTP were purchased from NEN Life Sciences Products (Boston, Mass.). A total RNA isolation system was purchased from Promega (Madison, Wis.). Magna-Graph nylon transfer membrane was purchased from Micron Separations (Westborough, Mass.). A rapid downward transfer system was purchased from Turboblotter (Schleicher & Schuell, Keene, N.H.). Rapid-hyb buffer was purchased from Amersham Life Science Products (Arlington Heights, Ill.). OPTIFLUOR was purchased from Packard BioScience B.V. (The Netherlands).

Cell lines

All established cell lines were obtained from the ATTC catalogue and have been described previously. The prostate cancer cell lines used were LNCaP [16], DU145 [39] and PC3 [18]. The breast cancer cell line used was MCF-7 [38] and was a gift from Cheryl Conover (Mayo Clinic and Mayo Foundation). For some control experiments (see Discussion) primary cultures of porcine vascular smooth muscle cells were utilized and have been previously described [21]. DNA probes c-*fos* and *Tieg* were a gift from T.C. Spelsberg (Mayo Clinic and Mayo Foundation).

Growth medium

Prostate cancer cell lines were maintained in RPMI 1640 (pH 7.4) containing 10% fetal bovine serum. Breast cancer cells were maintained in 45%/45% Ham's F12 nutrient mixture/DME containing 4.5 g/l glucose, 10% fetal bovine serum and 1.0 m*M* sodium pyruvate. Porcine vascular smooth muscle cells were maintained in phenol red-free M-199 medium containing Earle's salts, 200 m*M* L-glutamine and 10% fetal bovine serum. Serum-free medium was essentially the same as that described above but contained 1.0% penicillin-streptomycin.

Proliferation assays

The proliferation assays have been described previously [28]. Briefly, cell cultures were kept in incubators at 37 °C in an atmosphere containing 5% CO_2 for the total time of cell growth. Initial cell cultures were grown and maintained in medium until confluent. Confluent cells were trypsinized to facilitate cell detachment and

excess trypsin was removed by centrifugation followed by resuspension in fresh medium. Resuspended cells were placed in 24-well plates at a density of approximately 25,000 cells/well for prostate cancer cells and 1/10 dilution of a confluent flask for breast cancer cells and incubated for at least 24 h in serum-containing medium to allow cell attachment. LNCaP cells required 48 h incubation in serum-containing medium to achieve sufficient cell attachment. After the cells had attached to the wells, serum-containing medium was replaced with serum-free medium followed by incubation for 48 h before titration of effectors. Effectors were added either directly to each well or diluted to a final concentration in fresh serum-free medium and added to aspirated wells. In either case, the results were the same and are expressed as a percentage of control where no effectors were present.

Cells were grown with effectors for at least 24 h before the addition of ^{3}H-thymidine (1.0 μCi) for a minimum pulse of 6 h which was followed by determination of thymidine incorporation and protein content. For comparison, MCF-7 cells were pulsed for 18 h (data not shown) and the results were the same as with the 6-h pulse.

Thymidine incorporation was determined by methods previously published [28]. Briefly, the medium was aspirated from the wells and the cells were washed twice with ice-cold phosphate-buffered saline (PBS). When cell attachment was weak (LNCaP cells) the cells were washed once with PBS at 37 °C. Proteins were precipitated by the addition of 10% trichloroacetic acid (TCA) to the wells and the plates were kept on ice for a minimum of 10 min. Wells were rinsed once with 10% TCA and 0.1 *N* NaOH (500 μl) was added to the wells to solubilize the proteins. The plates were incubated for 20 min at 37 °C and then stored overnight at 4.0 °C. Aliquots (100 μl) were counted on a Beckman scintillation counter and the results were normalized for the protein content in each well. The protein content was determined by the method of Bradford [4]. The results of the thymidine uptake experiments were confirmed with cell counts.

Thyroid hormones were resuspended in NaOH (≤0.02 *N*) and the final concentration of NaOH was less than 4.0×10^{-4} *N*. Titrations of NaOH at concentrations reaching 4.0×10^{-3} *N* had no effect on cell proliferation in this system (data not shown). Thus, wells containing only vehicle were not included in the experiments.

Statistical analysis

All data were analyzed using one-way ANOVA and Students *t*-test to obtain *P*-values. Deviations of the mean are shown for all data and calculated using standard methods. The results are expressed as percentage of control where no effectors were added to the cells.

Northern blot analysis

Total RNA was isolated with the total RNA isolation system using the manufacturer's protocol with modifications. Briefly, a 75-cm flask of confluent cells was rinsed twice in ice-cold PBS and denaturing solution (1.0 ml) was added to the flask. The slurry (0.5 ml) was transferred to a tube, sodium acetate solution (60 μl) was added, the tube was vortexed. Phenol-chloroform solution (0.5 ml) was added to the tube, which was vortexed and left on ice for 15 min. The tube was centrifuged for 20 min at 13,000 *g*. The top phase was transferred to a new tube and isopropanol (0.5 ml) was added and the tube was stored overnight at −20 °C. The tube was centrifuged at 13,000 *g* for 20 min and the supernatant was discarded. The pellet was resuspended in denaturing solution (0.5 ml) and isopropanol (0.5 ml), and the tube vortexed and stored overnight at −20 °C. The tube was centrifuged at 13,000 *g* for 20 min, the pellet was resuspended in 75% ethanol/25% diethylpyrocarbonate (DEPC)-treated water and the tube was vortexed. The tube was centrifuged at 13,000 *g* for 20 min and the pellet was resuspended in DEPC water (50 μl). The RNA concentration was determined by absorbance at 260 and 280 nm.

RNA (20 μg/lane) was separated by electrophoresis on a 1.0% agarose formaldehyde (1 *M*) gel at a constant 80 V for a time sufficient for the dye front to reach the bottom of the gel according to a published protocol [6]. RNA was transferred to a nylon membrane using the rapid downward transfer system according to the manufacturer's protocol for neutral transfer. Total RNA (18 and 28 S) was visualized by staining the blot with 0.03% (w/v) methylene blue in 0.3 *M* sodium acetate [6]. The membrane was hybridized using Rapid-hyb buffer from Amersham with ^{32}P-labeled probes prepared with a RadPrime labeling system according to the manufacturer's specifications. Autoradiography was done at −70 °C using Kodak Scientific imaging film.

Results

Thyroid hormones, transferrin and PC3 cells

Transferrin, when added to cultures of prostate cancer PC3 cells, stimulated cell proliferation. Titrations of transferrin were done to determine the dilutions at which increased cell proliferation occurred and was linear with transferrin (data not shown), which ensured that the effects of additional compounds would be observed. Thus, at the dilutions used, transferrin was not saturating. The dilutions of transferrin used in the experiments increased proliferation by 100–150% (Fig. 1A, lane 5, 0.63 ng/ml; Fig. 1B, lane 5, 0.96 ng/ml) compared to control cultures (Fig. 1A–D, lane 6) but increases of up to 250% were attainable (data not shown). Titration of T3 (Fig. 1A) and T4 (Fig. 1D) to PC3 cell cultures caused a reduction in the stimulatory effect of transferrin (Fig. 1A,D, lane 5). Titration of RT3 to cell cultures had no effect on cells incubated with or without transferrin (Fig. 1B,C). At the highest concentrations of T3 and T4 (Fig. 1A,D, lane 4) cell proliferation was less than that of control cells (lanes 6) grown in serum-free medium.

Titration of T3 or T4 to PC3 cell cultures without transferrin decreased cell proliferation compared to control cells (data not shown) in a fashion that was similar to experiments with cell lines that were unresponsive to transferrin (see below).

Thyroid hormones, transferrin and DU145 cells

DU145 cells were tested for increased proliferation in the presence of transferrin. Transferrin had no effect when added to DU145 cell cultures (data not shown). Titration of T4 to DU145 cell cultures decreased cell proliferation (Fig. 2B, lanes 1–4) compared to control cultures (Fig. 2B, lanes 5 and 6). Titration of T3 or RT3 to DU145 cell cultures had no effect on proliferation compared to control cultures (Fig. 2A,C).

Thymidine uptake in duplicate sets of four wells each containing control cells was determined to evaluate the degree of experimental deviation between controls in the same experiment in addition to the experimental deviation between repeat experiments. This method was used in all subsequent experiments. The differences between control sets were small ($< 10\%$) and insignificant when compared only to each other (compare lanes 5 and 6, Fig. 2A–C).

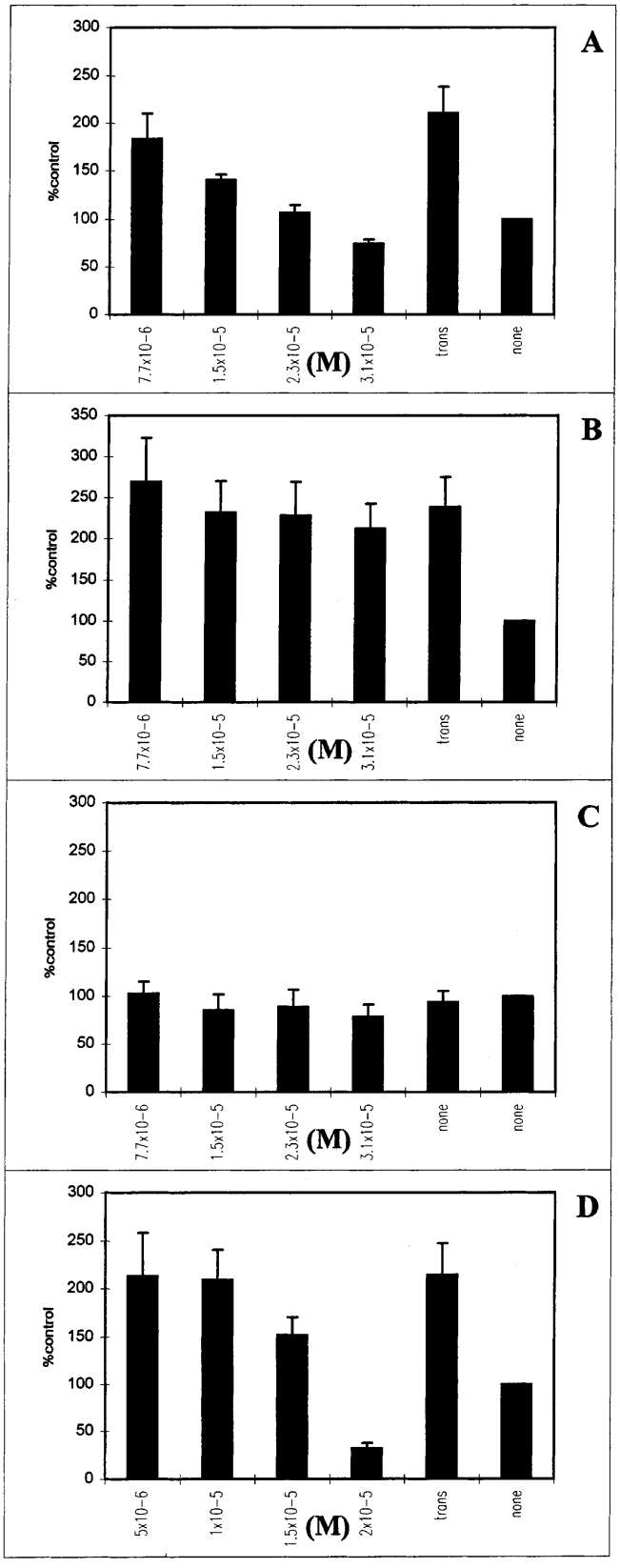

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Fig. 1A–D Thyroid hormones, transferrin and PC3 cells. PC3 cells were grown for 24 h in the presence of added effectors. Cells were pulsed for 6 h with ^{3}H-thymidine to determine differences in cell proliferation. All counted samples were normalized by protein content and plotted as a percentage of control to which no effectors were added. All effector concentrations were determined in quadruplicate for each experiment and n represents the number of experiments. Effectors used were: **A** transferrin (0.63 ng/ml) and titration of T3, $n = 4$ and $P < 0.001$; **B** transferrin (0.96 ng/ml) and titration of RT3, $n = 4$ and $P < 0.008$; **C** titration of RT3 alone, $n = 4$ and $P < 0.113$; **D** transferrin (0.63 ng/ml) and titration of T4, $n = 4$ and $P < 0.001$

Even though transferrin had no effect on DU145 cells, additional experiments were done to ensure that no interactions occurred between the thyroid hormone and transferrin (data not shown). The results of experiments done with or without transferrin were virtually identical. Thus, DU145 cells only responded to the addition of T4.

Thyroid hormones, transferrin and LNCaP cells

LNCaP cells were tested for increased proliferation in the presence of transferrin. Transferrin had no effect when added to LNCaP cell cultures (data not shown). LNCaP cells displayed two behaviors that were dependent on the concentrations of T3 or T4 added to the culture. At low concentrations of T3 or T4, proliferation was stimulated compared to control cultures (Fig. 3A,B, lanes 1 and 2). At higher concentrations of T3 or T4, proliferation was inhibited compared to control cultures (Fig. 3A,B, lane 4). Titration of RT3 to LNCaP cell cultures resulted in increased cell proliferation only (Fig. 3C).

The differences between control sets were slightly larger ($< 20\%$) in some cases than those observed in other cell lines (compare lanes 5 and 6, Figs. 1–4), but these differences did not affect the observed trends.

Even though transferrin had no effect on LNCaP cells, additional experiments were done to ensure that no interactions occurred between the thyroid hormone and transferrin (data not shown). The results of experiments done with or without transferrin were virtually identical. Thus LNCaP cells displayed two types of behavior following the addition of T3 or T4 to cell cultures, and only increased proliferation following the addition of RT3.

Thyroid hormones, transferrin and MCF-7 cells

MCF-7 cells were tested for increased proliferation in the presence of transferrin. Transferrin had no effect when added to MCF-7 cell cultures (data not shown). Titration of T3 or T4 to MCF-7 cell cultures decreased cell proliferation (Fig. 4A,B, lanes 1–4) compared to control cultures (Fig. 4A,B, lanes 5 and 6). Titration of RT3 to MCF-7 cell cultures had no effect on proliferation compared to control cultures (Fig. 4C).

The differences between control sets were small ($< 10\%$) and insignificant when compared only to each other (compare lanes 5 and 6, Fig. 4A–C).

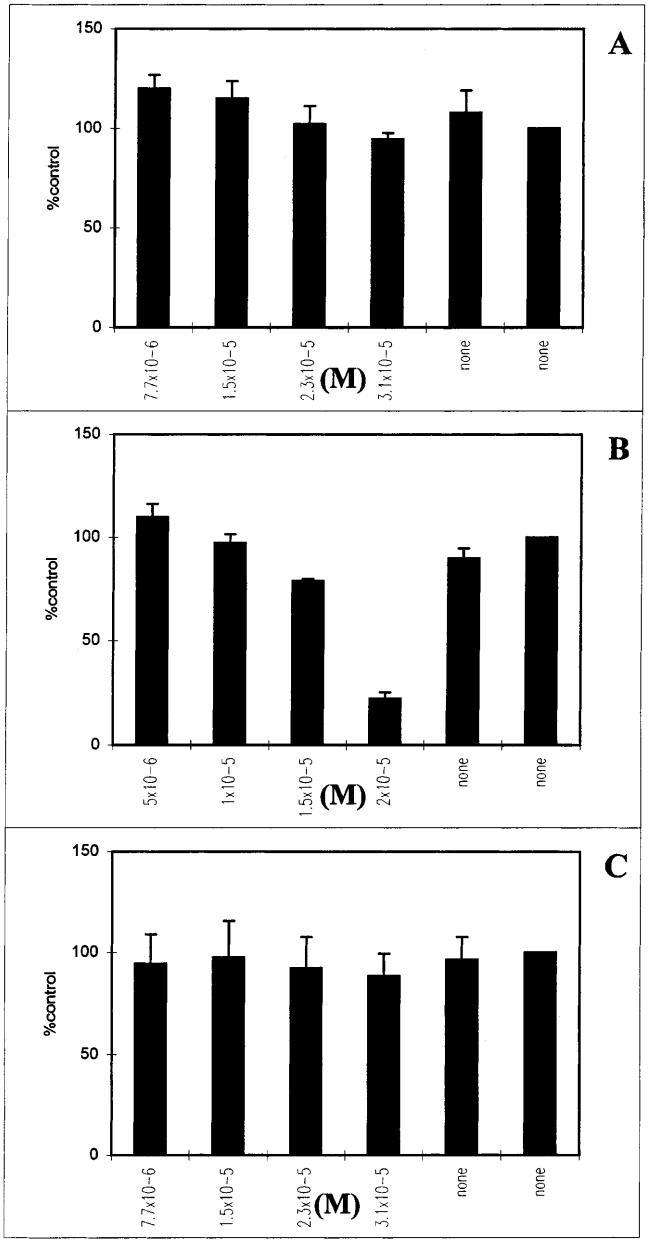


Fig. 2A–C Thyroid hormones, transferrin and DU145 cells. DU145 cells were grown for 24 h in the presence of added effectors. Cells were pulsed for 6 h with ^{3}H-thymidine to determine differences in cell proliferation. All counted samples were normalized by protein content and plotted as a percentage of control to which no effectors were added. All effector concentrations were determined in quadruplicate for each experiment and n represents the number of experiments. Effectors used were: **A** titration of T3, $n = 3$ and $P = 0.011$; **B** titration of T4, $n = 3$ and $P < 0.001$; **C** titration of RT3 where $n = 4$ and $P = 0.851$

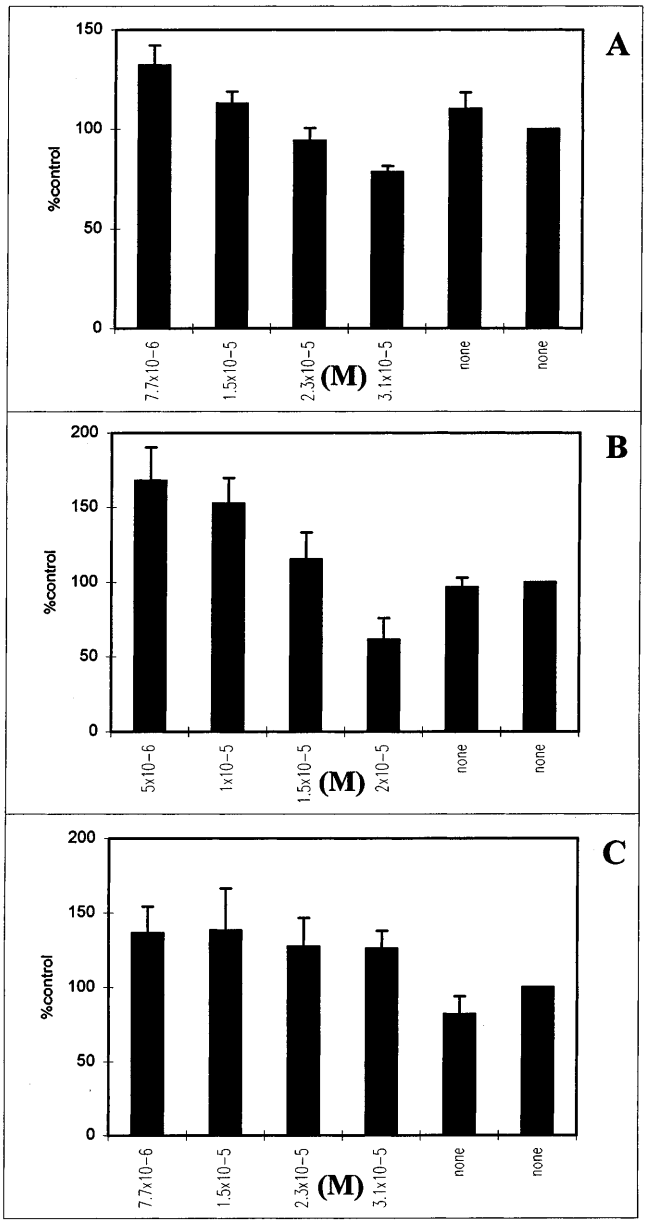
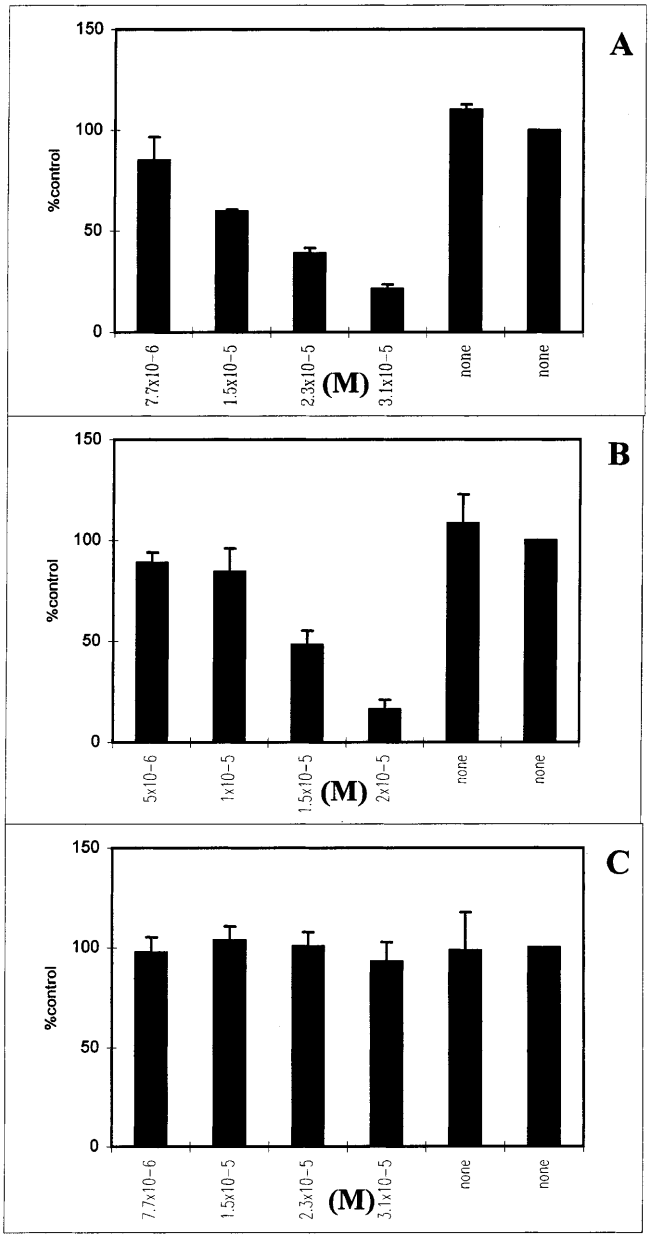


Fig. 3A–C Thyroid hormones, transferrin and LNCaP cells. LNCaP cells were grown for 24 h in the presence of added effectors. Cells were pulsed for 6 h with ^{3}H-thymidine to determine differences in cell proliferation. All counted samples were normalized by protein content and plotted as a percentage of control to which no effectors were added. All effector concentrations were determined in quadruplicate for each experiment and n represents the number of experiments. Effectors used were: **A** titration of T3, $n = 4$ and $P < 0.001$; **B** titration of T4, $n = 7$ and $P < 0.001$; **C** titration of RT3, $n = 4$ and $P = 0.001$

Even though transferrin had no effect on MCF-7 cells, additional experiments were done to ensure that no interactions occurred between the thyroid hormone and transferrin (data not shown). The results of experiments done with or without transferrin were virtually identical. Thus, MCF-7 cells responded to titration of T3 or T4 to cell cultures, while RT3 had no effect.

Titration of 3,3′-di-iodo-L-thyronine to cell cultures

T2 was tested on all the cell lines used in this study. The addition of T2 to LNCaP cells caused an increase in proliferation (Fig. 5D) greater than that observed with the addition of RT3 (Fig. 3C) but no decrease in proliferation was observed. The addition of T2 to PC3 and MCF-7 cell cultures decreased proliferation compared to



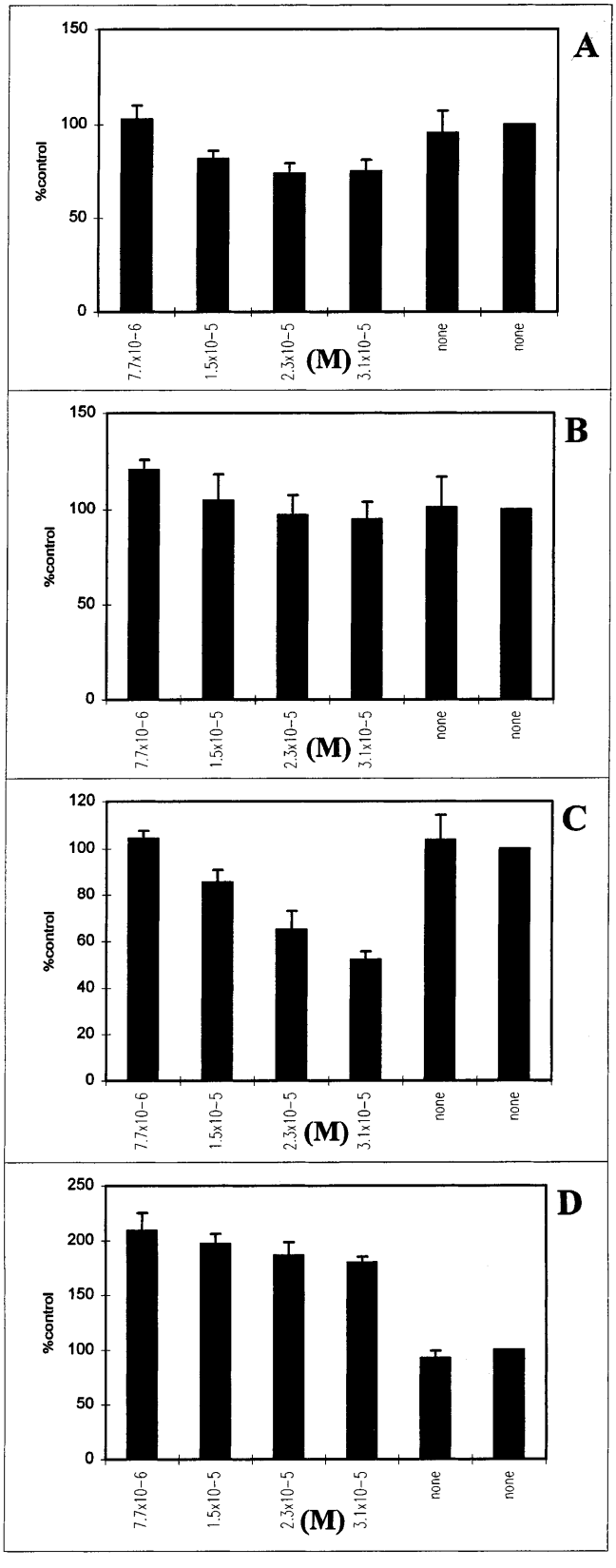


Fig. 4A–C Thyroid hormones, transferrin and MCF-7 cells. MCF-7 cells were grown for 24 h in the presence of added effectors. Cells were pulsed for 6 h with ^{3}H-thymidine to determine differences in cell proliferation. All counted samples were normalized by protein content and plotted as a percentage of control to which no effectors were added. All effector concentrations were determined in quadruplicate for each experiment and n represents the number of experiments. Effectors used were: **A** titration of T3, $n = 4$ and $P < 0.001$; **B** titration of T4, $n = 6$ and $P < 0.001$; **C** titration of RT3, $n = 4$ and $P = 0.79$

control cultures (Fig. 5A,C). The addition of T2 to DU145 cell cultures had no effect on cell proliferation (Fig. 5B).

Thyroid hormone comparison

Four different cancer cell lines responded differently to the addition of thyroid hormones. Binding of hormone to receptor protein may involve interaction of negatively charged iodine with positive charges on the receptor protein. These interactions may determine the specificity

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Fig. 5A–C Titration of 3,3′-di-iodo-L-thyronine to cell cultures. Cultures were grown for 24 h in the presence of T2. Cells were pulsed for 6 h with ^{3}H-thymidine to determine differences in cell proliferation. All counted samples were normalized by protein content and plotted as a percentage of control to which no effectors were added. All effector concentrations were determined in quadruplicate for each experiment and *n* represents the number of experiments. Cell lines were: **A** PC3, $n = 4$ and $P < 0.001$; **B** DU145, $n = 4$ and $P = 0.046$; **C** MCF-7, $n = 3$ and $P < 0.001$; **D** LNCaP, $n = 4$ and $P < 0.001$

for ligand-receptor binding so that a comparison of the responses to thyroid hormones in relation to the differences in the position and the number of iodines might indicate differences between receptor proteins.

All four cell lines responded to titration of T4. Titration of T3 was ineffective only in DU145 cells. Two responses were observed in LNCaP cells in response to titration of T3 or T4. RT3 had no effect on PC3, DU145, or MCF-7 cells, but resulted in increased proliferation of LNCaP cells. T2 was ineffective in DU145 cells only.

The thyroid hormones tested differ structurally in the position and number of iodines. A comparison of the responses of the cell lines tested in relation to the number and position of the iodines that are on each compound is shown in Table 1. Extrapolation of the results was used to determine the iodines that are essential (Table 1, bottom row) to the elicitation of the observed cellular responses. The prostate cancer cell line PC3 and breast cancer cell line MCF-7 may require a minimum of iodines in the 3 and 5 positions. The prostate cancer cell line DU145 may require a minimum of iodines in the 5′ and 5 positions of L-thyronine. The prostate cancer cell line LNCaP may require a minimum of iodines in the 3 and 5 positions to stimulate proliferation while an iodine in the 3′ position may inhibit proliferation.

Northern analysis

Cultures of the prostate cancer cell line DU145 were treated with transferrin (0.63 ng/ml), T4 (2×10^{-5} *M*), or transferrin and T4. Exposure times were 4, 2, 1 and 0.5 h. The nylon membrane containing the total RNA was probed with ^{32}P-labeled c-*fos* (Fig. 6A) or ^{32}P-labeled *Tieg* (Fig. 6B) cDNA probes. The autoradiographic results showed that both transferrin and T4 were effective in inducing c-*fos* mRNA with exposure times of 0.5–1 h (Fig. 6A, lanes 2–4 and 7) compared to control cultures (Fig. 6A, lane 1). With longer exposure times expression of c-*fos* mRNA decreased to zero. In contrast, no change in *Tieg* mRNA expression (Fig. 6B) compared with control cultures was observed with either compound with any exposure time.

Table 1 Proliferation responses of the cell lines to thyroid hormones in relation to the number and position of their iodines. The results were extrapolated to determine the iodines that are essential (*bottom row*) to the elicitation of the observed cellular responses

Hormone	Iodines	PC3	DU145	MCF-7	LNCaP
T4	3′, 5′, 3, 5	Neg	Neg	Neg	Pos/neg
T3	3′, 3, 5	Neg	None	Neg	Pos/neg
RT3	3′, 5′, 3	None	None	None	Pos
T2	3, 5	Neg	None	Neg	Pos
Essential iodines		3, 5	5′, 5	3, 5	3, 5 for stimulation; 3′ for inhibition

Discussion

The action of hormones on cancer cells has been a topic of great research interest and speculation as to the mechanism of hormone action has varied with different cell lines. In this study, we showed that non-physiological concentrations of thyroid hormones had a marked effect on prostate and breast cancer cell lines. Prostate and breast cancers may have common mutations in the thyroid hormone receptor leading to a cancerous state.

Thyroid hormones have several points where interaction of ligand and receptor might occur. In biological systems, T3 and T4 differ by one iodine but are quite different in cellular response with T3 being the more potent effector.

A logical explanation of the data presented in this study is that the ligand affinity for the thyroid hormone receptor has decreased. This has been observed previously in studies in which v-*erbA*, a viral oncogene, has been shown to result in a receptor protein with abolished ligand binding capability [31].

A unique situation exists with LNCaP cells which required testing compounds with iodines in various positions in L-thyronine to determine possible specific interactions between ligand and receptor. Two types of proliferative behavior could be accounted for by differences in the position of the iodines by extrapolation of the results from all the compounds tested. Comparison of cells grown with RT3 (Fig. 3C) or T2 (Fig. 5D) show

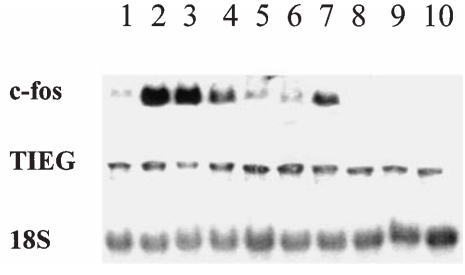

Fig. 6 Northern analysis. Cultures were untreated control (*lane 1*), or treated with transferrin and T4 (*lane 2*, see below for concentrations), T4 (2×10^{-5} *M*, *lanes 3–6*), or transferrin (0.63 ng/ml, *lanes 7–10*). Treatments were for 0.5 h (*lanes 2, 3 and 7*), 1 h (*lanes 4 and 8*), 2 h (*lanes 5 and 9*), 4 h (*lanes 6 and 10*). The membrane was probed with c-*fos* (**A**) or *Tieg* (**B**)

a greater stimulation of proliferation following the addition of T2 (iodines 3 and 5), so that even though proliferation was stimulated by RT3 (iodines 3′, 5′ and 3), a greater magnitude was achieved by inclusion of iodine 5 and exclusion of iodines at 3′ and 5′. Thus, the essential iodine that results in inhibition of cell proliferation, by extrapolation of these results, may be at position 3′. The iodines that contribute to increased cell proliferation are at positions 3 and 5.

Our data for LNCaP cells indicate that the two proliferation responses may be due to thyroid hormone binding on two different receptors where specificity is determined by interactions between specific iodines and charges on the receptor protein. Therefore, the term "biphasic" would be inappropriate for describing this situation.

The prostate cancer cell line PC3 and breast cancer cell line MCF-7 behaved in a similar fashion with proliferation inhibited by compounds containing iodines at position 5 and possibly position 3 (Fig. 5A,C). The behavior of the prostate cancer cell line DU145 was the easiest to delineate: iodines at positions 5 and 5′ were necessary to inhibit proliferation.

Other investigators have defined a medium for MCF-7 cells which supports cell growth and allows observation of the stimulatory effects of transferrin [14, 20]. For the continuity of this study we determined that a demonstration of the stimulatory effects of transferrin on breast cancer cells was unnecessary. In our system the effects of transferrin may be indistinguishable due to other factors including the absence of high-density lipoproteins. In any event, we expected that T2, T3 and T4 would counter the effects of transferrin in a fashion similar to the effects in PC3 cells.

Several lines of evidence suggest that the effects of thyroid hormones on cancer cells is not due to an inherent artifact of this system. Different thyroid hormones elicited different effects on different cell types. T4 was the only hormone that consistently decreased proliferation in all cell types. However, low concentrations of T4 stimulated proliferation of LNCaP cells. So that even though all cells ultimately responded to T4 by a decrease in proliferation, the behavior was not uniform across all cell lines. No hormone gave a uniform response across all cell lines. This latter observation would be expected since the cell lines tested are not uniform in their response to other hormones [11, 23, 35].

A further demonstration of nonuniformity is shown by the elicitation of a cell response to T2. These results suggest that changes in iodine position and number can give results that are explained by interactions between ligand and receptor. Further experimentation is necessary to delineate all mechanisms.

A concern was that high concentrations of thyroid hormones (10^{-5} *M*) may have been toxic to the cell cultures. Thyroid hormones (10^{-5} *M*) were added to cultures of vascular smooth muscle cells and serum (10% final concentration) was used to stimulate proliferation of cells grown in serum-free medium for 24 h. The results showed that the thyroid hormones did not inhibit proliferation compared to control cultures (data not shown). The vascular smooth muscle cells were primary cultures and should have been more sensitive to added effectors than the cell lines used in this study. Thus, it appeared that serum-containing medium attenuated the effects of nonphysiological concentrations of thyroid hormones. Furthermore, a nonuniform response of different cell lines to hormones suggests a more specific interaction not involving toxicity.

A concern was that cells displaying only inhibition of proliferation would require lower hormone concentrations than those used in this study to display stimulation of proliferation. PC3 and MCF-7 cells were tested for stimulation of proliferation by treatment with T3 and T4 at concentrations down to 10^{-8} *M* and no change was observed. Thus, proliferation trends could be used to determine the essential iodines.

Hormone receptor binding followed by receptor hormone complex-DNA interaction and finally RNA expression is a pathway expected if these nonphysiological concentrations of hormones are exerting their effect through the classical pathway. We were able to demonstrate with limited experiments that the conditions used for proliferation assays also affect mRNA expression of the proto-oncogene c-*fos*. This finding is important in proposing an explanation for the observed phenomena.

An upshot of these findings is that although transferrin did not affect proliferation of DU145 cells, it did affect c-*fos* mRNA expression. These results were unexpected and suggest that the effects of transferrin treatment on mRNA regulation should be tested in all cell lines. Experimental concentrations of transferrin were determined by titration to PC3 cells and choosing a point where increased proliferation was sufficient to observe countering effects. A concentration of 0.63 ng/ml transferrin is three orders of magnitude lower than physiological concentrations and the effects of transferrin may still need to be determined.

A unique aspect of this study was that we showed that nonphysiological concentrations of hormone can elicit responses that may occur at physiological concentrations in normal cells. A thyroid hormone concentration increase of two orders of magnitude is large for a biological system. However, from a biochemical view this increase would be expected for a receptor with significant change in affinity for ligand due to mutation in the ligand binding region. Further research is necessary to confirm thyroid receptor protein status and possible correlations with data from this study.

References

1. Aaron AD (1994) The management of cancer metastatic to bone. JAMA 272: 1206
2. Bodenner DL, McClaskey JH, Kim MK, Mixson AJ, Weintraub BD (1992) The pronto-oncogenes c-fos and

c-jun modulate thyroid hormone inhibition of human thyrotropin β subunit gene expression in opposite directions. Biochem Biophys Res Commun 189: 1050
3. Boring CC, Squires TS, Tong T (1993) Cancer statistics. CA Cancer J Clin 43: 7
4. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248
5. Braun MM, Helzlsouer KJ, Hollis BW, Comstock GW (1995) Prostate cancer and prediagnostic levels of serum vitamin D metabolites (Maryland, United States). Cancer Causes Control 6: 235
6. Brown T, Mackey K (1998) Analysis of RNA by Northern and slot blot hybridization. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Struhl K, Chanda VB (eds) Current protocols in molecular biology. John Wiley & Sons, New York
7. Cody V (1980) Role of iodine in thyroid hormones: molecular conformation of a halogen-free hormone analogue. J Med Chem 23: 584
8. Damm K, Thompson CC, Evans RM (1989) Protein encoded by v-*erbA* functions as a thyroid-hormone receptor antagonist. Nature 339: 593
9. Debois C, Aubert D, Legrand C, Pain B, Samarut J (1991) A novel mechanism of action for v-ErbA: abrogation of the inactivation of transcription factor AP-1 by retinoic acid and thyroid hormone receptors. Cell 67: 731
10. Donat SM, Powell CT, Israeli RS, Fair WR, Heston WDW (1995) Reversal by transferrin of growth-inhibitory effect of suramin on hormone-refractory human prostate cancer cells. J Natl Cancer Inst 87: 41
11. Esquenet M, Swinnen JV, Heyns W, Verhoeven G (1995) Triiodothyronine modulates growth, secretory function and androgen receptor concentration in the prostatic carcinoma cell line LNCaP. Mol Cell Endocrinol 109: 105
12. Gandrillon O, Jurdic P, Benchaibi M, Xiao J-H, Ghysdael J, Samarut J (1987) Expression of the *v-erbA* oncogene in chicken embryo fibroblasts stimulates their proliferation in vitro and enhances tumor growth in vivo. Cell 49: 687
13. Gann PH, Ma J, Hennekens CH, Hollis BW, Haddad JG, Stampfer MJ (1998) Circulating vitamin D metabolites in relation to subsequent development of prostate cancer. Cancer Epidemiol Biomarkers Prev 5: 121
14. Gospodarowicz D, Lui GM, Gonzalez R (1982) High-density lipoproteins and the proliferation of human tumor cells maintained on extracellular matrix-coated dishes and exposed to defined medium. Cancer Res 42: 3704
15. Hedlund TE, Moffatt KA, Miller GJ (1996) Stable expression of the nuclear vitamin D receptor in the human prostatic carcinoma cell line JCA-1: evidence that the antiproliferative effects of 1α25-dihydroxyvitamin D_3 are mediated exclusively through the genomic signaling pathway. Endocrinology 137: 1554
16. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, Murphy GP (1983) LNCaP model of human prostatic carcinoma. Cancer Res 43: 1809
17. Jacobs SC (1983) Spread of prostate cancer to bone. Urology 21: 337
18. Kaighn ME, Narayan KS, Ohnuki Y, Leehaer JF, Jones LW (1979) Establishment and characterization of a human prostatic carcinoma cell line (PC3). Investig Urol 17: 432
19. Lemaire M, Mahiel B (1986) Nuclear thyroid hormone receptors in human cancer tissues. Anticancer Res 6: 695
20. Lippman ME, Allegra JC, Strobl JS (1980) A hormone-dependent breast cancer cell line grown in defined medium. Recent Results Cancer Res 71: 69
21. Moraghan T, Antoniucci DM, Grenert JP, Sieck GC, Johnson C, Miller VA, Fitzpatrick LA (1996) Differential response in cell proliferation to beta estradiol in coronary arterial vascular smooth muscle cells obtained from mature female versus male animals. Endocrinology 137: 5174
22. Oppenheimer JH (1979) Thyroid hormone action at the cellular level. Science 203: 971
23. Peehl DM, Wong ST, Stamey TJ (1993) Vitamin A regulates proliferation and differentiation of human prostatic epithelial cells. Prostate 23: 69
24. Peehl DM, Skowronski RJ, Leung GK, Wong ST, Stamey TJ, Feldman D (1994) Antiproliferative effects of 1,25-dihydroxyvitamin D3 on primary cultures of human prostatic cells. Cancer Res 54: 805
25. Perez P, Schonthal A, Aranda A (1993) Repression of *c-fos* gene expression by thyroid hormone and retinoic acid receptors. J Biol Chem 268: 23538
26. Privalsky ML (1992) *v-erb A*, nuclear hormone receptors, and oncogenesis. Biochem Biophys Acta 1114: 51
27. Rijnders AWM, Korput JAGM van der, Steenbrugge GJ van, Romijn JC, Trapman J (1985) Expression of cellular oncogenes in human prostatic carcinoma cell lines. Biochem Biophys Res Commun 132: 548
28. Ritchie CK, Maercklein PB, Fitzpatrick LA (1994) Direct effect of calcium channel antagonists on osteoclast function: alterations in bone resorption and intracellular calcium concentrations. Endocrinology 135: 996
29. Rossi MC, Zetter BR (1992) Selective stimulation of prostatic carcinoma cell proliferation by transferrin. Proc Natl Acad Sci USA 89: 6197
30. Samuels HH, Forman BM, Horowitz ZD, Ye ZS (1988) Regulation of gene expression by thyroid hormone. J Clin Invest 81: 957
31. Sap J, Munoz A, Damm K, Goldberg Y, Ghysdael J, Leutz A, Beug H, Vennstrom B (1986) The c-*erb-A* protein is a high-affinity receptor for thyroid hormone. Nature 324: 635
32. Sato N, Sadar MD, Bruchovsky N, Saatcioglu F, Rennie PS, Sato S, Lange PH, Gleave ME (1997) Androgenic induction of prostate-specific antigen gene is repressed by protein-protein interaction between the androgen receptor and AP-1/c-jun in the human prostate cancer cell line LNCaP. J Biol Chem 272: 17485
33. Schmidt ED, Cramer SJ, Offringa R (1993) The thyroid hormone receptor interferes with transcriptional activation via the AP-1 complex. Biochem Biophys Res Commun 192: 151
34. Sharif M, Privalsky ML (1992) V-erbA and c-erbA proteins enhance transcriptional activation by c-jun. Oncogene 7: 953
35. Skowronski RJ, Peehl DM, Feldman D (1993) Vitamin D and prostate cancer: 1,25 dihydroxyvitamin D_3 receptors and actions in human prostate cancer cell lines. Endocrinology 132: 1952
36. Skowronski RJ, Peehl DM, Feldman D (1995) Actions of vitamin D_3 analogs on human prostate cancer cell lines: comparison with 1,25-dihydroxyvitamin D_3. Endocrinology 136: 20
37. Sonnenschein C, Olea N, Pasanen ME, Soto AM (1989) Negative controls of cell proliferation: human prostate cancer cells and androgens. Cancer Res 49: 3474
38. Soule HD, Varguez J, Long A, Albert S, Brennan M (1973) A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51: 1409
39. Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF (1978) Isolation of a human prostate carcinoma cell line {DU145}. Int J Cancer 21: 274
40. Varga F, Rumpler M, Luegmayr E, Fratzl-Zelman N, Glantschnig H, Klaushofer K (1997) Triiodothyronine, a regulator of osteoblastic differentiation: depression of histone H4, attenuation of c-fos/c-jun, and induction of osteocalcin expression. Calcif Tissue Int 61: 404
41. Wagner RL, Apriletti JW, McGrath ME, West BL, Baxter JD, Fletterick RJ (1995) A structural role for hormone in the thyroid hormone receptor. Nature 378: 690
42. Weinberger C, Thompson CC, Ong ES, Lebo R, Gruol DJ, Evans RM (1986) The c-*erb-A* gene encodes a thyroid hormone receptor. Nature 324: 641
43. Weiner PK, Langridge R, Blaney JF, Schaefer R, Kollman PA (1982) Electrostatic potential molecular surfaces. Proc Natl Acad Sci USA 79: 3754
44. Wondisford FE, Steinfelder HJ, Nations M, Radovick S (1993) AP-1 antagonizes thyroid hormone receptor action on the thyrotropin beta-subunit gene. J Biol Chem 268: 2749

45. Young CYF, Murtha PE, Zhang J (1994) Tumor-promoting phorbol ester-induced cell death and gene expression in a human prostate adenocarcinoma cell line. Oncol Res 6: 203
46. Zhang XK, Wills KN, Husmann M, Hermann T, Pfahl M (1991) Novel pathway for thyroid hormone action through interaction with jun and fos oncogene activities. Mol Cell Biol 11: 6016
47. Zhuang S-H, Schwartz GG, Cameron D, Burnstein KL (1997) Vitamin D receptor content and transcriptional activity do not fully predict antiproliferative effects of vitamin D in human prostate cancer cell lines. Mol Cell Endocrinol 126: 83
48. Zilliacus J, Wright AP, Carlstedt-Duke J, Gustafsson JA (1995) Structural determinants of DNA-binding specificity by steroid receptors. Mol Endocrinol 9: 389