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ANDROGEN REGULATES MMTV RNA IN THE SHORT-TERM IN S115 MOUSE MAMMARY TUMOUR CELLS

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Summary—This report demonstrates that androgens as well as glucocorticoids can regulate MMTV RNA production in the short term. In S115 mouse mammary tumour cells, MMTV RNA accumulation is regulated within hours by androgen, at a time before any increase in DNA synthesis can be detected, thus providing a marker of an early postreceptor molecular event in steroid action on these cells. Androgen acts via its own receptor and not by cross-binding to the glucocorticoid receptor. The effects are at transcription and not just on stabilisation of RNA because they are blocked by actinomycin D. However, the androgen action shows some partial dependence on simultaneous protein synthesis since cycloheximide is inhibitory. The androgen regulation of MMTV RNA is compared and contrasted with that by glucocorticoids in these cells.

INTRODUCTION

Steroid hormones regulate the growth of many normal and tumour cells. Early events involve binding of steroid to its specific receptor within the cell [1] and molecular studies have now defined regions in the DNA which bind the receptor molecules [2]. Late events involve general increased protein and RNA synthesis followed by increased DNA synthesis. However, events in between receptor interaction and these late events remain uncharacterised.

The Shionogi S115 cell line provides a model system in which to study postreceptor events *in vitro*. This cell line was derived from the Shionogi androgen-dependent mouse mammary carcinoma of a DD/Sio mouse [3] and exhibits a positive proliferative response to androgens in tissue culture (+A cells) [4]. Removal of testosterone (−T) from the cells, results within 1–3 days in a 50% reduction in growth rate [5] and a total change of all cells from fibroblastic to epithelial morphology [6]. The cells develop marked changes in cytoskeletal structure [7], an increased dependence on anchorage to the substrate for growth [8], loss of ability to grow in suspension culture [6] and an increased density regulation [9].

On the basis of these dramatic alterations in cell behaviour, it has been suggested that S115 cells resemble transformed cells in the presence of testosterone but are more like normal cells in its absence [7, 8]. These cells are, of course, all cancer cells and will form tumours in nude mice [10]. However, given this parallel to phenotypic change in transformation, it is a possibility that androgen action in these S115 cells might involve a postreceptor event of switching on production of transforming elements. In view of the well documented involvement of mouse mammary tumour virus (MMTV) in the development of mammary carcinomas in the mouse [11], MMTV might play such a role.

We have shown previously that MMTV RNA is made in S115 cells only in the long-term presence of androgen. Prolonged culture in the absence of androgen results not only in loss of the MMTV RNA but also in increased methylation of MMTV sequences in the DNA [12]. It is now well established that expression of MMTV proviral DNA is stimulated by glucocorticoids, and that this is a primary response to the hormone mediated by the glucocorticoid receptor which can bind to the long terminal repeat (LTR) region [2]. We have examined here whether the androgen regulation of MMTV RNA in the S115 cells is also a direct response to testosterone regulated by its own receptor and have compared the regulation by androgen with that by glucocorticoids.

EXPERIMENTAL

Culture of stock S115 cells

Stock of androgen maintained S115 cells (+A) were a cloned cell line exhibiting a positive proliferative response to androgens. Cells were grown routinely as monolayer cultures in DMEM supplemented with 2% foetal calf serum (FCS) [Gibco Bio-Cult], 40 mM HEPES buffer (Sigma) and 3.5×10^{-8} M testosterone in a humidified atmosphere of 10% carbon dioxide in air at 37°C. Cells were subcultured at weekly intervals.

Growth of S115 cells for RNA

Cells from stock plates were suspenced by treatment in 0.06% trypsin buffered with 0.02% EDTA

(pH 7.3) and counted on a haemocytometer. Cells were added to the overall required volume of DMEM containing 2% dextran-charcoal stripped foetal calf serum (DC-FCS), 40 mM HEPES buffer with or without 3.5×10^{-8} M testosterone. DC treatment of serum has been described previously [13]. Cells were plated in monolayer into 15 cm plastic tissue culture dishes (Falcon) in 45 ml aliquots at a density of 9×10^5 cells per dish. Medium was changed at appropriate times to contain 3.5×10^{-8} M testosterone (Steraloids Ltd, Croydon, England) or 10^{-7} M dexamethasone (Sigma). Testosterone and dexamethasone were dissolved in ethanol and added to culture medium such that the final concentration of ethanol was less than 0.01%. For cycloheximide and actinomycin D experiments, the medium was changed to contain cycloheximide alone (Sigma) [2 μg/ml] for 1 h to inhibit protein synthesis or actinomycin D alone (Sigma) [10 μg/ml] for 1 h to inhibit transcription. The medium was then changed again to contain testosterone or dexamethasone as well as cycloheximide or actinomycin D. For each RNA analysis, a minimum of 3 dishes were pooled.

Growth of MGR cells

MGR cells are mink lung cells infected with the GR strain of MMTV. Stock cells were grown as monolayer cultures in DMEM supplemented with 5% FCS in a humidified atmosphere of 10% carbon dioxide in air at 37°C. For RNA analysis, cells were grown as for stocks but in 5% DC-FCS in 15 cm plastic tissue culture dishes to a density of about 2/3 confluence. The medium was then changed to DMEM supplemented with 5% DC-FCS and 10^{-7} M dexamethasone or 3.5×10^{-8} M testosterone. The cells were harvested 24 h later.

Preparation of whole cell RNA

Cells were washed in monolayer 3 times with phosphate-buffered saline and harvested using a rubber policeman in phosphate-buffered saline. Whole cell RNA was prepared from the cell pellet by the guanidinium–caesium chloride method described in detail by Maniatis *et al.*[14].

Analysis of RNA by Northern blotting

RNA was subjected to electrophoresis in 1.5% agarose-formaldehyde gels [14] at about 40 μg total RNA per track. The RNA was transferred to nitrocellulose by blotting in 20 × SSC and hybridised to 5×10^6 cpm of ^{32}P-labelled probe per 10 ml. Hybridisation was in 1 × SSC, 10 × Denhardt's, 0.1% SDS, 50 μg/ml single-stranded calf thymus DNA and 10% dextran sulphate at 65% for 18 h. Blots were washed at a stringency of 0.2 × SSC, 0.1% SDS for 1 h at 65°C and autoradiographed using Kodak film XAR 5. The cloned DNA probes used were MMTV-LTR (the 1.4 kb PstI fragment cloned into pAT 153) and actin (the 2.3 kb PstI actin fragment). To make the ^{32}P-labelled probe, the cloned DNA fragments were separated from the plasmid and then labelled with ^{32}P by nick translation [14].

Measurement of total protein synthesis

Total protein synthesis was measured in 15 cm dishes with and without cycloheximide to monitor the effectiveness of the drug. Each dish of cells was washed three times with a balanced salt solution and 15 ml of DMEM lacking methionine was added together with 50×10^6 cpm of [^{35}S]methionine. The cells were incubated for 1 h at 37°C, washed and harvested in phosphate-buffered saline and then lysed in 0.3N NaOH and added to 20 vol of 5% TCA containing 0.1% methionine. The TCA precipitate was spun down, collected on a glass fibre disc, washed consecutively with 5% TCA, ethanol, ethanol–ether (1:1), ether and dried at 60°C. The precipitate was counted by liquid scintillation.

Receptor assays

S115 cells were incubated in serum-free medium for 3 days prior to harvesting in order to deplete hormone levels in the cells. Glucocorticoid receptor was assayed as described by Sica *et al.*[15] except that the samples were equilibrated overnight at 4°C and the unbound steroid removed with 0.25% charcoal, 0.025% dextran for 30 min at 4°C. The glucocorticoid receptor content of the cytosol was 235 fmol/mg protein.

Androgen receptor was measured by the protamine method of Zava *et al.*[16]. The concentration was 70 fmol/mg protein.

RESULTS

Short-term steroid regulation of MMTV RNA in S115 cells

The species of MMTV-related RNA produced in androgen-responsive S115 +A cells have been described previously [12]. The short-term effects on these RNAs of testosterone (−T) removal followed by readdition of either testosterone (+T) or dexamethasone (+D) are shown in Fig. 1 (tracks 1–10). Removal of steroid and use of DC-stripped serum (to remove endogenous steroids from serum) resulted in significant loss of these RNAs after 4–5 days (tracks 2, 3). Readdition of testosterone for as little as 2 h resulted in an increase in RNAs (track 4) which returned to the maximal levels of +A cells (track 1) after 24 h (track 7). Readdition of dexamethasone appeared to stimulate these RNAs even faster than did testosterone (tracks 8–10), such that after 24 h (track 10) the RNA levels were even higher than in original +A cells (track 1).

Effects of actinomycin D on short-term steroid regulation of MMTV RNA in S115 cells

The accumulation of MMTV RNA in hormone-treated cells may be due to an increase in RNA synthesis or a decrease in its rate of degradation or

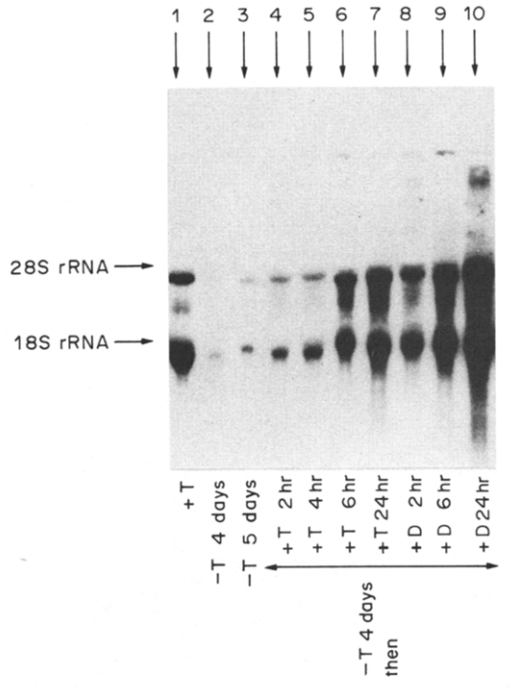

Fig. 1. Short-term effects of testosterone (T) and dexamethasone (D) on MMTV-LTR-related whole cell RNA of +A S115 cells. Cells were grown as follows: +T (1), −T 4 days (2), −T 5 days (3), −T 4 days then +T 2 h (4), +T 4 h (5), +T 6 h (6), +T 24 h (7), +D 2 h (8), +D 6 h (9), +D 24 h (10). Testosterone concentration was 3.5 × × 10^{-8} M, dexamethasone 10^{-7} M. RNA was loaded at 30 μg/track and autoradiography was for 66 h.

a combination of the two. Thus the effects of actinomycin D (10 μg/ml, sufficient to inhibit [^{3}H]uridine incorporation by 98%) on the induction have been examined. Figure 2 shows that a 1 h preincubation of S115 cells in actinomycin D, followed by a 6 h exposure to androgen of glucocorticoid in the continued presence of the inhibitor, completely prevents accumulation of MMTV RNA.

Effects of actinomycin D on short-term steroid regulation of MMTV RNA in S115 cells

We then tested the effect of an inhibitor of protein synthesis, cycloheximide, on the induced accumulation. Cells were pretreated for 1 h with cycloheximide (2 μg/ml) such that total protein synthesis was inhibited by >75% (data not shown). Cycloheximide alone weakly inhibits the basal levels of MMTV RNA in S115 cells (Fig. 3, track 4), suggesting that when no hormone is present, the RNA is less stable in the absence of protein synthesis. Treatment of S115 cells with cycloheximide for 1 h followed by dexamethasone + cycloheximide for 5 h did not alter significantly the MMTV RNA accumulation (Fig. 3, track 7) compared to that in the presence of dexamethasone alone (Fig. 3, track 6). Thus, as has been documented previously [17], dexamethasone-induced accumulation of MMTV RNA is not dependent upon protein synthesis. Treatment of cells for 1 h with cycloheximide followed by testosterone + cycloheximide for 5 h partially inhibited MMTV RNA accumulation (track 5). Thus, the story appears different for testosterone, although

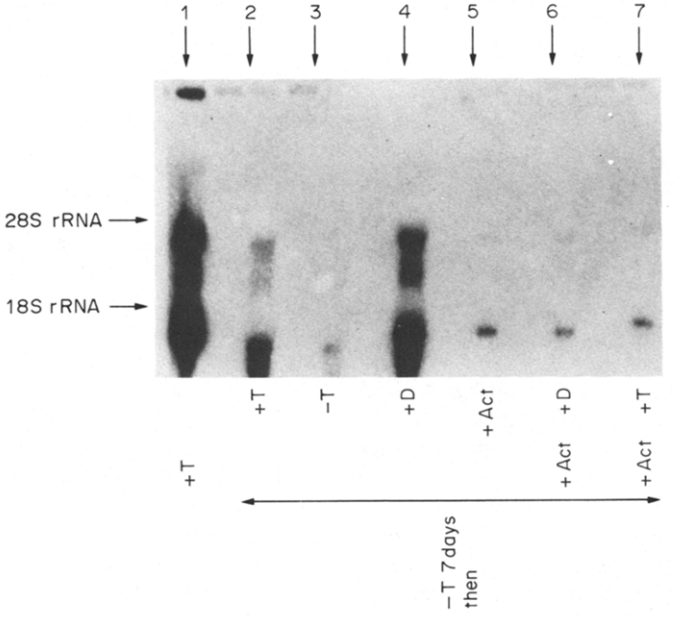

Fig. 2. Effects of actinomycin D (Act) on short-term regulation of MMTV-LTR-related whole cell RNA of +A S115 cells. Cells were grown as follows: +T (1), −T 7 days (3), −T 7 days then +T 7 h (2), +D 7 h (4), +Act 7 h (5), +Act 1 h then +Act + D 6 h (6), +Act 1 h then +Act + T 6 h (7). Testosterone (T) concentration was 3.5 × 10^{-8} M, dexamethasone (D) 10^{-7} M, actinomycin D 10 μg/ml. RNA was loaded at 40 μg/track and autoradiography was for 72 h.

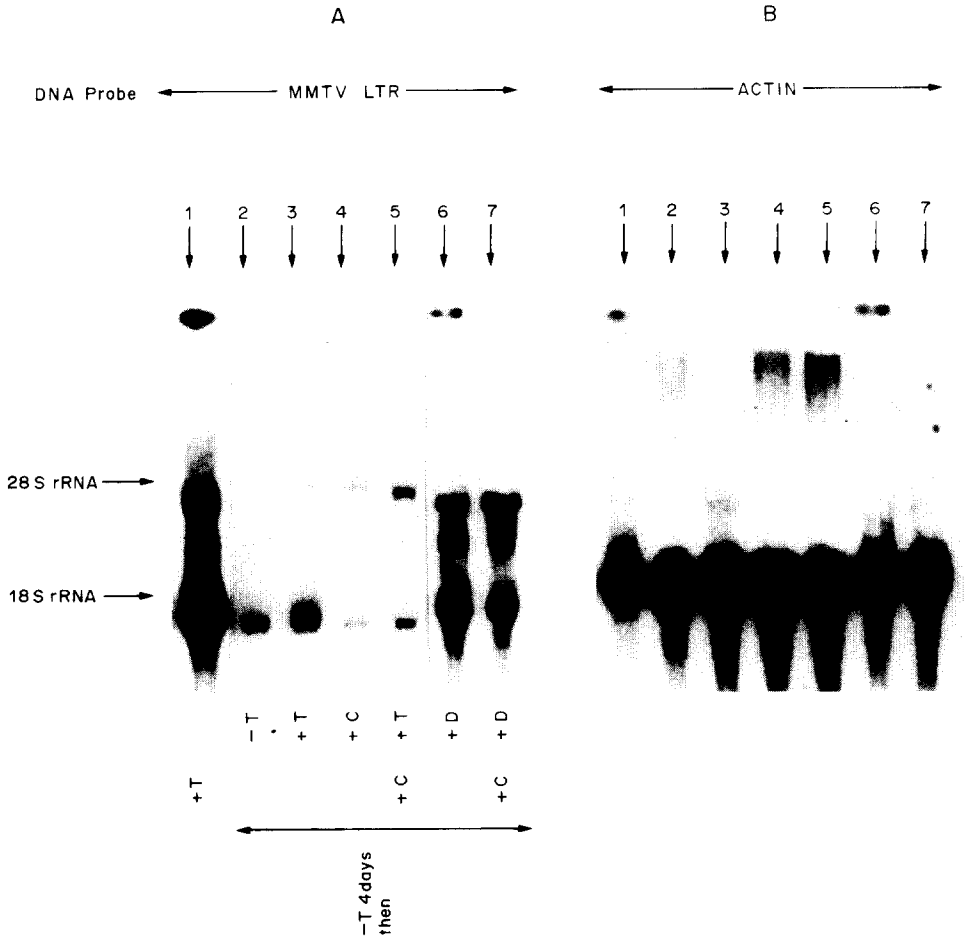

Fig. 3. A. Effects of cycloheximide (C) on short-term regulation of MMTV-LTR-related whole cell RNA of +A S115 cells. Cells were grown as follows: +T (1), −T 4 days (2), −T 4 days then +T 5 h (3), +C 6 h (4), +C 1 h then +T+C 5 h (5), +D 5 h (6), +C 1 h then +D+C 5 h (7). Testosterone concentration was 3.5×10^{-8} M, dexamethasone 10^{-7} M, cycloheximide 2 μg/ml. RNA was loaded at 40 μg/track and autoradiography was for 48 h. B. Effects of testosterone, dexamethasone and cycloheximide on short-term regulation of whole cell actin RNA of +A S115 cells. This was the same Northern blot as used for A. The ^{32}P was left to decay for 3 months and the same blot re-probed with ^{32}P-labelled actin DNA. Autoradiography was for 10 h.

the data are hard to interpret in the presence of an inhibitory effect of cycloheximide alone.

Specificity of steroid regulation of MMTV RNA in S115 cells

It is known that androgens do not alter gross RNA levels per S115 cell [18], suggesting that the modulation of MMTV RNA levels was a specific effect. However, to confirm the specificity of the steroid hormone effects on the MMTV transcripts, actin *m*-RNA levels have been studied also. Figure 3 shows that actin *m*-RNA levels were unaffected by steroid hormones or cycloheximide treatment of the cells.

Does androgen act via the glucocorticoid receptor?

The glucocorticoid receptor complex is known to induce MMTV synthesis by direct action on the long terminal repeat (LTR) of the provirus [2]. We wanted to distinguish between the possibilities that testosterone was acting via the androgen receptor or indirectly via the glucocorticoid receptor. The cycloheximide data shown above would not support the latter model but additional experiments were performed.

The ligand specificity of the glucocorticoid and androgen receptor of S115 cells strongly supports the view that separate receptors are involved. Binding of [^{3}H]dexamethasone to the glucocorticoid receptor is strongly inhibited by cortisol but not by testosterone or any of its known metabolites in S115 cells [19] except dihydrotestosterone (DHT); even this steroid had only a small inhibitory effect (Fig. 4A). The small inhibition of binding of the synthetic androgen [^{3}H]R1881 (methyltrienolone) to androgen receptor by cortisol and dexamethasone (Fig. 4B) is probably non-specific as it is not dose-dependent.

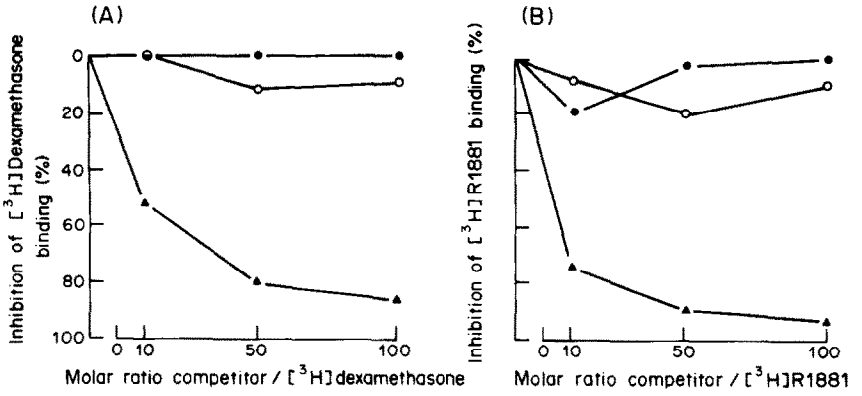

Fig. 4. Ligand specificity of the glucocorticoid receptor (A) and androgen receptor (B) from S115 cells. A. 10^{-8} M [^{3}H]dexamethasone was incubated with cytosol plus the stated molar excess of unlabelled cortisol (▲) or dihydrotestosterone (○). Testosterone, 5α-androstan-3α-17β diol, 5α-androstane-3β-ol-17-one, 5α-androstane-dione or androstenedione were all ineffective (●). B. 5×10^{-9} M [^{3}H]R1881 plus 5×10^{-6} M triamcinolone acetonide were incubated with cytosol plus the stated molar excess of unlabelled testosterone (▲), cortisol (●) or dexamethasone (○).

Additional data in support of the two receptor model was obtained with mink lung cells infected with the GR strain of MMTV (MGR cells). These cells possess glucocorticoid but not androgen receptors (data not shown) and we have studied the effects of 24 h exposure to testosterone or dexamethasone on MMTV RNA accumulation. The pattern of MMTV RNA stimulated in these cells by dexamethasone has been described previously [12]. Only dexamethasone was able to stimulate MMTV RNA production and not testosterone (data not shown), indicating that testosterone does not act by binding to the glucocorticoid receptor which then binds to the LTR.

DISCUSSION

These results demonstrate that androgen can regulate MMTV RNA production in an early post-receptor event in S115 cells. It has been shown previously that when testosterone is added back to S115 cells after deprivation for 3 days, there is a lag phase of 8 h before DNA synthesis increases and total recovery takes up to 16 h [20]. It can be up to 2 or 3 days before this is reflected in changes in cell number and cell morphology. The data reported here show that both androgen and glucocorticoid act within this lag period to increase MMTV-related RNAs, demonstrating a clear molecular marker of postreceptor events.

The mechanism of androgen stimulation of MMTV RNA in S115 cells is not merely by stabilisation of messenger RNA since effects are blocked by actinomycin D. Thus, the androgen regulation must involve transcription, as for the glucocorticoid-mediated effects [17]. Other evidence suggesting a similarity in action between testosterone and dexamethasone is that the effects of the two hormones are not additive. Dexamethasone does not increase MMTV RNA levels in S115 cells grown in the presence of testosterone, and in long-term androgen deprived cells which have become unresponsive to androgens MMTV RNA is not produced after exposure to either hormone [12]. In the very long-term this may reflect increased DNA methylation of proviral copies but the short-term MMTV RNA response to either steroid is lost before DNA methylation changes are detectable [21] indicating that other factors may be involved. Indeed, it appears that the only situation in which dexamethasone alone stimulates MMTV RNA production in S115 cells is following short term androgen deprivation, at which time either steroid readded will increase MMTV RNA. The fact that the response to dexamethasone is faster than the response to testosterone could be due to several factors and does not necessarily imply separate modes of action. For example, it could result from the lower levels of androgen receptor compared to glucocorticoid receptor in S115 cells (see the Experimental section) or alternatively there could be differences in affinity of the two receptors for the LTR region.

There are, however, some important differences in action of these two steroids. Firstly, the two hormones do not act via the same receptor. Testosterone does not bind to the glucocorticoid receptor nor dexamethasone to the androgen receptor, confirming a previous report [5] that androgen effects on S115 cell growth were mediated by the androgen receptor. All these data, together with the lack of androgen effect on MMTV RNA in MGR cells, indicate that testosterone stimulates MMTV RNA via the androgen receptor and not by cross-binding to the glucocorticoid receptor. Furthermore, it is already known that androgen effects in S115 cells cannot be explained by metabolic conversion of testosterone to glucocorticoid [19]. Secondly, whilst dexamethasone acts directly without dependence on protein syn-

thesis, the testosterone-induced MMTV RNA accumulation shows a partial dependence on protein synthesis. Recent transfection experiments with the LTR of MMTV in S115 cells have shown that androgen regulation is indeed via the LTR region and not through adjacent chromosomal DNA sequences [22]. However, it remains in question as to whether there is direct binding of the steroid receptor or an intermediate protein to the LTR region. The former could be true and the cycloheximide results could be explained as instability of the androgen receptor itself in the absence of protein synthesis.

These results clearly demonstrate that glucocorticoids are not the only steroids which can regulate MMTV RNA production in cells. Indeed, the critical factor in determining steroid hormone regulation on the LTR may well prove to be the presence of adequate amounts of the appropriate receptor within the cell. We have demonstrated that in S115 cells, which possess receptors for androgen and glucocorticoid but not for oestrogen or progesterone, hormonal regulation on the LTR corresponds to the receptor status [22]. Other recent data imply some structural similarity in the DNA sequences needed for binding of different steroid hormone receptors [23]. If this turns out to be true, receptor status of the cell may turn out to be a major determinant.

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