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Isolation of pMGT1: a Gene that is Repressed by Oestrogen and Increased by Antioestrogens and Antiprogestins

D.L. Manning and R.I. Nicholson

In order to isolate additional markers of oestrogen responsiveness in breast cancer and to study the mechanisms associated with the development of endocrine resistance, we have searched for oestrogen regulated genes. Differential hybridisation analysis of a cDNA library prepared from oestrogen-stimulated T-47D cells has led to the isolation of a sequence (pMGT1) whose expression is repressed (up to 8-fold) by oestrogen (10^{-9} mol/l) and represents the first down-regulated gene to be identified by this methodology. Further studies of pMGT1 expression in MCF-7 cells has revealed that the pure antioestrogens, ICI164384 (10^{-7} mol/l) and ICI182780 (10^{-7} mol/l) and the antiprogestin Ru38486 (10^{-7} mol/l), increase pMGT1 mRNA levels by approximately 40–50-fold relative to the value seen in cells exposed to oestrogens. Under the same conditions, pS2(pLIV2), a gene which is positively regulated by oestradiol, was almost undectable. Significantly, both tamoxifen (10^{-7} mol/l), and 4-hydroxytamoxifen (10^{-7} mol/l), failed to increase pMGT1 mRNA levels. Since cell culture studies have indicated that ICI164384 and ICI182780 are more effective than tamoxifen and 4-hydroxytamoxifen at inhibiting the growth of MCF-7 cells by mechanisms that lower their viability and sensitivity to growth factors, it is feasible that pMGT1 plays a central role in mediating these events and instigating pathways associated with cell death.
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INTRODUCTION

DIFFERENTIAL HYBRIDISATION strategies have proved extremely useful in the isolation of oestrogen regulated genes in breast cancer cells. The technique usually involves the screening of cDNA libraries, prepared from oestrogen-stimulated cells, with two cDNA probes; one prepared from the mRNA of hormone-stimulated cells and the other from hormone withdrawn cells. Its sensitivity is limited however, since oestrogen withdrawal procedures have been inadequate. Consequently, the differential expression of responsive genes is not maximised.

The exhaustive removal of oestrogens from serum [1] and the culturing of cells in phenol red-free media [2] have led to improvements in the withdrawal procedure, leading to the isolation of several oestrogen regulated genes by ourselves [3, 4] and others [5]. Indeed, at least two genes, pS2 [5], identical to the pLIV2 and pNR2 transcripts isolated by Manning *et al.* [3] and May and Westley [1], respectively, and pLIV1 [3], are expressed predominantly in oestrogen receptor (ER) positive breast cancers and may serve as prognostic indicators of oestrogen responsiveness [6, 7].

Despite the improvements in screening strategies, the isolation of oestrogen-repressed sequences has remained elusive. We have previously described the cloning of eight oestrogen-induced sequences from the T-47D human breast cancer cell line [4]. At the same time, we also identified several clones that appeared to contain sequences which were repressed by oestrogen but by amounts (less than 2-fold) that excluded them from further analysis. We have now re-screened this group against RNA from oestrogen-stimulated or withdrawn MCF-7 cells (which are more responsive to oestrogen than the T-47D cell line) and isolated a further clone, pMGT1, which is significantly repressed by oestrogen in this cell line compared to the levels observed in T-47D cells. Furthermore, when MCF-7 cells are grown in the presence of either of the pure antioestrogens ICI164384 and ICI182780 or the antiprogestin RU38486, pMGT1 levels are dramatically increased.

This is the first report of an oestrogen-repressed sequence identified by differential hybridisation analysis.

MATERIALS AND METHODS

cDNA library construction and screening for oestrogen-regulated sequences

Complementary DNA library construction and screening for oestrogen regulated sequences by sequential rounds of colony, Southern- and northern-blot hybridisation, using cDNA probes prepared from T-47D cells grown for 7 days either in the presence (+E) or absence (−E) of oestrogen was performed as described previously [4]. At each round, only those clones that demonstrated at least a 2-fold stronger hybridisation signal with either the +E or −E probe were isolated.

Tissue culture

T-47D and MCF-7 cells were grown with or without oestrogen (10^{-9} mol/l) for 7 days as described previously [2]. In order to determine the effect of different antioestrogens (transtamoxifen, 4-hydroxytamoxifen, trioxifene, LY139481, LY117018, ICI164384, ICI182780), *cis*-tamoxifen and the antiprogestin, RU38486 on pMGT1 expression, cells were grown in the presence of each drug (10^{-7} mol/l) for 7 days.

RNA isolation and northern analysis

Cells were harvested by the addition of 4 mol/l guanidinium thiocyanate containing 1% 2-mercaptoethanol directly to the tissue culture flask. Total RNA was obtained by sedimentation through 5.7 mol/l caesium chloride and stored in RNAse free water at −70°C. Northern analysis was performed using pS2, pMGT1 and actin probes labelled with ^{32}P-[CTP] as described previously [2]. Filters were washed extensively and exposed to X-ray film prior to densitometric analysis using a video densitometer (Biorad 620).

RESULTS

The retrospective isolation of pMGT1 is shown in Fig. 1 following sequential rounds of colony (Fig. 1a), and Southern-blot hybridisation analysis (Fig. 1b). While our previous results have shown that the majority of clones were oestrogen inducible, i.e. they showed a stronger hybridisation to the +E probe (prepared from T-47D cells grown in the presence of oestrogen) compared to the −E probe (prepared from oestrogen-withdrawn T-47D cells) in both the colony and Southern-blot screens (Fig. 1a and Ref. 4). The pMGT1 clone (Fig. 1a, arrowed) contained sequences which were approximately 4–6-fold more abundant in oestrogen-withdrawn compared to oestrogen-stimulated T-47D cells. However, the difference in pMGT1 expression was not upheld to the same degree as detected by northern analysis (Fig. 1c). pMGT1 levels were only 2-fold greater in cells grown in the absence (lane 2) compared to presence (lane 1) of oestrogen. Interestingly, pMGT1 levels were increased 40–50-fold in cells grown for 7 days in the presence of ICI164384 (lane 3), an effect that could be reversed following the re-addition of oestrogen for 3 days (lane 4). Actin mRNA levels were unaffected by ICI164384 treatment as demonstrated by washing and reprobing filters (Fig. 1d).

In the MCF-7 cell line, the difference in pMGT1 expression, as determined by northern analysis, between oestrogen-treated and -withdrawn cells was more evident than in T-47D cells, with an 8-fold increase observed following oestrogen depletion (Fig. 2a). Furthermore, pMGT1 mRNA levels were increased 40–50-fold (compared to the levels observed in oestrogen maintained cells) by the pure antioestrogens ICI164384 and ICI182780. In contrast, the partial antioestrogens which frequently show oestrogen-like activity [8] did not increase pMGT1 levels above the oestrogen deprived value. Significantly, the induction of pMGT1 is not restricted to pure antioestrogens but was also increased by the antiprogestin RU38486.

Reprobing the same filter with the oestrogen inducible pS2 gene showed suppressive effects with all the antioestrogens studied, except tamoxifen. No pS2 expression could be observed with ICI164384 and ICI182780 even after long exposure times (Fig. 2b). Interestingly, the antiprogestin RU38486 also reduced pS2 levels to below the oestrogen deprived value.

DISCUSSION

The observation that not all ER positive breast cancers respond to antioestrogen therapy has led to the search for other markers of oestrogen responsiveness. Oestrogen regulated genes are ideal candidates and several inducible sequences have been isolated from cDNA libraries by differential screening [4]. Although these have been of value in assessing the likelihood of patient response to endocrine therapies, recent studies by our group [7] have suggested that a failure to detect oestrogen inducible genes may have two diverse biological explanations. Firstly, they may truly reflect an inability of the tumour cells to respond to oestrogens and therefore provide an accurate marker for the loss of hormone sensitivity. Alternatively, in indolent and metabolically less active tumours, they may result from the down-regulation of oestrogen regulated gene products to values that are not readily detected by routine methodologies [9]. Since clinical experience indicates that good quality tumour remissions to endocrine based therapies are frequently observed in ER positive tumours with low growth fractions [10], failure to detect

Correspondence to D.L. Manning.
The authors are at the Breast Cancer Laboratory, Tenovus Cancer Research Centre, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XX, U.K.
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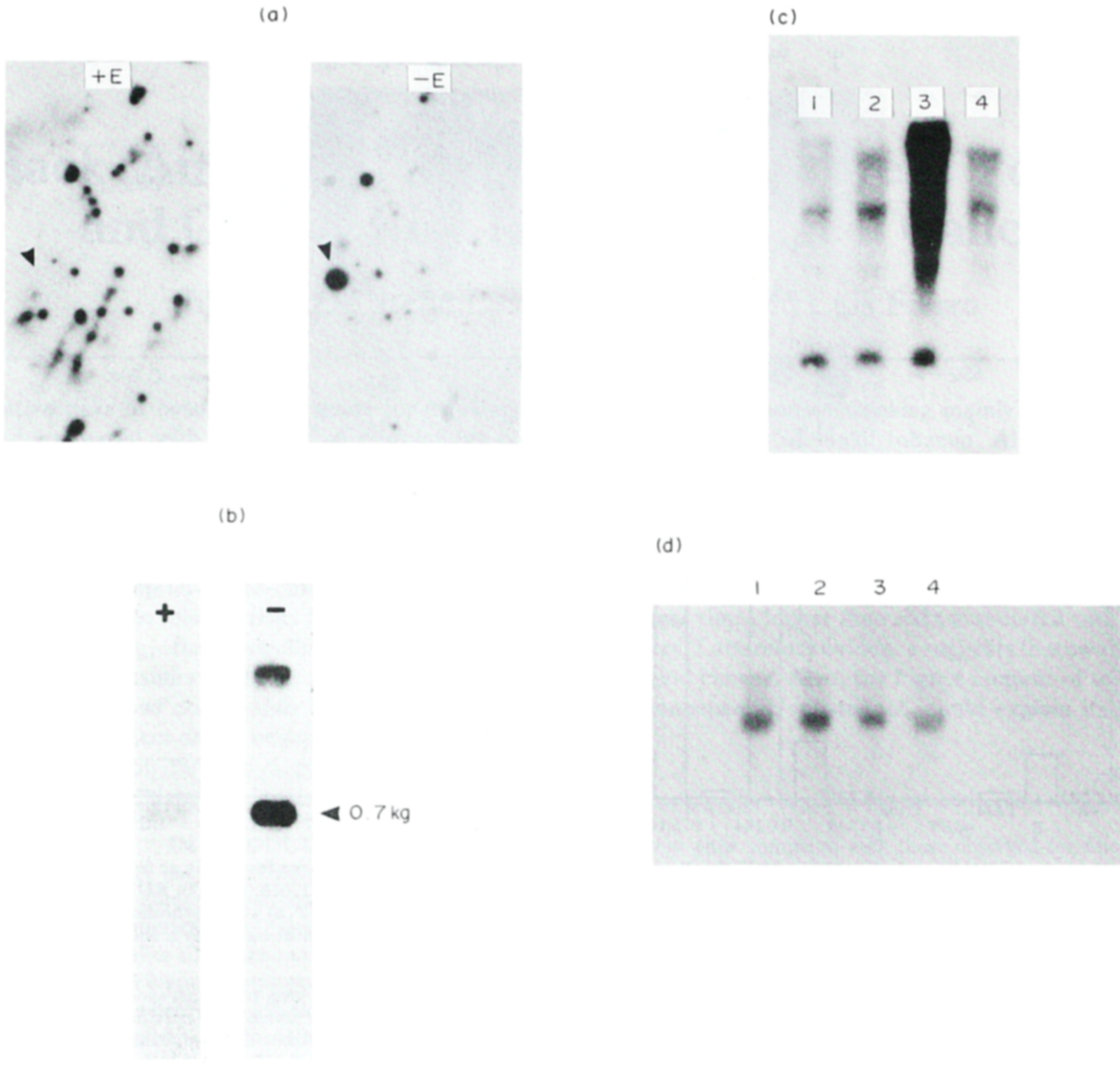

Fig. 1. Sequential rounds of colony (a), and Southern-blot (b) differential hybridisation analysis was performed on a cDNA library prepared from oestrogen re-stimulated T-47D cells as previously described [4]. The pMGT1 colony (arrowed), which showed a stronger signal to the cDNA probe prepared from the mRNA from cells grown in the absence of oestrogen (−E) compared to the presence of oestrogen (+E) was isolated (a). Plasmid DNA was prepared, digested with *Bam*H1 and probed by differential Southern analysis (b). Finally (c), the pMGT1 sequence (0.7kb) was labelled and used as probe against RNA (10μg) from cells grown for 7 days with or without 10^{-9} oestrogen (lanes 1 and 2), 10^{-7} ICI164384 (lane 3) and ICI164384 treated cells followed by the re-addition of oestrogen for 3 days (lane 4). Filters were washed and reprobed with actin cDNA (d).

oestrogen regulated gene products in these tumour types clearly may not reflect an autonomous condition. Consequently, this has stimulated our search for gene sequences that are negatively regulated by oestrogen and which may be induced in hormone sensitive resting cell populations.

Although a number of genes have been identified which are repressed by oestrogen including transforming growth factor β (TGFβ) [11] and the ER gene itself [12], this is the first report of an oestrogen repressed sequence isolated by differential hybridisation of a cDNA library. Our current success in this area has been aided by the employment of a low-density hybridisation protocol [4], growing cells in an oestrogen-depleted environment [2] and the availability of pure antioestrogens. The latter compounds bind to ER [13] producing a receptor complex devoid of biological activity [14], thereby promoting total oestrogen deprivation [15]. Using this approach we report a sequence which is suppressed approximately 8-fold by oestradiol but increased approximately 50-fold by the pure antioestrogens ICI164384 and ICI182780 in MCF7 cells, an effect that can be reversed by oestrogen. At present, however, the identity of the gene awaits elucidation by sequence analysis.

Interestingly, the increase in expression of the pMGT1 mRNA species is not specific for pure antioestrogens, with the antiprogestin RU38486 producing an equivalent rise. Since antioestrogens and antiprogestins have additive antitumour properties [16] and the antiprogestin RU38486 has been shown to inhibit the oestrogen stimulated growth of mammary tumours in mice [17], our current data may indicate a molecular point of interaction between these diverse classes of drugs in mechanisms that lead to tumour cell death.

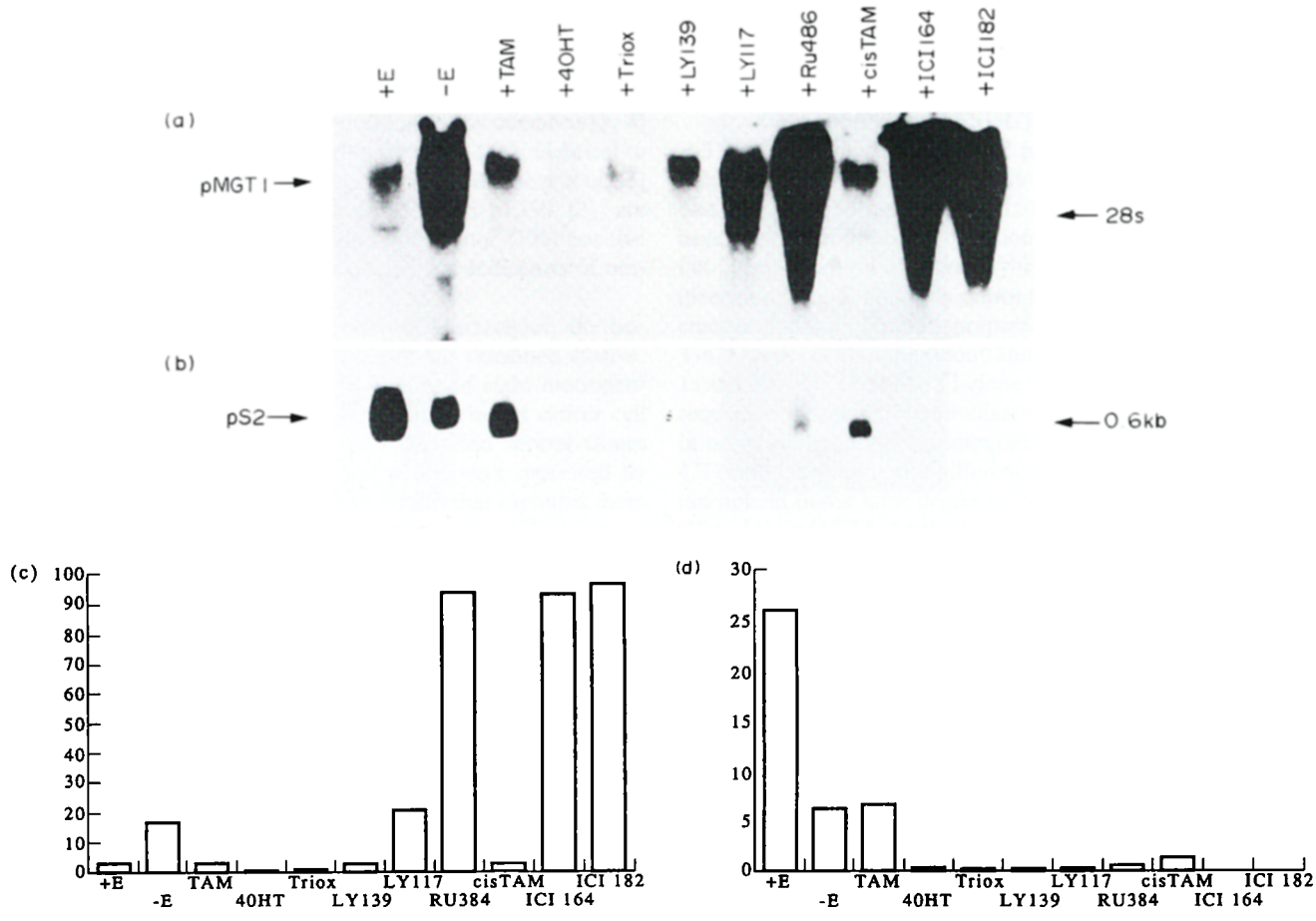

Fig. 2. 10μg of total RNA was isolated from MCF-7 cells grown with (+E) or without (−E) oestrogen for 7 days or from cells grown with trans-tamoxifen (TAM), 4-hydroxytamoxifen (4OHT), trioxifene (Triox), LY139481 (LY139), LY117018 (LY117), RU38486 (RU384), *cis*-tamoxifen (CisTAM), ICI164383 (ICI164) or ICI182780 (ICI182) and probed with pMGT1 (a) or pS2 (b) which hybridise to mRNA species >10 kb and 0.6 kb in size, respectively. Filters were exposed to X-ray film for 1–6 h and densitometrically scanned (c, d).

Finally, the fold induction of pMGT1 by ICI164384 and ICI182780 is much greater than that achieved with a series of antioestrogens with partial oestrogen-like activity. This observation again correlates with the relative antitumour activity of these compounds against the growth of MCF-7 cells [15, 18].

In conclusion, we have isolated an oestrogen repressed sequence which may be of value in selecting patients for endocrine based therapies and monitoring the antitumour activities of antiprogestins and antioestrogens, furthering our molecular understanding of growth controls in hormone related cancers.

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