Identification of novel oestrogen receptor target genes in human ZR75–1 breast cancer cells by expression profiling

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ABSTRACT

Oligonucleotide microarrays were used to analyse gene expression profiles in human ZR75–1 breast cancer cells in the presence of 17β-oestradiol and oestrogen antagonists. Differential gene expression of a number of genes was confirmed by quantitative RNA analysis. In addition to known oestrogen-responsive genes, an appreciable number of novel targets were identified, including growth factors and components of the cell cycle, adhesion molecules, enzymes, signalling molecules and transcription factors. The most pronounced oestrogen-sensitive gene was that for the cytochrome P450-IIB enzyme, involved in metabolising steroids and xenobiotics, which was increased 100-fold over a 24 h period. It is a direct target gene for oestrogens, because its expression was increased in the presence of cyclohexamide. In contrast, expression of cytochrome P450-IIB was not detected in human MCF7 breast cancer cells. Expressions of the cationic amino acid transporter E16, gap junction protein and insulin-like growth factor binding protein 4 were also markedly increased by oestrogens, but the kinetics of induction varied according to the target gene. With the exception of the cationic amino acid transporter E16 and the insulin-like growth factor binding protein 4, the expression of the majority of the genes was unaffected by antioestrogen treatment. Further analysis of this set of markers will provide alternative approaches to the investigation of the mitogenicity of oestrogens in breast cancer cells.

Journal of Molecular Endocrinology (2001) **27,** 259–274

INTRODUCTION

The oestrogen receptor (ER) is an important pharmaceutical target for hormone replacement treatment in menopausal women and for endocrine adjuvant therapy for the management and, recently, the prevention of breast cancer (Jordon & Morrow 2000). A wide repertoire of structurally distinct compounds bind to ER with differing degrees of affinity and potency. Some of these compounds, such as the natural ligand, 17β-oestradiol, act solely as receptor agonists, whereas others, such as faslodex (ICI 182,780), function as pure antagonists (Wakeling & Bowler 1988, Howell *et al*. 2000). A third category, termed selective ER modulators (SERMs), are capable of acting as either agonists or antagonists, depending on the target cell type or tissue (Dhingra 1999, Jordon & Morrow 2000). For example, tamoxifen and raloxifen function as antagonists in the breast and as agonists in bone, but differ in their effects in the endometrium, where tamoxifen is an agonist and raloxifen is an antagonist (Levenson & Jordan 1999). Responses also vary according to the promoter and the ER isoform targeted. Thus, although both 17β-oestradiol and antioestrogens are capable of stimulating transcription from activator protein-1-containing genes, this seems to be achieved primarily by means of ERα in the presence of 17β-oestradiol, but by either ERα or ERβ in the presence of antioestrogen (Paech *et al*. 1997).

To date, a relatively small number of oestrogen target genes have been identified in tissues or in cell lines. These include *pS2*, identified by differential screening of a cDNA library from MCF7 breast cancer cells (Masiakowski *et al*. 1982), and cathepsin D (Westley & May 1987). More recently, antioestrogens were shown to stimulate

0952–5041/01/027–259

the expression of quinone reductase by using the technique of RNA differential display (Montano & Katzenellenbogen 1997). New approaches based on DNA microarrays have been used to characterise distinctive gene expression patterns in human mammary epithelial cells and breast cancers (Perou *et al*. 1999). A similar approach has been used to monitor gene expression during breast cancer progression (Sgroi *et al*. 1999) and to characterise breast tumours (Nacht *et al*. 1999, Bertucci *et al*. 2000, Perou *et al*. 2000). In this way, it is possible to identify clusters of genes that show similar patterns of expression when cells are grown under different conditions.

We have used a gene expression profiling approach to identify ER target genes in human ZR75–1 breast cancer cells. Complementary RNA generated from cells grown in the presence of 17β-oestradiol, 4-hydroxytamoxifen, raloxifen or faslodex were analysed for hybridisation to Affymetrix HuGeneFL oligonucleotide microarrays and differential expression was confirmed by subsequent analysis of RNA from ZR75–1 and MCF-7 cells. In this way, we have identified known and novel oestrogen target genes.

MATERIALS AND METHODS

Cell culture

Human ZR75–1 and MCF-7 breast cancer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco Brl, Paisley, UK) and 10^{-8} M 17β-oestradiol. For MCF7 cell culture, insulin (10 μg/ml) and glutamine (4 mM) were added to the medium. The cells were plated in 175 cm^2 flasks (Tissue Culture Flask 750 ml 3045, Falcon, London, UK). To prepare total RNA, each cell line was cultured for 5 days in phenol-free medium containing 10% charcoal–dextran-stripped fetal bovine serum (CSS) and then maintained in phenol-free medium containing 10% CSS and 10 μg/ml cycloheximide, with no hormone, 10^{-8} M 17β-oestradiol, 10^{-7} M 4-hydroxytamoxifen, 10^{-7} M raloxifen or 10^{-7} M faslodex for defined periods as specified below.

Expression profiling

Total RNA was extracted from cells using Trizol reagent (Life Technologies, Paisley, UK). Polyadenylate (poly(A)+) RNA was purified from total RNA with oligo-dT-linked Oligodetex resin (Qiagen, Crawley, UK). Two micrograms poly(A)+ RNA were converted into double-stranded cDNA (ds-cDNA) by using Superscript Choice System (Life Technologies) with an oligo-dT primer containing a T7 RNA polymerase promoter. After second-strand synthesis, the reaction mixture was extracted with phenol–chloroform–isoamyl alcohol, and ds-cDNA was recovered by ethanol precipitation. Subsequently, complementary RNA (cRNA) was generated by *in vitro* transcription using a T7 Megascript Kit (Ambion AMS, Abingdon, UK) with 25% of the ds-cDNA template in the presence of a mixture of unlabelled ATP, CTP, GTP and UTP and biotin-labelled CTP and UTP (bio-11-CTP and bio-16 UTP (Enz, Farmingdale, CN, USA)). Biotin-labelled cRNA was purified using an RNeasy affinity column (Qiagen), and fragmented randomly to sizes ranging from 35 to 200 bases by incubating at 94 °C for 35 min in 1 × Affymetrix fragmentation buffer (Affymetrix, Stanford, CA, USA). The hybridisation solution contained 100 mM 2-[N-morpholino]ethanesulfonic acid (MES), 1 M Na^+, 20 mM EDTA and 0·01% Tween 20 and was 0·1 μg/μl (final concentration) of fragmented cRNA. After hybridisation at 45 °C overnight, Affymetrix HuGeneFL arrays were washed in a fluidics station with stringent buffer (100 mM MES, 0·1 M Na+, 0·01% Tween 20) and non-stringent buffer (6 × SSPE, 0·01% Tween 20) and stained with streptavidin–phycoerythrin after antibody amplification. DNA chips were read at a resolution of 6 μm with an Affymetrix GeneArray scanner.

Data analysis

The arrays were analysed using GeneChip 3.1 software, comparing arrays with similar signal-to-noise ratios and similar backgrounds. These arrays showed similar percentages of genes expressed. The average fluorescence intensity for each Affymetrix HuGeneFL chip was scaled to the same value so that every chip could be compared directly. For each time point, the data were analysed using the six possible comparisons (four different treatments compared two-by-two). The results were sorted using Access application and each gene selected was analysed individually in a global way (logic results for the following six comparisons: 4-hydroxytamoxifen-, raloxifen- and faslodex-treated with 17β-oestradiol-treated, 4-hydroxytamoxifen-treated with raloxifen- and faslodex-treated, and raloxifen-treated with faslodex-treated).

RNA analysis

Specific probes for each gene were designed, based on a BLAST search with mRNA, PCR fragment and primer sequences. Primers were selected using Primer3 (Rozen & Skaletsky 2000).

A 367 bp fragment from the human transducer of ErbB2,1 (Tob) cDNA and a 291 bp fragment from the human receptor interacting protein (RIP) 140 cDNA were amplified by PCR using pfu DNA polymerase (Stratagene, Amsterdam, The Netherlands) and the following primers: Tob-F: taccactgccacttttgctg; Tob-R: actgaggttaaggggctgt; RIP-F: cctggcagatgattcagaca; RIP-R: gcccaactcctgt gttcact. They were then cloned into pCR-Blunt II-Topo vector (Invitrogen, Paisley, UK). RNase protection probes were generated by *in vitro* transcription of the plasmids described above (linearized with BamHI) and of the pTRI-β actin-125-human plasmid (Ambion), using T7 RNA polymerase. RNase protection assays were carried out using the RPA III Kit (Ambion). Total RNA was hybridised with specific probes at 42 °C overnight, followed by digestion with RNase A/T1. Finally, protected fragments were ethanol precipitated and separated on a 5% polyacrylamide sequencing gel.

Northern blots and hybridisations were performed by standard techniques using 10 μg total RNA and radioactive probes generated by random priming of sequence-verified PCR products amplified with elongase (Gibco Brl) and obtained with the following primers: E16-F: ctgttctgggaggtggacat; E16-R: cgtttgtcagtggagtgtgg; inositol 1,3,4 triphosphate 5/6 kinase (IP3K)-F: cagactcaccccatcacctt; IP3K-R: ttgttgggtcctcagtttcc; gap junction protein (Gap)-F: ctcctcctgggtacaagctg; Gap-R: cctccaccggat caaaatta; insulin-like growth factor binding protein 4 (IGFBP4)-F: gcccaagaggactgagactg; IGFBP4-R: tggtgcaacaaccagaccta; cytochrome P450-IIB (Cyt)-F: tcctttctgaggttccgaga; Cyt-R: tggaatggaagaggaaggtg; glucose-6-phosphate dehydrogenase (G6PD)-F: atttgccaacaggatcttcg; G6PD-R: tcccacctctcattctccac; immediate early response 3 (IEX)-F: gacctcagcactttc ctcca; IEX-R: tacagcagggggaacatctc; tropomyosin 4 (TPM4)-F: cttaagacggaggccatttg; TPM4-R: gatgttt gtcctgcatggtg. The amount of TPM4 was used as an internal standard.

RESULTS

We compared gene expression profiles in human breast cancer ZR75–1 cells grown in the presence of four different ligands for the ER by exploiting GeneChip methodology. The ligands tested were the natural hormone 17β-oestradiol, the SERMs 4-hydroxytamoxifen and raloxifen, and the pure antagonist faslodex. As a first step to the identification of clusters of genes of which the expression is regulated by each ligand, we analysed patterns of expression by using Affymetrix HuGeneFL oligonucleotide arrays representing 5600 human genes. Cells were initially maintained in medium deprived of oestrogens for 5 days and then treated with ligand for 6 or 24 h in the presence of cyclohexamide to ensure that changes in gene expression were primary responses. RNA was isolated from the cells and analysed for *pS2* gene expression, a well-characterised target gene for 17β-oestradiol (Brown *et al*. 1984, Chalbos *et al*. 1993). This marker of oestrogen action was significantly increased by 17β-oestradiol relative to the oestrogen antagonists (data not shown). Poly(A)-containing RNA was isolated and used to prepare cDNA samples that were then transcribed to generate biotin-labelled cRNA to hybridise with the GeneChip HuGeneFL probe array. Expression profiles were determined using cRNA generated from cells treated with ligands for 6 or 24 h. Hybridisation signals were first analysed using GeneChip 3.1 software and genes of interest were selected using Access software. The results were then analysed for each gene in six different comparisons at two different time-points (see Materials and Methods). This last selection allowed us to discard a large number of false-positive results.

A total of 53 genes was identified, the relative expression of which differed by more than 1·5-fold (Table 1; parts 1 and 2). The samples derived from cells treated with 17β-oestradiol were used as a baseline in the differential gene expression comparisons, as this highlighted the effects of the different antioestrogens. The majority of these genes were more highly expressed in the presence of the hormone, so that the fold change in the presence of antioestrogens was negative. The expression of several known oestrogen-target genes (*pS2*, *cyclin D1*, *cathepsin D*) and many novel genes was greater in cells grown in the presence of 17β-oestradiol. The most pronounced change observed was that for *Cyt* (Fig. 1a), expression of which in the presence of 17β-oestradiol was 20-fold that in the presence of 4-hydroxytamoxifen, 45-fold that with raloxifen and 70-fold that with faslodex. Inspection of the 20 probe pairs (Fig. 1a) corresponding to perfect-match and mismatch oligonucleotides indicated that the majority exhibited similar changes. Under these circumstances, differences in gene expression of as little as 1·5-fold were subsequently confirmed by quantitative RNA analysis (see below).

Expression of potential ER target genes, predicted from the profiling analysis, was then analysed by northern blotting and RNase protection experiments. As an internal control we monitored TPM4 mRNA levels which, as confirmed by the DNA chip analysis, were unaffected by any of the ligands. Expression levels were compared with those in a

TABLE 1. PART 1. Identification of candidate ER target genes by hybridisation to oligonucleotide arrays. Summary of results from hybridisation to oligonucleotide arrays representing 5600 expressed sequences. Labelled RNA probes derived from ZR75-1 cells grown in the presence of cyclohexamide and 17β-oestradiol (E), 4-hydroxytamoxifen (T), raloxifen (R) or faslodex (F) for 6 h were hybridised to arrays. For each sample, the average difference (AD) in hybridisation intensity between the perfect-match and the mismatch oligonucleotides represent the transcript level. The fold changes (FC) indicated were calculated by GeneChip 3.1 software using the 17β-oestradiol sample as a baseline. The negative and positive FCs correspond to decreases and increases respectively. One gene, MSX-2, is represented by two different probe sets on the Chip array

			6 h						
		Action	E (AD)	T (AD)	**FC**	R (AD)	**FC**	F (AD)	**FC**
Gene/category									
Adhesion protein membranire migration									
X52003	pS2 protein (trefoil factor 1)	Promotes cell migration (motogen)	33 997	3826	**−11·0**	5047	**−8·4**	5374	**−7·9**
U78735	ABC3 (ATP-binding cassette)	Involved in resistance to xenobiotics	9215	1593	**−5·2**	1468	**−5·6**	1330	**−6·2**
X52947	Gap junction	Permits the direct exchange of ions and small molecules between cells	3446	645	**−5·7**	542	**−6·4**	257	**−8·3**
M80244	E16, SLC7A5	Cationic amino acid transporter	7987	3468	**−2·3**	3054	**−2·6**	1941	**−4·1**
L20814	Glutamate receptor 2 (AMPA2)	Channel	2089	571	**−4·4**	689	**−4·1**	712	**−3·1**
S53911	CD34	Possible adhesion molecule	6003	1468	**−4·1**	1979	**−3·0**	1523	**−3·9**
L08044 (L15203)	Trefoil factor 3	Promotes cell migration (motogen)	5564	4276	**−1·9**	1821	**−3·1**	2446	**−2·3**
U81375	Equilibrative nucleoside transporter 1 (ENT1, SLC29A1)	Transporter essential for nucleotide synthesis	6274	3292	**−1·9**	3737	**−1·7**	2638	**−2·4**
X76180	Sodium channel, non-voltage-gated 1 alpha	Electrodiffusion of the luminal sodium through apical membrane of epithelial cells	38 096	19 801	**−1·9**	21 479	**−1·7**	18 223	**−2·0**
U66711 (U56145)	Lymphocyte antigen 6 complex, locus E; retinoic acid-induced gene E	Glycosylphosphatidylinositol (GPI)-anchored cell-surface glycoprotein	12 276	12 257	**NC**	9914	**NC**	9417	**NC**
Z29083	5T4 antigen	Modulates cell adhesion, shape and motility	32 739	16 039	**−1·8**	18 002	**−1·6**	15 575	**−1·9**
D28364	Annexin II	Expressed at the surface of human brease carcinoma	1324	2538	**1·9**	2457	**2·1**	2330	**1·8**
L38608	ALCAM	Adhesion molecule, binds to CD6	3348	5917	**1·8**	6981	**2·1**	6006	**1·8**
U23070	NMA (putative transmembrane protein)	Unknown	3308	5780	**1·7**	5708	**2·0**	4786	**1·7**
X92098	Transmembrane protein RNP24	May have a role in vesicular trafficking	742	1823	**2·5**	2367	**3·2**	2084	**2·8**

ALCAM, activated leucocyte cell adhesion molecule; NC, no change.

TABLE 1. PART 1. *Continued*

Gene/category		Action	6 h E (AD)	6 h T (AD)	6 h FC	6 h R (AD)	6 h FC	6 h F (AD)	6 h FC
Cell cycle signal transduction									
M62403 (U20982)	Insulin-like growth factor binding protein 4 (IGFBP4)	Prolongs the half-life of IGF and modifies its growth-promoting effect	43 508	8615	**−4·8**	7690	**−5·1**	5362	**−7·9**
Z35491	BAG1 (BCL2 associated athanogene)	Multifunctional protein that blocks apoptosis and interacts with several types of protein, including steroid receptors	2090	1179	**−2·4**	772	**−4·9**	663	**−4·3**
D38305	Tob (TROB1)	Transducer of ErbB2,1	8499	3305	**−4·2**	3913	**−3·6**	4051	**−3·6**
U22376 (X52125, U22376)	c-myb	Transcriptional activator, control of proliferation	7930	2954	**−2·4**	2284	**−3·1**	2800	**−2·3**
M30703 (M30704)	Amphiregulin (AR)	Epithelial cell growth regulator (EGF family) and a tumour-inhibitory activity	5837	2316	**−2·5**	2364	**−2·5**	2550	**−2·3**
S81914	IEX-1 (immediate-early response 3)		20 391	13 495	**−1·5**	12 088	**−1·7**	8248	**−2·5**
X59798	Cyclin D1	Regulator of progression through G1 phase during cell cycle	45 588	23 597	**−1·9**	24 664	**−1·8**	23 389	**−1·9**
L27624 (D29992)	Tissue factor pathway inhibitor-2 (TFPI2)	Regulator	120	−22	**NC**	476	**NC**	117	**NC**
HG4264-HT4534 (U18420)	Guanine nucleotide binding protein, Rab5c-like protein	Involved in endocytosis	29 146	12 569	**−2·0**	15 066	**−1·7**	29 765	**NC**
M36430 (X04526)	Guanine nucleotide binding protein (G protein), beta polypeptide 1	Modulator or transducer in various transmembrane signalling systems	1744	3551	**2·0**	4836	**2·8**	4216	**2·4**

TABLE 1. PART 1. *Continued*

			6 h						
		Action	E (AD)	T (AD)	**FC**	R (AD)	**FC**	F (AD)	**FC**
Gene/category									
Enzyme									
M29874 (M29873, HG1875-HT1912)	Cytochrome P450-IIB	Oxidation of steroids, fatty acids, xenobiotics	30 850	1531	**−20·5**	700	**−44·9**	172	**−69·6**
M68516	PCI, protein C inhibitor (plasminogen activator inhibitor 3)	Proteinase inhibitor, belongs to the serpin family	1556	511	**−3·0**	88	**−3·8**	−164	**−4·8**
K01396	α-1-antitrypsin	Inhibitor of serine proteases (elastease but also plasmin and thrombin), belongs to the serpin family	6951	1648	**−3·9**	1883	**−3·7**	1246	**−5·2**
D29805	β-1,4-galactosyltransferase	Function in lactose biosynthesis	24 391	7582	**−2·9**	4161	**−5·3**	5912	**−3·8**
U51336	Inositol 1,3,4-triphosphate 5/6-kinase		30 579	9203	**−3·0**	9378	**−3·0**	9779	**−2·9**
X55448 (M65234)	Glucose-6-phosphate dehydrogenase	Produces pentose sugars for nucleic acid synthesis and main producer of NADPH reducing power	33 770	16 440	**−2·2**	22 870	**−1·6**	23 082	**−1·5**
X69878	FLT4 (FMS-related tyrosine kinase 4)	Receptor tyrosine kinase, class V	6776	3798	**−1·8**	3484	**−1·9**	3736	**−1·8**
D50840	Ceramide glucosyltransferase	Catalyses the first glycosylation step of glycosphingolipid synthesis	10 084	4110	**−2·0**	6076	**−1·4**	5203	**−1·9**
M63138	Cathespin D	Acid protease active in intracellular protein breakdown	20 171	18 807	**−1·1**	20 517	**−1·1**	19 935	**−1·1**
HG1862-HT1897	Calmodulin type I (phosphorylase kinase)	Calcium modulated protein	11 066	6249	**−2·5**	7298	**−2·2**	7168	**−2·2**
X75091	12PP2A (PHAPII SET)	Inhibitor of protein phosphatase 2A	1194	3262	**2·8**	3081	**2·6**	3047	**2·6**
HG4747-HT5195	NADH-ubiquinone oxidoreductase, 51 kDa subunit	Transfer of electrons from NADH to the respiratory chain	4784	6710	**1·6**	9438	**2·3**	8221	**2·1**
HG3141-HT3317	NADH-ubiquinone oxidoreductase, 39 kDa subunit	Transfer of electrons from NADH to the respiratory chain	748	3122	**8·7**	4017	**10·6**	3869	**10·0**

FMS, colony-stimulating factor I receptor.

TABLE 1. PART 1. *Continued*

			6 h						
		Action	E (AD)	T (AD)	**FC**	R (AD)	**FC**	F (AD)	**FC**
Gene/category									
Transcription factor									
X84373	RIP140 (NRIP1)	Modulates transcriptional activation of the oestrogen receptor	754	93	**− 2·7**	177	**− 2·5**	165	**− 2·5**
U37408	CtBP, C-terminal binding protein 1	Transcriptional repressor	5817	2187	**− 2·2**	1368	**− 3·5**	407	**− 10·6**
U70370	PTX1 (paired-like homeodomain transcription factor 1)	Transcription factor	13 143	8063	**− 2·2**	7764	**− 2·4**	6989	**− 3·0**
D89377 (X69295)	MSX-2	Involved in epithelial–mesenchymal signalling	5930	1429	**− 4·1**	1062	**− 3·6**	2482	**− 3·1**
X06614	Receptor of retinoic acid	Controls cell function by directly regulating gene expression	12 398	5669	**− 2·5**	6087	**− 2·6**	4729	**− 3·1**
D89377	MSX-2	Involved in epithelial–mesenchymal signalling	1901	842	**− 2·3**	1298	**− 1·5**	622	**− 3·1**
U72066	CtIP, retinoblastoma-binding protein 8	Interacts with CtBP, BRCA1, mediates transcriptional regulation	12 498	6021	**− 2·1**	6365	**− 2·0**	4896	**− 2·6**
AF009674	AXIN 1 (axin)	Negative regulator of the WNT signalling pathway, promotes the GSK3B-dependent phosphorylation of β catenin	11 363	3818	**− 2·1**	4162	**− 2·1**	5780	**− 2·0**
Other									
M87789 (J00231)	IGHG3 (immunoglobulin γ 3)	Gm marker	2015	− 1230	**− 8·5**	− 1394	**− 8·7**	− 3176	**− 12·4**
L22214	Adenosine A1 receptor	Receptor for adenosine	13 180	2553	**− 8·9**	− 239	**− 28·6**	1513	**− 18·7**
J02943	Corticosteroid binding globulin	Transports protein for glucocorticoids and progestins, belongs to the serpin family	4119	1754	**− 2·3**	2109	**− 2·0**	1721	**− 2·7**
M60784 (X06347)	snRNP-specific protein A	Involved in splicing	14 272	5879	**− 2·4**	5151	**− 2·8**	5395	**− 2·6**
HG2855-HT2995	Heat shock 70 kDa protein 2	Acts as a molecular chaperone	3222	938	**− 4·1**	1569	**− 2·5**	1163	**− 3·3**
Unknown									
D87468	KIAA0278, ARC: activity-regulated cytoskeleton-associated protein	Unknown	9171	− 1199	**− 24·2**	− 518	**− 25·6**	− 440	**− 24·7**
L18972	Anonymous gene (PK1.3)	Unknown	4634	962	**− 4·8**	1269	**− 3·7**	752	**− 6·2**
D63487	KIAA0153 gene	Unknown	8566	6595	**− 2·0**	2485	**− 3·4**	3155	**− 2·7**

TABLE 1. PART 2. Identification of candidate ER target genes by hybridisation to oligonucleotide arrays. Summary of results from hybridisation to oligonucleotide arrays representing 5600 expressed sequences. Labelled RNA probes derived from ZR75-1 cells grown in the presence of cyclohexamide and 17β-oestradiol (E), 4-hydroxytamoxifen (T), raloxifen (R) or faslodex (F) for 24 h were hybridised to arrays. For each sample, the average difference (AD) in hybridisation intensity between the perfect-match and the mismatch oligonucleotides represent the transcript level. The fold changes (FC) indicated were calculated by GeneChip 3.1 software using the 17β-oestradiol sample as a baseline. The negative and positive FCs correspond to decreases and increases respectively. One gene, MSX-2, is represented by two different probe sets on the Chip array

			24 h						
		Action	E (AD)	T (AD)	**FC**	R (AD)	**FC**	F (AD)	**FC**
Gene/category									
Adhesion protein membranire migration									
X52003	pS2 protein (trefoil factor 1)	Promotes cell migration (motogen)	28 009	4603	**−6·1**	6375	**−4·4**	4504	**−6·2**
U78735	ABC3 (ATP-binding cassette)	Involved in resistance to xenobiotics	13 469	2058	**−6·3**	2063	**−6·5**	1924	**−6·8**
X52947	Gap junction	Permits the direct exchange of ions and small molecules between cells	5362	1186	**−4·5**	1530	**−3·5**	607	**−7·3**
M80244	E16, SLC7A5	Cationic amino acid transporter	13 568	4020	**−3·1**	3571	**−3·1**	1286	**−8·6**
L20814	Glutamate receptor 2 (AMPA2)	Channel	1112	462	**−3·3**	886	**−1·5**	158	**−5·4**
S53911	CD34	Possible adhesion molecule	4444	1482	**−3·0**	2108	**−2·2**	1836	**−2·4**
L08044 (L15203)	Trefoil factor 3	Promotes cell migration (motogen)	10 437	3471	**−3·0**	5340	**−2·0**	4518	**−2·5**
U81375	Equilibrative nucleoside transporter 1 (ENT1, SLC29A1)	Transporter essential for nucleotide synthesis	4367	2052	**−2·4**	1775	**−2·5**	1904	**−2·3**
X76180	Sodium channel, non-voltage-gated 1 alpha	Electrodiffusion of the luminal sodium through apical membrane of epithelial cells	31 992	15 456	**−2·1**	15 256	**−2·1**	12 342	**−2·6**
U66711 (U56145)	Lymphocyte antigen 6 complex, locus E; retinoic acid-induced gene E	Glycosylphosphatidylinositol (GPI)-anchored cell-surface glycoprotein	10 862	4299	**−2·1**	3620	**−3·0**	2464	**−3·7**
Z29083	5T4 antigen	Modulates cell adhesion, shape and motility	13 198	9095	**NC**	10 630	**NC**	9657	**−1·4**
D28364	Annexin II	Expressed at the surface of human breast carcinoma	5843	10 499	**1·8**	12 251	**2·1**	7151	**1·4**
L38608	ALCAM	Adhesion molecule, binds to CD6	3925	6877	**1·8**	7755	**2·0**	6634	**1·7**
U23070	NMA (putative transmembrane protein)	Unknown	1064	2358	**2·2**	2893	**2·9**	2373	**2·2**
X92098	Transmembrane protein RNP24	May have a role in vesicular trafficking	1172	2299	**2·0**	3091	**2·6**	2211	**1·9**

TABLE 1. PART 2. *Continued*

			24 h						
		Action	E (AD)	T (AD)	**FC**	R (AD)	**FC**	F (AD)	**FC**
Gene/category									
Cell cycle signal transduction									
M62403 (U20982)	Insulin-like growth factor binding protein 4 (IGFBP4)	Prolongs the half-life of IGF and modifies its growth-promoting effect	32 707	11 568	**−2·8**	12 449	**−2·6**	6490	**−5·0**
Z35491	BAG1 (BCL2 associated athanogene)	Multifunctional protein that blocks apoptosis and interacts with several types of protein, including steroid receptors	3467	1352	**−2·6**	1680	**−2·1**	2166	**−1·9**
D38305	Tob (TROB1)	Transducer of ErbB2,1	9711	6754	**−1·4**	4229	**−1·8**	3340	**−2·5**
U22376 (X52125, U22376)	c-myb	Transcriptional activator, control of proliferation	4229	2339	**−1·8**	2669	**−1·6**	2242	**−2·3**
M30703 (M30704)	Amphiregulin (AR)	Epithelial cell growth regulator (EGF family) and a tumour-inhibitory activity	11 626	4782	**−2·1**	6771	**−1·7**	4524	**−1·8**
S81914	IEX-1 (immediate-early response 3)		8838	5043	**−1·8**	4822	**−1·8**	3641	**−2·4**
X59798	Cyclin D1	Regulator of progression through G1 phase during cell cycle	27 543	20 294	**−1·4**	17 828	**−1·5**	23 704	**−1·2**
L27624 (D29992)	Tissue factor pathway inhibitor-2 (TFPI2)	Regulator	2360	698	**−3·4**	728	**−3·2**	340	**−6·9**
HG4264-HT4534 (U18420)	Guanine nucleotide binding protein, Rab5c-like protein	Involved in endocytosis	21 630	11 157	**−1·8**	14 724	**−1·5**	12 717	**−1·6**
M36430 (X04526)	Guanine nucleotide binding protein (G protein), beta polypeptide 1	Modulator or transducer in various transmembrane signalling systems	4713	6540	**1·4**	6934	**1·5**	8632	**1·7**

TABLE 1. PART 2. *Continued*

Gene/category		Action	24 h						
			E (AD)	T (AD)	FC	R (AD)	FC	F (AD)	FC
Enzyme									
M29874 (M29873, HG1875-HT1912)	Cytochrome P450-IIB	Oxidation of steroids, fatty acids, xenobiotics	39 159	759	**−49·9**	876	**−44·4**	610	**−63·8**
M68516	PCI, protein C inhibitor (plasminogen activator inhibitor 3)	Proteinase inhibitor, belongs to the serpin family	1448	−7	**−11·4**	17	**−8·5**	−35	**−8·3**
K01396	α-1-antitrypsin	Inhibitor of serine proteases (elastease but also plasmin and thrombin), belongs to the serpin family	7638	860	**−8·1**	1560	**−4·5**	1060	**−6·7**
D29805	β-1,4-galactosyltransferase	Function in lactose biosynthesis	16 680	7184	**−2·2**	8138	**−2·0**	7465	**−2·2**
U51336	Inositol 1,3,4-triphosphate 5/6-kinase		15 751	7005	**−2·0**	8614	**−1·8**	8272	**−1·7**
X55448 (M65234)	Glucose-6-phosphate dehydrogenase	Produces pentose sugars for nucleic acid synthesis and main producer of NADPH reducing power	24 349	9936	**−2·7**	12 731	**−2·1**	13 017	**−2·0**
X69878	FLT4 (FMS-related tyrosine kinase 4)	Receptor tyrosine kinase, class V	3736	1252	**−3·0**	3118	**−1·2**	2423	**−1·5**
D50840	Ceramide glucosyltransferase	Catalyses the first glycosylation step of glycosphingolipid synthesis	4885	2551	**−1·9**	2930	**−1·8**	2384	**−2·0**
M63138	Cathespin D	Acid protease active in intracellular protein breakdown	13 294	9376	**−1·6**	3893	**−2·9**	11 332	**−1·3**
HG1862-HT1897	Calmodulin type I (phosphorylase kinase)	Calcium modulated protein	20 642	10 626	**−1·9**	14 374	**NC**	10 676	**−1·8**
X75091	12PP2A (PHAPII SET)	Inhibitor of protein phosphatase 2A	4818	5639	**NC**	7937	**NC**	3900	**NC**
HG4747-HT5195	NADH-ubiquinone oxidoreductase, 51 kDa subunit	Transfer of electrons from NADH to the respiratory chain	5270	7708	**1·5**	10 804	**2·0**	8773	**1·6**
HG3141-HT3317	NADH-ubiquinone oxidoreductase, 39 kDa subunit	Transfer of electrons from NADH to the respiratory chain	4550	10 217	**2·2**	10 599	**2·3**	10 699	**2·4**

TABLE 1. PART 2. *Continued*

			24 h						
		Action	E (AD)	T (AD)	**FC**	R (AD)	**FC**	F (AD)	**FC**
Gene/category									
Transcription factor									
X84373	RIP140 (NRIP1)	Modulates transcriptional activation of the oestrogen receptor	1839	325	**−5·0**	173	**−7·5**	119	**−7·0**
U37408	CtBP, C-terminal binding protein 1	Transcriptional repressor	5399	3611	**−1·5**	4204	**−1·3**	3266	**−1·7**
U70370	PTX1 (paired-like homeodomain transcription factor 1)	Transcription factor	1511	5182	**−1·9**	709	**−5·5**	3367	**−3·7**
D89377 (X69295)	MSX-2	Involved in epithelial–mesenchymal signalling	6496	2264	**−2·9**	3712	**−2·2**	2640	**−2·5**
X06614	Receptor of retinoic acid	Controls cell function by directly regulating gene expression	15 427	5169	**−3·3**	7991	**−2·1**	5200	**−3·6**
D89377	MSX-2	Involved in epithelial–mesenchymal signalling	2331	874	**−2·7**	1612	**−1·4**	743	**−3·1**
U72066	CtIP, retinoblastoma-binding protein 8	Interacts with CtBP, BRCA1, mediates transcriptional regulation	16 909	8414	**−2·0**	10 718	**−1·6**	9751	**−1·7**
AF009674	AXIN 1 (axin)	Negative regulator of the WNT signalling pathway, promotes the GSK3B-dependent phosphorylation of β catenin	8196	4302	**−1·4**	4026	**−2·2**	5117	**−1·6**
Other									
M87789 (J00231)	IGHG3 (immunoglobulin γ 3)	Gm marker	5745	−584	**−29·6**	−1384	**−28·2**	−744	**−21·9**
L22214	Adenosine A1 receptor	Receptor for adenosine	6283	713	**−7·7**	1839	**−3·4**	1667	**−3·8**
J02943	Corticosteroid binding globulin	Transports protein for glucocorticoids and progestins, belongs to the serpin family	3810	1566	**−2·9**	2059	**−2·2**	1293	**−4·0**
M60784 (X06347)	snRNP-specific protein A	Involved in splicing	6199	2417	**−2·6**	3392	**−1·8**	1720	**−2·8**
HG2855-HT2995	Heat shock 70 kDa protein 2	Acts as a molecular chaperone	7387	6432	**NC**	6846	**NC**	4030	**−1·5**
Unknown									
D87468	KIAA0278, ARC: activity-regulated cytoskeleton-associated protein	Unknown	1293	−441	**−17·0**	850	**−2·5**	−1013	**−14·7**
L18972	Anonymous gene (PK1.3)	Unknown	7626	620	**−12·1**	352	**−21·7**	1381	**−11·1**
D63487	KIAA0153 gene	Unknown	5624	3835	**NC**	3230	**NC**	3730	**NC**

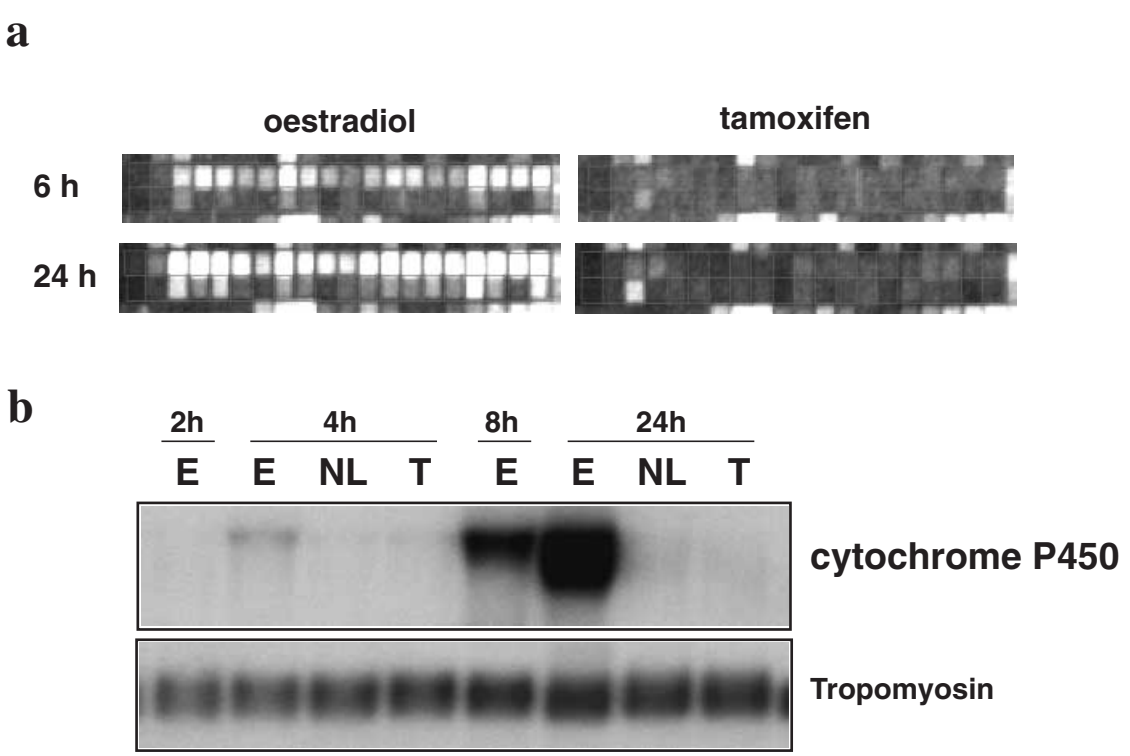

FIGURE 1. Regulation of Cyt (hIIB3) mRNA expression by ER ligands. (a) Transcript monitoring by hybridisation to oligonucleotide arrays. Each gene represented on the array is detected by 20 matched vertical pairs of 25-mer oligonucleotides. The top oligonucleotide in each pair is a perfect-match (PM) 25-mer, and the bottom oligonucleotide has a single base mismatch (MM) at the central position and serves as an internal control for hybridisation specificity. The intensity signals for each oligonucleotide are shown here for the 20 oligonucleotide pairs that detected the gene encoding Cyt. The gene is expressed more in cells cultured in the presence of 17β-oestradiol than in those cultured with 4-hydroxytamoxifen and its expression increases with time in 17β-oestradiol-stimulated cells (compare 24 and 6 h 17β-oestradiol induction). The average difference in hybridisation intensity between the PM and the MM oligonucleotides is used to determine transcript level. The comparison for each probe pair of the average difference obtained for two samples is used to calculate the fold difference. (b) Time course of Cyt (hIIB3) mRNA induction by oestradiol. Northern blot analysis of *Cyt* (hIIB3) gene expression in ZR75–1 cells treated by different ligands in the presence of cyclohexamide. The cells were cultured in the presence of 17β-oestradiol (E) or 4-hydroxytamoxifen (T), or in the absence of any ligand (NL). The oligonucleotides spotted on the GeneChip arrays are specific for two different transcripts of the *Cyt* gene. After RT-PCR and subcloning of cytochrome P450-IIB fragment, we obtained only clones corresponding to the hIIB3 isoform.

control sample from cells grown in the presence of cyclohexamide but in the absence of any ER ligand, to investigate the hormonal regulation of expression of each gene. Consistent with the DNA chip analysis we found that *Cyt* gene expression was markedly stimulated by 17β-oestradiol relative to that in antioestrogens (Fig. 1b), increasing with time to approximately 100-fold after 24 h. Surprisingly, we were not able to detect expression of *Cyt* in the human MCF-7 breast cancer cell line (data not shown).

We then analysed expression of a number of additional genes in ZR75–1 cells predicted to be differentially expressed in the presence of different ER ligands according to the HuGeneFL chip analysis, as shown in Figures2 and 3a. Thus 17β-oestradiol increased the expression of the cationic amino acid transporter *E16* by approximately fivefold after 6 h and 10-fold over a 24 h period, whereas tamoxifen and raloxifen, but not faslodex, increased expression up to twofold during this period. Levels of mRNA for *Gap* were markedly increased by 17β-oestradiol (10- to 15-fold), but were completely unaffected by antagonist. 17β-Oestradiol also increased the expression of *G6PD* and *IP3K*, both increasing by up to

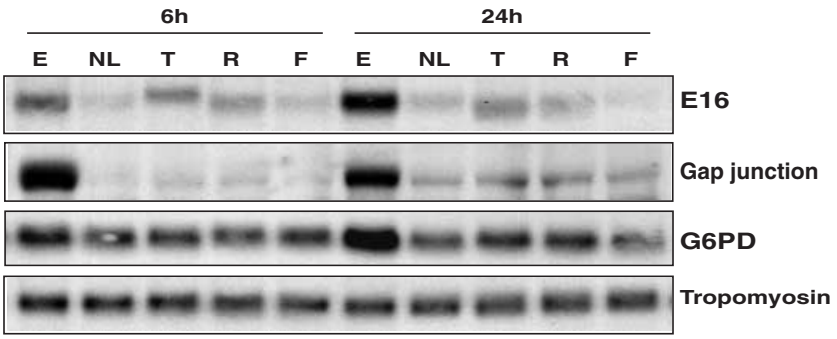

FIGURE 2. Northern blot analysis of E16, Gap (alpha 1) and G6PD mRNA expression in ZR75–1 treated by different ER ligands in the presence of cyclohexamide. The cells were cultured for 6 or 24 h in the presence of 17β-oestradiol (E), 4-hydroxytamoxifen (T), raloxifen (R) or faslodex (F), or in the absence of any ligand (NL). The Gap transcript is 3038 bases long; another transcript corresponding to connexin 43 was detected with the same probe, but its expression level was constant (data not shown).

fivefold but with different kinetics. We next examined the regulation of a number of genes in both ZR75–1 and MCF7 cells (Fig. 3b). *IGFBP4* gene was stimulated rapidly and markedly by 10 to 15-fold in both cell lines and the *IEX-1* gene was also regulated similarly in both cell lines, although the absolute levels of expression differed significantly. Finally, we examined the expression of the nuclear receptor cofactor *RIP140* and *Tob* by RNase mapping and found that they were also increased by up to threefold in the presence of 17β-oestradiol (Fig. 4). Although these genes are all oestrogen target genes, their expression was regulated with different kinetics. Increases in

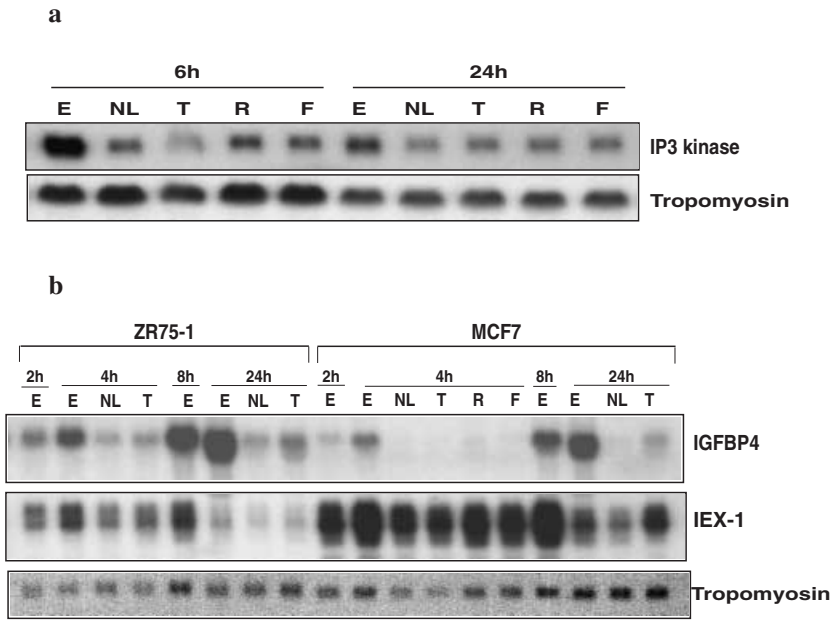

FIGURE 3. Northern blot analysis of (a) IP3K expression in ZR75–1 cells and (b) IGFBP4 and IEX-1 expression in ZR75–1 and MCF7 cells treated by different ER ligands in the presence of cyclohexamide. The cells were cultured for defined periods (as specified) in presence of 17β-oestradiol (E), 4-hydroxytamoxifen (T), raloxifen (R) or faslodex (F), or in the absence of any ligand (NL).

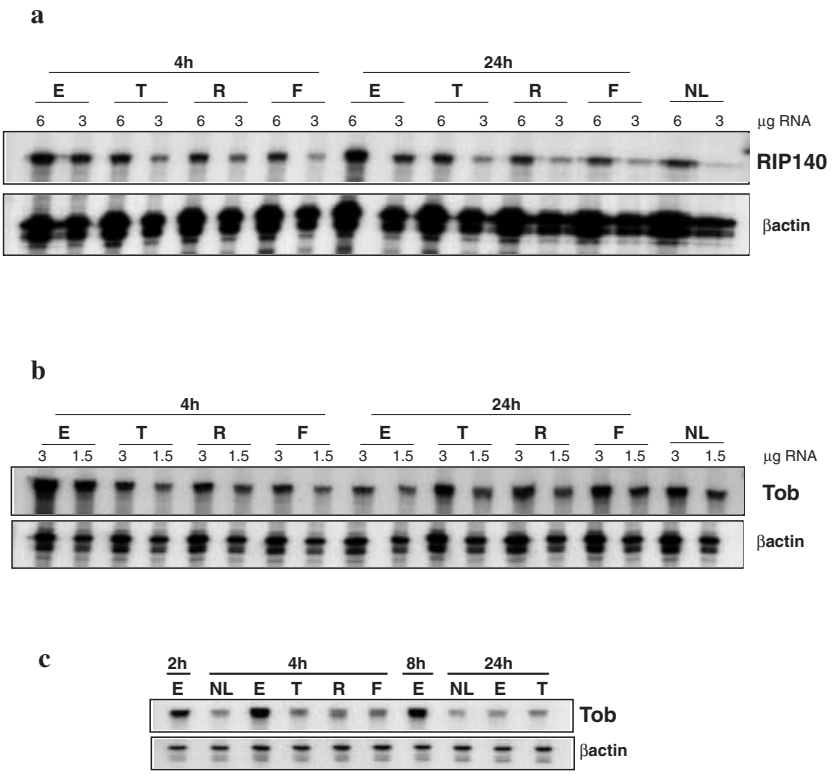

FIGURE 4. Regulation of RIP140 and Tob mRNA expression by ER ligands. (a) RNase protection analysis of RIP140 in ZR75–1 cells. (b) Tob expression in ZR75–1 cells. (c) Tob expression in MCF7 cells. Cells were cultured for 4 or 24 h in the presence of 17β-oestradiol (E), 4-hydroxytamoxifen (T), raloxifen (R) or faslodex (F), or in the absence of any ligand (NL). RNase protection experiments were carried out using different amounts of total RNA to determine accurate quantification and calculation of fold inductions. The results shown for MCF7 were obtained with 1·5 µg total RNA; those results are consistent with the results obtained with 3 µg.

mRNAs for G6PD, IP3K, Gap, IEX-1 and Tob were apparent within 6 h and there was no further increase after 24 h. In contrast, although E16 and IGFBP4 mRNAs were increased within 6 h, they were substantially greater after 24 h, whereas the increases in IEX-1 and Tob mRNA were transient. The majority of these genes were unaffected by antioestrogen treatment, with the exception of *E16* and *IGFBP4*, which increased about twofold in the presence of 4-hydroxytamoxifen and raloxifen.

DISCUSSION

The characterisation of gene expression profiles for tumour cell lines provides a framework for assessing the biological significance of expression patterns in a specific tumour (Perou *et al*. 1999, 2000, Ross *et al*. 2000). We focused on target genes for 17β-oestradiol in view of its importance as a mitogen in a subset of ER-positive cell lines and tumours. In this study we identified a number of oestrogen-regulated genes, all of which were direct targets, as they were differentially expressed in the presence of cyclohexamide. We have organised the genes into clusters according to their function.

Several genes are implicated in cell proliferation. Thus Tob, which was first identified by its ability to interact with ErbB-2, may have antiproliferative activity (Matsuda *et al*. 1996, Yoshida *et al*. 1997). IGFBP4 has been proposed to modulate binding of IGFs to their receptors and thereby regulate IGF action. Interestingly, IGFBP4 induction by 17β-oestradiol has recently been described by other groups (Glantschnig *et al*. 1998, Zazzi *et al*. 1998). Given that IGFBP4 has been found to inhibit the

growth of breast cancer cells driven by IGF pathways (Corcoran *et al*. 1996), it is conceivable that IGFBP4 expression alters the sensitivity of breast cancers to the mitogenic effects of oestrogens. IEX-1, which is an immediate early response gene induced by serum growth factor, is a member of the nuclear factor-κB/Rel (NF-κB) family of transcription factors and may be important in cell survival (Wu *et al*. 1998). Finally, the identification of cyclin D1 was expected, as it clearly has an important role in cell cycle progression in response to mitogens, including oestrogens (Planas-Silva & Weinberg 1997, Prall *et al*. 1997).

A number of differentially expressed genes encode membrane-associated proteins or proteins involved in adhesion, migration or communication between cells. For example, Gap alpha 1, a member of the connexin gene family, is a component of gap junctions that are important in cellular differentiation and organogenesis, and loss of its function is correlated with progression to a malignant phenotype (Locke 1998). The observation that its expression is upregulated by oestrogens in ZR75–1 cells is consistent with the relatively well differentiated phenotype of this ER-positive breast cancer cell line.

A third group consists of genes that encode enzymes involved in cell metabolism. The *G6PD* gene encodes a cytosolic enzyme the main function of which is to produce NADPH, a key electron donor in the defence against oxidising agents and in reductive biosynthetic reactions. Interestingly, a positive effect of 17β-oestradiol on G6PD activity in MCF7 cells has previously been described (Monet *et al*. 1987, Thomas *et al*. 1990). The *Cyt* family of enzymes are involved in steroid metabolism and in the metabolism of various xenobiotics (Nelson *et al*. 1993, Honkakoski & Negishi 2000). These enymes are highly expressed in the liver, where they have been shown to be induced by a number of xenobiotics acting through nuclear receptors such as constitutively acting receptor, pregnane X receptor and peroxisome proliferator activating receptor alpha. It is difficult to judge whether the expression of this enzyme in breast cancer cells may affect their senstivity to xenobiotics, and the importance of environmental oestrogens as a risk factor for breast cancer remains rather speculative.

The relevance of the majority of oestrogen target genes to oestrogen-dependent growth is unclear, but further analysis may provide new markers for the analysis of hormone responses in specific tumours. Thus it would be informative to examine them in a large number of ER-positive tumour samples to determine if their expression correlates with their response to endocrine therapy.

ACKNOWLEDGEMENTS

We wish to thank Affymetrix for the opportunity to use the GeneChip technology and for supplying the microarrays within the Academic User Center Program (this research was supported, in part, by an NIH Grant (PO1HG01323) to the Affymetrix User Center Program), and especially Gene Tanimoto (Affymetrix, Santa Clara) for his continuous help with the microarray experiments and data analysis. We are grateful to Alan Wakeling and Maurice Needham (AstraZeneca Pharmaceuticals, Macclesfield, UK) for providing the antioestrogens used in this study. We also thanks John Sgouros in the Computational Genome Analysis lab (ICRF) for help with data analysis and bioinformatics, and Roger White, Mark Uden and Clive Dickson for suggestions and comments on the manuscript.

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RECEIVED IN FINAL FORM 2 August 2001
ACCEPTED 7 August 2001