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

M Soulez and M G Parker

Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

(Requests for offprints should be addressed to M G Parker, Molecular Endocrinology Laboratory, Institute of Reproductive and Developmental Biology, Faculty of Medicine, Imperial College, Hammersmith Campus, Du Cane Road, London W12 0NN, UK; Email: m.parker@ic.ac.uk)

ABSTRACT

Oligonucleotide microarrays were used to analyse gene expression profiles in human ZR75–1 breast cancer cells in the presence of 17 β -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, 17 β -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 17 β -oestradiol and antioestrogens are capable of stimulating transcription from activator protein-1-containing genes, this seems to be achieved primarily by means of ER α in the presence of 17 β -oestradiol, but by either ER α or ER β 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

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 17 β -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 17 β -oestradiol. For MCF7 cell culture, insulin (10 μ g/ml) and glutamine (4 mM) were added to the medium. The cells were plated in 175 cm² 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 μ g/ml cycloheximide, with no hormone, 10^{-8} M 17 β -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 $\times$ 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 μ g/ μ l (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 $\times$ SSPE, 0.01% Tween 20) and stained with streptavidin-phycoerythrin after antibody amplification. DNA chips were read at a resolution of 6 μ m 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 17 β -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: taccactgccacttttctgtg; Tob-R: actgaggttaaggggctgtg; RIP-F: cctggcagatgattcagaca; RIP-R: gcccaactctgtgttact. 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 μ g 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: cgtttgcagtggagtgtgg; inositol 1,3,4 triphosphate 5/6 kinase (IP3K)-F: cagactcacccatcacct; IP3K-R: ttgttgggtcctcagtttcc; gap junction protein (Gap)-F: ctctctcctgggtacaagctg; Gap-R: cctccaccggatcaaaatta; insulin-like growth factor binding protein 4 (IGFBP4)-F: gcccaagaggactgagactg; IGFBP4-R: tgggtcaacaaccagacct; cytochrome P450-IIB (Cyt)-F: tccttctgaggttccgaga; Cyt-R: tggaatggaagaggaggtg; glucose-6-phosphate dehydrogenase (G6PD)-F: atttgccaacaggatcttcg; G6PD-R: tccacacctctattctccac; immediate early response 3 (IEX)-F: gacctcagcacttctcca; IEX-R: tacagcaggggaacatctc; tropomyosin 4 (TPM4)-F: cttaagacggaggccatttg; TPM4-R: gatgttgcctgcattggtg. 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 17 β -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 oligo-

nucleotide 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 17 β -oestradiol (Brown *et al.* 1984, Chalbos *et al.* 1993). This marker of oestrogen action was significantly increased by 17 β -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 17 β -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 17 β -oestradiol. The most pronounced change observed was that for *Cyt* (Fig. 1a), expression of which in the presence of 17 β -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 17 β -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 17 β -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

Gene/category		6 h						
		E (AD)	T (AD)	FC	R (AD)	FC	F (AD)	FC
Adhesion protein membrane migration								
X52003	pS2 protein (trefoil factor 1)	33 997	3826	-11·0	5047	-8·4	5374	-7·9
U78735	ABC3 (ATP-binding cassette)	9215	1593	-5·2	1468	-5·6	1330	-6·2
X52947	Gap junction	3446	645	-5·7	542	-6·4	257	-8·3
M80244	E16, SLC7A5	7987	3468	-2·3	3054	-2·6	1941	-4·1
L20814	Glutamate receptor 2 (AMPA2)	2089	571	-4·4	689	-4·1	712	-3·1
S53911	CD34	6003	1468	-4·1	1979	-3·0	1523	-3·9
L08044 (L15203)	Trefoil factor 3	5564	4276	-1·9	1821	-3·1	2446	-2·3
U81375	Equilibrative nucleoside transporter 1 (ENT1, SLC29A1)	6274	3292	-1·9	3737	-1·7	2638	-2·4
XX76180	Sodium channel, non-voltage-gated 1 alpha	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	12 276	12 257	NC	9914	NC	9417	NC
Z29083	5T4 antigen	32 739	16 039	-1·8	18 002	-1·6	15 575	-1·9
D28364	Annexin II	1324	2538	1·9	2457	2·1	2330	1·8
L38608	ALCAM	3348	5917	1·8	6981	2·1	6006	1·8
U23070	NMA (putative transmembrane protein)	3308	5780	1·7	5708	2·0	4786	1·7
XX92098	Transmembrane protein RNP24	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)	T (AD)	FC	R (AD)	FC	F (AD)
	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
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
D38305	Tob (TROB1)	Transducer of ErbB2,1	8499	3305	-4·2	3913	-3·6	4051
U22376	c-myc	Transcriptional activator, control of proliferation	7930	2954	-2·4	2284	-3·1	2800
M30703 (M30704)	Amphiregulin (AR)	Epithelial cell growth regulator (EGF family) and a tumour-inhibitory activity	5837	2316	-2·5	2364	-2·5	2550
S81914	IEX-1 (immediate-early response 3)	Regulator of progression through G1 phase during cell cycle	20 391	13 495	-1·5	12 088	-1·7	8248
X59798	Cyclin D1	Regulator	45 588	23 597	-1·9	24 664	-1·8	23 389
L27624 (D29992)	Tissue factor pathway inhibitor-2 (TFPI2)	Involved in endocytosis	120	-22	NC	476	NC	117
HG4264-HT4534 (U18420)	Guanine nucleotide binding protein, Rab5c-like protein	Modulator or transducer in various transmembrane signalling systems	29 146	12 569	-2·0	15 066	-1·7	29 765
M36430 (X04526)	Guanine nucleotide binding protein (G protein), beta polypeptide 1		1744	3551	2·0	4836	2·8	4216

TABLE 1. PART 1. Continued

		6 h					
Gene/category	Action	E (AD)	T (AD)	FC	R (AD)	F (AD)	FC
Enzyme							
M29874 (M29873, HG1875-HT1912)	Cytochrome P450-IIIB	30 850	1531	- 20.5	700	- 44.9	172 - 69.6
M68516	PCI, protein C inhibitor (plasminogen activator inhibitor 3)	1556	511	- 3.0	88	- 3.8	- 4.8
K01396	α -1-antitrypsin	6951	1648	- 3.9	1883	- 3.7	- 5.2
D29805	β -1,4-galactosyltransferase	24 391	7582	- 2.9	4161	- 5.3	- 3.8
U51336	Inositol 1,3,4-triphosphate 5/6-kinase	30 379	9203	- 3.0	9378	- 3.0	- 2.9
X55448 (M65234)	Glucose-6-phosphate dehydrogenase	33 770	16 440	- 2.2	22 870	- 1.6	- 1.5
X69878	FLT4 (FMS-related tyrosine kinase 4)	6776	3798	- 1.8	3484	- 1.9	- 1.8
D50840	Ceramide glucosyltransferase	10 084	4110	- 2.0	6076	- 1.4	- 1.9
M63138	Cathespin D	20 171	18 807	- 1.1	20 517	- 1.1	- 1.1
HG1862-HT1897	Calmodulin type I (phosphorylase kinase)	11 066	6249	- 2.5	7298	- 2.2	- 2.2
X75091	12PP2A (PHAPII SET)	1194	3262	2.8	3081	2.6	2.6
HG4747-HT5195	NADH-ubiquinone oxidoreductase, 51 kDa subunit	4784	6710	1.6	9438	2.3	2.1
HG3141-HT3317	NADH-ubiquinone oxidoreductase, 39 kDa subunit	748	3122	8.7	4017	10.6	10.0

FMS, colony-stimulating factor 1 receptor.

TABLE 1. PART 1. Continued

6 h							
Gene/category	Action	E		T		R	
		(AD)	(AD)	(AD)	(AD)	(AD)	(AD)
Transcription factor							
X84373	RIP140 (NRIPI)	754	93	— 2·7	177	— 2·5	165
U37408	CtBP, C-terminal binding protein 1	5817	2187	— 2·2	1368	— 3·5	407
U70370	PTX1 (paired-like homeodomain transcription factor 1)	13 143	8063	— 2·2	7764	— 2·4	6989
D89377 (X69295)	MSX-2	5930	1429	— 4·1	1062	— 3·6	2482
X06614	Receptor of retinoic acid	12 398	5669	— 2·5	6087	— 2·6	4729
D89377	MSX-2	1901	842	— 2·3	1298	— 1·5	622
U72066	CtIP, retinoblastoma-binding protein 8	12 498	6021	— 2·1	6365	— 2·0	4896
AF009674	AXIN 1 (axin)	11 363	3818	— 2·1	4162	— 2·1	5780
Other							
M87789 (J00231)	IGHG3 (immunoglobulin γ 3)	2015	— 1230	— 8·5	— 1394	— 8·7	— 3176
L22214	Adenosine A1 receptor	13 180	2553	— 8·9	— 239	— 28·6	1513
J02943	Corticosteroid binding globulin	4119	1754	— 2·3	2109	— 2·0	1721
M60784 (X06347)	snRNP-specific protein A	14 272	5879	— 2·4	5151	— 2·8	5395
HG2855-HT2995	Heat shock 70 kDa protein 2	3222	938	— 4·1	1569	— 2·5	1163
Unknown							
D87468	KIAA0278, ARC: activity-regulated cytoskeleton-associated protein	9171	— 1199	— 24·2	— 518	— 25·6	— 440
L18972	Anonymous gene (PK1.3)	4634	962	— 4·8	1269	— 3·7	752
D63487	KIAA0153 gene	8566	6595	— 2·0	2485	— 3·4	3155

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						
Gene/category	Action	E (AD)	T (AD)	FC	R (AD)	FC	F (AD)	FC
Adhesion protein membrane migration								
X52003	pS2 protein (trefoil factor 1)	28 009	4603	-6.1	6375	-4.4	4504	-6.2
U78735	ABC3 (ATP-binding cassette)	13 469	2058	-6.3	2063	-6.5	1924	-6.8
X52947	Gap junction	5362	1186	-4.5	1530	-3.5	607	-7.3
M80244	E16, SLC7A5	13 568	4020	-3.1	3571	-3.1	1286	-8.6
L20814	Glutamate receptor 2 (AMPA2)	1112	462	-3.3	886	-1.5	158	-5.4
S53911	CD34	4444	1482	-3.0	2108	-2.2	1836	-2.4
L08044 (L15203)	Trefoil factor 3	10 437	3471	-3.0	5340	-2.0	4518	-2.5
U81375	Equilibrative nucleoside transporter 1 (ENT1, SLC29A1)	4367	2052	-2.4	1775	-2.5	1904	-2.3
X76180	Sodium channel, non-voltage-gated 1 alpha	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	10 862	4299	-2.1	3620	-3.0	2464	-3.7
Z29083	5T4 antigen	13 198	9095	NC	10 630	NC	9657	-1.4
D28364	Annexin II	5843	10 499	1.8	12 251	2.1	7151	1.4
L38608	ALCAM	3925	6877	1.8	7755	2.0	6634	1.7
U23070	NMA (putative transmembrane protein)	1064	2358	2.2	2893	2.9	2373	2.2
XX92098	Transmembrane protein RNP24	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 Multifunctional protein that blocks apoptosis and interacts with several types of protein, including steroid receptors	32 707	11 568	-2·8	12 449	-2·6	6490	-5·0
Z35491	BAG1 (BCL2 associated athanogene)								
D38305	Tob (TROB1)	Transducer of ErbB2,1	9711	6754	-1·4	4229	-1·8	3340	-2·5
U22376 (X52125, U22376)	c-myc								
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)								
X59798	Cyclin D1	Regulator of progression through G1 phase during cell cycle	8838	5043	-1·8	4822	-1·8	3641	-2·4
L27624 (D29992)	Tissue factor pathway inhibitor-2 (TFPI2)								
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

		24 h					
Gene/category	Action	E (AD)	T (AD)	FC	R (AD)	F (AD)	FC
Enzyme							
MM29873, HG1875-HT1912)	Cytochrome P450-IIIB	39 159	759	- 49·9	876	610	- 63·8
MM68516	PCI, protein C inhibitor (plasminogen activator inhibitor 3)	1448	- 7	- 11·4	17	- 35	- 8·3
K01396	α -1-antitrypsin	7638	860	- 8·1	1560	1060	- 6·7
D29805	β -1,4-galactosyltransferase	16 680	7184	- 2·2	8138	7465	- 2·2
U51336	Inositol 1,3,4-trisphosphate 5/6-kinase	15 751	7005	- 2·0	8614	8272	- 1·7
XX55448 (M65234)	Glucose-6-phosphate dehydrogenase	24 349	9936	- 2·7	12 731	13 017	- 2·0
XX69878	FLT4 (FMS-related tyrosine kinase 4)	3736	1252	- 3·0	3118	2423	- 1·5
D50840	Ceramide glucosyltransferase	4885	2551	- 1·9	2930	2384	- 2·0
MM6138	Cathespin D	13 294	9376	- 1·6	3893	11 332	- 1·3
HG1862-HT1897	Calmodulin type I (phosphorylase kinase)	20 642	10 626	- 1·9	14 374	10 676	- 1·8
XX75091	12PP2A (PHAPII SET)	4818	5639	NC	7937	3900	NC
HG4747-HT5195	NADH-ubiquinone oxidoreductase, 51 kDa subunit	5270	7708	1·5	10 804	8773	1·6
HG3141-HT3317	NADH-ubiquinone oxidoreductase, 39 kDa subunit	4550	10 217	2·2	10 599	10 699	2·4

TABLE 1. PART 2. Continued

24 h							
Gene/category	Action	E		T		R	
		(AD)	(AD)	(AD)	(AD)	(AD)	(AD)
Transcription factor							
X84373	RIP140 (NRIP1)	1839	325	−5·0	173	−7·5	119
U37408	CtBP, C-terminal binding protein 1	5399	3611	−1·5	4204	−1·3	3266
U70370	PTX1 (paired-like homeodomain transcription factor 1)	1511	5182	−1·9	709	−5·5	3367
D89377 (X69295)	MSX-2	6496	2264	−2·9	3712	−2·2	2640
X06614	Receptor of retinoic acid	15 427	5169	−3·3	7991	−2·1	5200
D89377	MSX-2	2331	874	−2·7	1612	−1·4	743
U72066	CtIP, retinoblastoma-binding protein 8	16 909	8414	−2·0	10 718	−1·6	9751
AF009674	AXIN 1 (axin)	8196	4302	−1·4	4026	−2·2	5117
	Modulates transcriptional activation of the oestrogen receptor						−7·0
	Transcriptional repressor						−1·7
	Transcription factor						−3·7
	Involved in epithelial–mesenchymal signalling						−2·5
	Controls cell function by directly regulating gene expression						−3·6
	Involved in epithelial–mesenchymal signalling						−3·1
	Interacts with CtBP, BRCA1, mediates transcriptional regulation						−1·7
	Negative regulator of the WNT signalling pathway, promotes the GSK3B-dependent phosphorylation of β catenin						−1·6
Other							
M87789 (J00231)	IGHG3 (immunoglobulin γ 3)	5745	−584	−29·6	−1384	−28·2	−21·9
L22214	Adenosine A1 receptor	6283	713	−7·7	1839	−3·4	−3·8
J02943	Corticosteroid binding globulin	3810	1566	−2·9	2059	−2·2	−4·0
	Gm marker						
	Receptor for adenosine						
	Transports protein for glucocorticoids and progestins, belongs to the serpin family						
	Involved in splicing						
	Acts as a molecular chaperone						
M60784 (X06347)	snRNP-specific protein A	6199	2417	−2·6	3392	−1·8	−2·8
HG2855-HTT2995	Heat shock 70 kDa protein 2	7387	6432	NC	6846	NC	−1·5
Unknown							
D87468	KIAA0278, ARC: activity-regulated cytoskeleton-associated protein	1293	−441	−17·0	850	−2·5	−14·7
L18972	Anonymous gene (PK1.3)	7626	620	−12·1	352	−21·7	−11·1
D63487	KIAA0153 gene	5624	3835	NC	3230	NC	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 17 β -oestradiol than in those cultured with 4-hydroxytamoxifen and its expression increases with time in 17 β -oestradiol-stimulated cells (compare 24 and 6 h 17 β -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 17 β -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 17 β -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 Figures 2 and 3a. Thus 17 β -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 17 β -oestradiol (10- to 15-fold), but were completely unaffected by antagonist. 17 β -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 17 β -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 signifi-

cantly. 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 17 β -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 17 β -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 μ g total RNA; those results are consistent with the results obtained with 3 μ g.

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- κ B/Rel (NF- κ B) 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 enzymes 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 sensitivity 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.

REFERENCES

- Bertucci F, Houlgatte R, Benziane A, Granjeaud S, Adelaide J, Tagett R, Loriod B, Jacquemier J, Viens P, Jordan B, Birnbaum D & Nguyen C 2000 Gene expression profiling of primary breast carcinomas using arrays of candidate genes. *Human Molecular Genetics* **9** 2981–2991.
- Brown AM, Jeltsch JM, Roberts M & Chambon P 1984 Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7. *PNAS* **81** 6344–6348.
- Chalbos D, Philips A, Galtier F & Rochefort H 1993 Synthetic antiestrogens modulate induction of pS2 and cathepsin D messenger ribonucleic acid by growth factors and adenosine 3', 5'-monophosphate in MCF-7 cells. *Endocrinology* **133** 571–576.
- Corcoran D, Perachiotti A & Darbre PD 1996 Increased autocrine production of insulin-like growth factor II (IGF-II) alters serum sensitivity of MCF-7 human breast cancer cell proliferation. *Cell Proliferation* **29** 479–493.
- Dhingra K 1999 Antiestrogens – tamoxifen, SERMs and beyond. *Investigation in New Drugs* **17** 285–311.
- Glantschnig H, Varga F, Luegmayr E & Klaushofer K 1998 Characterization of the mouse insulin-like growth factor binding protein 4 gene regulatory region and expression studies. *DNA Cell Biology* **17** 51–60.
- Honkakoski P & Negishi M 2000 Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *Biochemical Journal* **347** 321–337.
- Howell A, Osborne CK, Morris C & Wakeling AC 2000 ICI 182,780 (faslodex): development of a novel, 'pure' antiestrogen. *Cancer* **89** 817–825.
- Jordan VC & Morrow M 2000 Tamoxifen, raloxifen, and the prevention of breast cancer. *Endocrine Reviews* **20** 253–278.
- Levenson AS & Jordan VC 1999 Selective oestrogen receptor modulation: molecular pharmacology for the millennium. *European Journal of Cancer* **35** 1628–1639.
- Locke D 1998 Gap junctions in normal and neoplastic mammary gland. *Journal of Pathology* **186** 343–349.
- Masiakowski P, Breathnach R, Bloch J, Gannon F, Krust A & Chambon P 1982 Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line. *Nucleic Acids Research* **10** 7895–7903.

- Matsuda S, Kawamura-Tsuzuku J, Ohsugi M, Yoshida M, Emi M, Nakamura Y, Onda M, Yoshida Y, Nishiyama A & Yamamoto T 1996 Tob, a novel protein that interacts with p185 erbB2, is associated with anti-proliferative activity. *Oncogene* **12** 705–713.
- Monet J, Thomas M, Dautigny N, Brami M & Bader C 1987 Effects of 17 beta-estradiol and R5020 on glucose-6-phosphate dehydrogenase activity in MCF-7 human breast cancer cells: a cytochemical assay. *Cancer Research* **47** 5116–5119.
- Montano MM & Katzenellenbogen BS 1997 The quinone reductase gene: a unique estrogen receptor-regulated gene that is activated by antiestrogens. *PNAS* **94** 2581–2586.
- Nacht M, Ferguson AT, Zhang W, Petroziello JM, Cook BP, Gao YH, Maguire S, Riley D, Coppola G, Landes GM, Madden SL & Sukumar S 1999 Combining serial analysis of gene expression and array technologies to identify genes differentially expressed in breast cancer. *Cancer Research* **59** 5464–5470.
- Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC, Gotoh O, Okuda K & Nebert DW 1993 The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biology* **12** 1–51.
- Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson JA, Kushner PJ & Scanlan TS 1997 Differential ligand activation of estrogen receptors ER α and ER β at AP1 sites. *Science* **277** 1508–1510.
- Perou CM, Jeffrey SS, van de Rijn M, Rees CA, Eisen MB, Ross DT, Pergamenschikov A, Williams CF, Zhu SX, Lee JC, Lashkari D, Shalon D, Brown PO & Botstein D 1999 Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. *PNAS* **96** 9212–9217.
- Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO & Botstein D 2000 Molecular portraits of human breast tumours. *Nature* **406** 747–752.
- Planas-Silva MD & Weinberg RA 1997 Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution. *Molecular and Cellular Biology* **17** 4059–4069.
- Prall O, Sarcevic B, Musgrove E, Watts C & Sutherland R 1997 Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. *Journal of Biological Chemistry* **272** 10882–10894.
- Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, Van de Rijn M, Waltham M, Pergamenschikov A, Lee JC, Lashkari D, Shalon D, Myers TG, Weinstein JN, Botstein D & Brown PO 2000 Systematic variation in gene expression patterns in human cancer cell lines. *Nature Genetics* **24** 227–235.
- Rozen S & Skaletsky H 2000 Primer 3 on the WWW for general users and for biologist programmers. *Methods in Molecular Biology* **132** 365–386.
- Sgroi DC, Teng S, Robinson G, LeVangie R, Hudson JR & Elkahoul AG 1999 *In vivo* gene expression profile analysis of human breast cancer progression. *Cancer Research* **59** 5656–5661.
- Thomas M, Bader C & Monet J 1990 Sex steroid hormone modulation of NADPH pathways in MCF-7 cells. *Cancer Research* **50** 1195–1200.
- Wakeling AE & Bowler J 1988 Biology and mode of action of pure antioestrogens. *Journal of Steroid Biochemistry* **30** 141–148.
- Westley BR & May FE 1987 Oestrogen regulates cathepsin D mRNA levels in oestrogen responsive human breast cancer cells. *Nucleic Acids Research* **15** 3773–3786.
- Wu MX, Ao Z, Prasad KV, Wu R & Schlossman SF 1998 IEX-1L, an apoptosis inhibitor involved in NF-kappaB-mediated cell survival. *Science* **281** 998–1001.
- Yoshida Y, Matsuda S & Yamamoto T 1997 Cloning and characterization of the mouse tob gene. *Gene* **191** 109–113.
- Zazzi H, Nikoshkov A, Hall K & Luthman H 1998 Structure and transcription regulation of the human insulin-like growth factor binding protein 4 gene (IGFBP4). *Genomics* **49** 401–410.

RECEIVED IN FINAL FORM 2 August 2001

ACCEPTED 7 August 2001