Evidence that the mouse insulin receptor substrate-1 belongs to the gene family on which the promoter is activated by estrogen receptor α through its interaction with Sp1

M L Panno^{1*}, L Mauro^{1*}, S Marsico³, D Bellizzi¹, P Rizza³, C Morelli², M Salerno¹, F Giordano¹ and S Ando^{1,2,3}

¹Department of Cellular Biology, ²Faculty of Pharmacy and ³Centro Sanitario, University of Calabria, Via Pietro Bucci, cubo 4c, 87030 Arcavacata di Rende (CS), Italy

(Requests for offprints should be addressed to S Ando^{*} at the Department of Cellular Biology, University of Calabria; Email: sebastiano.ando@unical.it)

^{*}(M L Panno and L Mauro contributed equally to this work)

Abstract

In the present study, the molecular mechanism underlying the up-regulatory effect of estradiol (E_2) on mouse insulin receptor substrate-1 (IRS-1) promoter was investigated in CHO cells on which the same promoter had first been functionally characterized. The mouse IRS-1 promoter bears four consensus half Estrogen Responsive Elements (ERE) sequences and thirteen AP-1- and ten Sp1-binding elements. We performed molecular dissection of this promoter gene providing 3' different deleted constructs, containing the same AP-1 rich region with a progressively increased number of ERE half sites located downstream. None of these constructs was responsive to E_2 , while a downstream region (nt -1420 to -160) rich in GC elements was induced by E_2 . However, the latter region lost its intrinsic E_2 responsiveness when the whole IRS-1 promoter was mutated for deletion in all four ERE half sites. Deletion analysis of the ERE half sites demonstrated that only ERE located at the position -1500 to -1495, close to the GC-rich region, was able to maintain the induced activatory effect of E_2 on the IRS-1 gene. Electrophoretic mobility shift and chromatin immunoprecipitation assays identified the region containing the half ERE/Sp1 (nt -1500 to -1477) as the one conferring E_2 responsiveness to the whole promoter. This effect occurs through the functional interaction between E_2 /ER α and Sp1.

Journal of Molecular Endocrinology (2006) **36**, 91–105

Introduction

Estrogen and insulin-like growth factor (IGF) regulate breast cancer cell growth and survival through the activation of distinct transductional pathways (Dickson & Lippman 1987, 1995, Surmacz 2000). However, evidence of a cross-talk between estrogen and growth factors (such as IGFs) has been documented, addressing an additive effect of these two mitogenic systems on breast cancer cell growth and survival (Molloy *et al.* 2000, Yee & Lee 2000).

The previous findings of ourselves and others have disclosed novel arguments to sustain the cross-talk between the two signals, demonstrating how exposure of breast cancer cells to estradiol (E_2) enhances the expression of insulin receptor substrate-1 (IRS-1), a key molecule linked to phosphatidylinositol-3 kinase (PI-3K)/Akt and ERK1/ERK2 pathways, crucial for cell proliferative response and survival (Surmacz 2000, Molloy *et al.* 2000, Yee & Lee 2000, Mauro *et al.* 2001). This deduction fits well with previous evidence reporting on how the mitogenic effects of insulin or IGF-I were

amplified by exposure to estrogen (Ando' *et al.* 1998, Lee *et al.* 1999). In addition, breast cancer cells overexpressing IRS-1 show a marked growth advantage and reduced or abrogated estrogen growth requirements (Guvakova & Surmacz 1997). Our recent data have demonstrated that E_2 is able to increase IRS-1 mRNA level through activation of the regulatory region of the IRS-1 gene (Mauro *et al.* 2001). Mouse IRS-1 promoter, characterized for the first time by Araki *et al.* (1995) in CHO cells, has furthermore been analyzed and our results show four consensus half Estrogen Responsive Elements (ERE) sequences and thirteen AP-1- and ten Sp1-binding elements. These might be important regulatory sites for the actions of estrogen. The up-regulatory effect induced by E_2 on this promoter activity in both MCF-7 and CHO cells, expressing estrogen receptor α (ER α), seems to underscore a general mechanism which is not strictly related to the cell type (Mauro *et al.* 2001).

In the present study, we have demonstrated, through a molecular dissection of the IRS-1 promoter, how the region bearing the ERE half site, separated by 12

nucleotides from the Sp1 site (5'-AGGTCA(N)₁₂CCG CCC-3') within nt -1500 to -1477, is responsible for the E₂-induced activation of the whole IRS-1 promoter. Both electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay confirmed that the above-mentioned sequence is functionally involved in mediating the up-regulatory effect induced by E₂ on IRS-1 expression. The effect, as documented for other E₂-responsive genes, occurs through the interaction between ER α and Sp1 proteins, bound separately to the ERE half sequence and Sp1 responsive element respectively and present in the ERE/Sp1 region of the IRS-1 promoter (Dubik & Shiu 1992, Wu-Peng *et al.* 1992, Krishnan *et al.* 1994, Rishi *et al.* 1995, Porter *et al.* 1996, 1997, Scholz *et al.* 1998, Petz & Nardulli 2000, Saville *et al.* 2000, Khan *et al.* 2003).

Materials and methods

Materials

Dulbecco's modified essential medium (DMEM)/Ham's F-12, L-glutamine, penicillin/streptomycin, calf serum (CS), bovine serum albumin (BSA), aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, 4-OH-tamoxifen and E₂ were purchased from Sigma (Milan, Italy). FuGENE 6 and poly (dI-dC) were from Roche Applied Science (Milan, Italy). Taq DNA polymerase, T4 polynucleotide kinase, 1 kb DNA ladder, dual luciferase kit, pGL₂ basic vector and timidine kinase promoter (TK) Renilla luciferase plasmid were provided by Promega (Madison, WI, USA). [γ ³²P]ATP, Sephadex G50 spin columns and the enhanced chemoluminescence (ECL) system were from Amersham Biosciences. Sp1 (1C6), ER α F10 and β -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IRS-1 antibody was from UpState Biotechnology (New York, NY, USA). Human recombinant ER α and Sp1 proteins were obtained from Invitrogen (Carlsbad, CA, USA) and Alexis (Lausen, Switzerland) respectively.

The plasmid pBluescript SKII containing mouse IRS-1 promoter (3.3 kb) and a coding region of the IRS-1 gene (3.4 kb) was kindly given by Dr Kaku Tsuruzoe (Research Division, Joslin Diabetes, Boston, MA, USA). pHEGO plasmid, containing the full length of ER α cDNA was generously provided by Dr Didier Picard (Department of Cellular Biology, University of Geneva, Geneva, Switzerland).

Plasmids

The sequence of the IRS-1 mouse promoter was analyzed by MatInspector V2.2 software to identify potential transcriptional regulatory sites, such as AP-1, Sp1 and ERE, other than that characterized by Araki

et al. (1995). The MatInspector V2.2 software is available at the web url <http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl>; it allows the identification of consensus sequences of transcriptional factors through the TRANSFAC data base (<http://transfact.gbf.de/TRANSFAC/>) (Quandt *et al.* 1995).

Plasmid pIRS-1-luciferase (luc) was generated by inserting the 3.3 kb fragment of the mouse IRS-1 gene promoter into the pGL₂ expression vector containing a luciferase gene. The sequence was confirmed by automated sequencing analysis with a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA).

IRS-1 promoter fragments (pIRS-1A-luc, pIRS-1B-luc, pIRS-1C-luc) were synthesized by unidirectional deletion using the exonuclease III reaction (Ausubel *et al.* 1988). pIRS-1D-luc was obtained by PCR using 5'-CCC TCCCTCACTCCTGCGT-3' and 5'-GGAAGATAG CCTGATCCGAG-3' as the sense and antisense primers respectively.

All ligation products were transformed into competent *Escherichia coli* cells (Invitrogen). Plasmids were isolated, and clones were confirmed by DNA sequencing. pHEGO plasmid contains the full-length ER α cDNA. The Renilla luciferase reporter vector pRL-TK (Promega) was used as a transfection standard to normalize transfection efficiency.

PCR mutagenesis

The plasmids pEREmut-luc, pERE1,2,3,mu-luc and pERE4 mu-luc were generated by PCR mutagenesis (Clackson *et al.* 1991).

pEREmut-luc contained mutation of all ERE half sites, located at sites nt -2218 to -2213, -2128 to -2123, -2050 to -2045 and -1500 to -1495. To generate this plasmid, PCRs were performed using the primers 1s (3'-GGCACCTCAGAGCAGATGG-5') and 2a (5'-TGGGGGGCGCTGGGGCGGAGGGGACGAC CCAACAGGTAAAGGCGACCCCG-3') to obtain the amplified product A (nt -3350 to -2240); the non-mutagenic external primer 3s (3'-CGTCTAATGCTCGTGCAAAC-5') and the mutagenic internal primer 6a (5'-TGGGGGGCGCTGGGGCGGAGGGGACGAC CCAACAGGTAAAGGCGACCCCG-3') to obtain the amplified product B (nt -2026 to -1466); the mutagenic internal primer 5s (5'-CGGGGGTTCGCTTTACCTGTTGGGTTCGTCCTCCGCCCCAGC GCCCCA-3') and the non-mutagenic external primer 4a (3'-AGGAAGATAGCCTGATCCGA-5') to obtain the amplified product C (nt -1536 to -170). Products B and C were used as templates in a second PCR using the primers 3s and 4a (product D, nt -2026 to -170). Products A and D were restricted by BamHI (specific sites were present in primers 2a and 3s) and ligated to obtain product E, lacking all ERE half sites, which was further

digested by SacI and HindIII (specific sites were present in primers 1s and 4a) and inserted in the pGL₂ plasmid.

pERE1,2,3,mut-luc contained mutations of the three ERE half sites, located at the sites nt -2218 to -2213, -2128 to -2123 and -2050 to -2045, and was generated using product A, as described above, and product F (nt -2026 to -170) obtained by PCR using primers 3s and 4a. Products A and F were restricted by BamHI, ligated to obtain product G, lacking the three ERE half sites, which was further digested by SacI and HindIII and inserted in the pGL₂ plasmid.

pERE4 mut-luc was mutated in the ERE half sites corresponding to nt -1500 to -1495, and was constructed using product C, as described above, and product H (nt -3350 to -1466) obtained by PCR using the primers 1s and 6a. Products C and H were used as templates in a second PCR using the primers 1s and 4a to obtain product L. The latter product was digested by SacI and HindIII and inserted in the pGL₂ plasmid.

Point mutations were done by PCR-mediated site-specific mutagenesis using degenerate primers, replacing one G with T in the ERE sequences. Namely, the ERE half site was located at position nt -2218 to -2213: AGtTCA; the ERE half site was located at position nt -2128 to -2123: TtACCC; the ERE half site was located at position nt -2050 to -2045: TCAAtG; the ERE half site was located at position nt -1500 to -1495: AGtTCA (mutations are shown as lower case letters).

Cell lines and culture conditions

CHO cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Wild-type human breast cancer (MCF-7) cells were a gift from Dr E Surmacz (Kimmel Cancer Institute, Philadelphia, PA, USA). The cell lines were cultured in DMEM/Ham's F12 (1:1) medium supplemented with 5% CS, 1% L-glutamine and 1% penicillin/streptomycin. The cells were cultured in phenol red-free, serum free medium, DMEM (PRF-SFM-DMEM) containing 0.5% BSA, 1% L-glutamine and 1% penicillin/streptomycin, 24 h before each experiment.

Transfections and luciferase assay

CHO and MCF-7 cells were seeded (1×10^5 cells/well) in DMEM/F-12 supplemented with 5% CS in 24-well plates. CHO cells were cotransfected with pIRS-1-luc promoter construct and pHEGO. Cells were transfected in SFM using FuGENE6 according to the manufacturer's instructions with a mixture containing 1 μ g/well of each specific plasmid and 25 ng/well of TK Renilla luciferase plasmid. An empty pGL₂ vector was used as the control vector to measure basal activity. Twenty-four

hours after the transfection the medium was changed and the cells were treated in PRF-SFM-DMEM in the presence of 10 pM and 1, 10 and 100 nM E₂. The firefly and Renilla luciferase activities were measured by using a dual luciferase kit. The firefly luciferase data for each sample were normalized on the basis of the transfection efficiency measured by Renilla luciferase activity.

Western blotting

CHO and MCF-7 cells were grown in 100 mm dishes to 70–80% confluence, shifted to SFM for 24 h and lysed. Protein lysates were obtained with a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 10 mM EGTA, pH 7.5, 10% glycerol, 1% Triton X-100 and protease inhibitors (2 μ M Na₃VO₄, 1% PMSF and 20 μ g/ml aprotinin).

The expression of IRS-1 was tested by Western blotting in 50 μ g protein lysates using an anti-IRS-1 antibody. Proteins were separated by SDS-PAGE and then transferred to a nitrocellulose membrane, probed with primary antibody and then stripped and reprobed with β -actin antibody. The antigen-antibody complex was detected by incubation of the membranes for 1 h at room temperature with a peroxidase-coupled anti-IgG antibody and revealed using the ECL system. Blots were then exposed to film and bands were quantified by densitometer. The results obtained are expressed in terms of arbitrary densitometric units.

Gel mobility shift assay

Nuclear extracts were prepared from CHO cells as previously described (Andrews & Faller 1991). The probe was generated by annealing single stranded oligonucleotides and labeled with [γ^{32} P]ATP and T4 polynucleotide kinase, and then purified using Sephadex G50 spin columns. The DNA sequences used as probe or as cold competitors were as follows (the nucleotide motifs of interest are underlined, mutations are shown as lower case letters): Sp1 5'-GTTGGGACTTGGCAGCt CGCCTCCCCCTGCCCAAG-3'; ERE/Sp1 5'-GAG AGCTAGCAGtTCAACCGCGTCCCTCtGCCCCA GCGCCCCACCCCTC-3'. The DNA sequence used as ERE cold competitor was as follows: 5'-TCCCC TGCAAGGTCACGGTGGCCACCCCGTG-3'. Oligonucleotides were synthesized by MWGBiotech (Ebersberg, Germany). The protein binding reactions were carried out in 20 μ l buffer (20 mM HEPES, pH 8, 1 mM EDTA, 50 mM KCl, 10 mM dithiothreitol (DTT), 10% glycerol, 1 mg/ml BSA, 50 μ g/ml poly (dI-dC)) with 50 000 c.p.m. labeled probe, 20 μ g CHO nuclear protein or an appropriate amount of ER α or Sp1 human recombinant proteins, and 5 μ g poly (dI-dC). The mixtures were incubated at room temperature for 20 min in the presence or absence of unlabeled

competitor oligonucleotides. The specificity of the binding was tested by adding specific antibodies (anti-ER α and anti-Sp1) to the reaction mixture. The entire reaction mixture was electrophoresed through a 4% polyacrylamide gel in 0.25 $\times$ Tris borate-EDTA for 3 h at 150 V. The gel was dried and subjected to autoradiography at -70°C .

ChIP and Reverse (Re)-ChIP assays

We followed the ChIP methodology described by Morelli *et al.* (2004). CHO cells were transiently transfected with pHEGO plasmid and treated with 100 nM E₂ for 1 h, or left untreated in SFM. The cells were then cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleared with sonicated salmon DNA/protein A agarose (Upstate Biotechnology Inc., New York, NY, USA) and immunoprecipitated with anti-ER α antibody (Ab) F-10 (Santa Cruz Biotechnology). Pellets were washed as reported in Morelli *et al.* (2004), eluted with elution buffer (1% SDS and 0.1 M NaHCO₃) and digested with proteinase K (Morelli *et al.* 2004). DNA was obtained by phenol/chloroform extractions and precipitated with EtOH. PCR was carried out with the ERE/Sp1 primers: upstream 5'-CTCACCCAGACACCGACATC-3' and downstream 5'-ACGCCCCGTGCCACCCAGAGC-3', or with the primers amplifying the IRS-1 promoter region containing the three upstream and non-functional ERE half sequences: upstream 5'-CAGGCAGTCTAGTGGATTGA-3' and downstream 5'-TGTGTATATGTTAGCAGATGTTTG-3'.

In Re-ChIP experiments, complexes from ER α immunoprecipitations (IPs) were eluted in RE-ChIP buffer (0.5 mM DTT, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris-HCl, pH 8.1) and subjected again to the ChIP procedure by using anti-Sp1 antibody PEP2 (Santa Cruz Biotechnology). Inputs were used as loading control and were obtained by eluting DNA from 5 μ l cell lysates prior to the IP step. Negative control was performed using normal IgGs in place of the primary antibody.

Statistical analysis

Each data point represents the means $\pm$ S.D. of at least three experiments. The data were analyzed by ANOVA using the STATPAC computer program (Statpac Inc., Bloomington, MN, USA).

Results

Analysis of the IRS-1 mouse promoter sequence

Sequencing of the IRS-1 mouse promoter by MatInspector V2.2 software (see Materials and methods)

led us to the identification of new potential transcriptional regulatory sites, in addition to those characterized by Araki *et al.* (1995), namely thirteen AP-1, ten Sp1 and four ERE half sites (Fig. 1). All these sites could be potential targets of E₂ action resulting in the activation of the IRS-1 promoter.

Identification of an E₂-activated IRS-1 promoter region

In CHO cells transiently co-transfected with pHEGO and pIRS-1-luc, encoding the full length of IRS-1 gene promoter linked to the firefly luciferase reporter gene, E₂ (10 pM and 1, 10 and 100 nM) was able to induce luciferase activity (Fig. 2A). The same results were obtained in ER-positive human breast cancer MCF-7 cells transfected with pIRS-1-luc (Fig. 2B). E₂ up-regulated IRS-1 protein content in both CHO and MCF-7 cells in a dose-dependent manner (Fig. 3).

Previous findings have demonstrated that ER may indirectly modulate transcription by interaction with the AP-1 sites (Umayahara *et al.* 1994, Paech *et al.* 1997, Webb *et al.* 1999); thus, we checked the potential role of the AP-1-rich region in mediating the activatory effect induced by E₂ on the IRS-1 promoter. In the same vein, we checked how the four ERE half sites may work, together with AP-1, in mediating the up-regulatory effect of E₂ on the activity of the whole IRS-1 promoter. With this aim, we transiently co-transfected CHO cells with pHEGO and three different 3'-deleted constructs conjugated with a luciferase reporter gene containing the same AP-1-rich region with a progressively increased number of ERE half sites, namely: (i) pIRS-1A-luc, containing one ERE half site (nt - 3370 to - 2180), (ii) pIRS-1B-luc, containing three ERE half sites (nt - 3370 to - 1650) and (iii) pIRS-1C-luc, containing four ERE half sites (nt - 3370 to - 1308). None of these constructs was stimulated by E₂ (Fig. 4).

On the contrary, the plasmid pIRS-1D-luc, containing the residual downstream region (nt - 1420 to - 160), rich in GC elements, was responsive to E₂ action (Fig. 4). The potential positive influence of AP-1 sites in the construct rich in Sp1 sequences has been ruled out since in previous experiments the deletion of the AP-1 site in the construct pIRS-1D-luc did not elicit substantial effects in the E₂-responsiveness (data not shown). Earlier reports (Saville *et al.* 2000) have shown how Sp1 *cis*-elements located in the promoter region of some genes are responsive to E₂; we therefore ascertained if the IRS-1 CG-rich region, present in the full length of IRS-1 promoter, was able to yield estrogen responsiveness independently on the four ERE half sites. Consequently, we performed site-specific mutagenesis at the ERE half sites of the IRS-1 mouse promoter located upstream to the CG-rich region. The effects of the site-specific mutagenesis on basal activity and E₂


```

-3370 ggatccaatc cagggccttg gcacctcaga gcagatggtc tacttcttct tcagtcagaa
-3310 taacattact tttatatcat aaacacccta atgttctgtg aagatgactt ataaaaatct
-3250 tccaaattaa attgtttttt ttgttgtctc ctcttcaag aaaatacttt cctccataaa
-3190 ggtacctoct ttttccccc ctctcttttt tggttggtct tggttggtga cagaggtcag
-3130 gaaggaagta attttaaaag ttaaagtctg gatttgaagt gctttcctac ataatgcgag
-3070 gtcccccctc tgtggactaa tctagattaa gcagttgcta gactagacta gctgtttatt
-3010 cccccccccc ccccacacac acactgttat tctgtacac taacttcaga tatctctagt
-2950 tgctatcctc ccaaccctta atgcatttca aatctttaga gtoccttctt tgctattgca
-2890 gtctccgtac cttttccaaa ttacttgcta tttatagcct tcctgtagtg ggcacatgtg
-2830 ccgcgcatc acagctgtca gaggggaaaa atgaaggcta tcaattcaat gtgtaccgca
-2770 taatccatta accagggtta ggagaaactg catgataaag gaggtgaaaa gagaaaacag
-2710 aggtcctcga gctttcgaga ggaggtgaca cctacagatg cggaataccc caattcaagt
-2650 accgcaagca tctgaatggc gccagtgccct acatctgtac cctctccctc tctccccttt
-2590 ccgcgcatct tgcaagacag gaaagacagg caactccacc aatcagcagg gctcccagg
-2530 aacctcaqcc cgcc tctcta gccgcattat gctgaggcgc gcagccccct ttaccatcc
-2470 gtctctcccc tactccaagc caggcagtcct agtggttgga gcatgccag cacctttact
-2410 gctgcgtctc actgcaaaac acctgcagtg ctggagagcg ggaggctgag aggttctctg
-2350 gcttaggaaa agaggtggaa aactagaa cgcc atggct agagtagaat ggctttaagc
-2290 actgggagca acgtcacatt tgtaggtctt taatgccggg tgggctagac agggacacc
-2230 tgcttgattt ataggtcatc ttaaaaagaa aaagaaaaaa aaaaactttg gggagcgtct
ERE

-2170 gcttgtggtg agactaagaa ataggctttg caaagaggct gctgacccgt tttcatcaag
ERE

-2110 aagtaccat tttcctgtgc agcctagcca cttatccttg tctccactt atcctt gtgg

-2050 tcaaggttaa aagtgtatag ggtcgtctaa tgctcgtgca aactatgaaa caaacatctg
ERE

-1990 ctaacatata cacagtaagg gtgtacactt aggaactctc tgaaggtcct ttacatcttt
-1930 aaaaaaaatc acaaatagac ttacgaatgt gataaggggt gccaggtttc ctccagcccc
-1870 cgcccccac cttttgcact aactaaccct taagtgtgaa tccctcttca cctgcatgaa
-1810 ggaagagaaa tggtgacttt tacattttat ttcttagtg atcagcggct aatagtcca
-1750 ggtgtgagat cccagggtcg gattccgaga gacctaaccc tagccaacag tcattgccct
-1690 tgggcgtccc tctctgtttc ttccaggtgc ttcaccacgg tacgtccgga gattccaggt
-1630 ggaagactgg ctcagaagtc ctttagaaaa tcttcaaaaa atgtagatct cctaccagg
-1570 gcctctcacc gagacacga catccagctg cctcccgggg gtccgcttta cctgttgggt
-1510 gagagctagc aggtcacccc cgtccctcc gccc bagcgc cccacccctc tcggggctct
ERE

-1450 gcagcccgag tgcacccccc ctctcactgc cccctccctc actcctcgct cttccccaga
-1390 tccccccctc cgtttttccc agactccctc cctgctttgc ggtagccacc caaacaagcc
-1330 tgccctaccg ccgcgggatt cctacgggac gggggcgctc tgggtggcac gggcgtggga

-1270 gacacagcgc cccgggctct gcggccacgc agggcgctgc cgagcgtgca gcaaagggcc
-1210 agcgcgcgcg ctctgcggca cgccagggg gtggagag gg cgggcgct ggcgggctcg
-1150 gcgcggaatg tagagcgagc agggagaggg agagagaccc gggctggacc gtcccgcgg
-1090 cgccagggct gctgagcgca gaggtccgt cacgtgtttt gctcctctg agtgagacgg
-1030 cggcgcggtc tgaagagggc cagcgcgcg tgccagccgc cgcagccgcc gcttgttttg
-970 gttggggctc tcggcaactc tccgaggagg aggaaggagg agggaggagg agggaggagg
-910 agaaggagg agggagagag aagtaactgc agcggtgctg cctcgggagg accgggctc
-850 tccccgac tcttcccaaa cctccccgc tcccgcctc cctccctct cccctccct
-790 cctcccttgc ggactggagc taggggcagg gatgagcctg tccctctgga ccgtccctg
-730 ctccccagc tgccagtag cgttgcgct ggaaaagcca ctttcccgc ccgcgagat
-670 gggctcgagt gcgggtgca gaggacgcgt cctcgggcgg cagcagcagc agcagcagca
-610 gcaacagccg cagcgcgcg ctctctgcga ttgagctggt atttgggcgg ctggtggcga
-550 cggggacggt tgaagggtgg gaggaggcag agggaggagg ggagaaggag gagggagaa
-490 ccggtgcaac gttgggactt ggcagccgc ctccccctgc ccaaggatat ttaatttgc
-430 tcgggaatcg ctacttccag aggggaactc gggagggaag gcgcgcgcgc ctagagagcc
-370 aagcggggac tctcgggcc atcgtgcct cctacatcgc gactcgacca ggggcgpaa
-310 gggatgcacc atagctcct ctctgtgca gggactggg gagacatagt cctcggaaga
-250 ttgcgctgc actacccta gacccactgc cctccctct ggcatgaaac gccttaaac
-190 tcggtacagg ctatcttctt tggcgcagtt acctcgtcct tcggctgccc ctccccagct
-130 ccaggaaacag cgtgaatttt ggagtcagaa tttctgcgag cttctccag ccggagtgc
-70 atgtgcgggg ccgcactgag aagccacccc tcaccagtt tttcaacacc tccctctgct
-10 ccgcagcagc

```

Figure 1 Regulatory sites of the IRS-1 mouse promoter. The analysis of the IRS-1 mouse promoter sequence by MatInspector V2.2 software allowed identification of 13 AP-1 (underlined nucleotides), ten Sp1 (nucleotides in the boxes) and four half ERE (nucleotides in grey boxes) potential regulatory sites.

Figure 2 E₂ enhances IRS-1 promoter activity. (A) CHO cells were co-transfected with pIRS-1-luc and pHEGO together with pRL-TK. (B) MCF-7 cells were transiently transfected with DNA mixture containing pIRS-1-luc and pRL-TK. The transfectants were treated in the absence (C, control) or in the presence of 10 pM and 1, 10 and 100 nM E₂ for 24 h. These results represent the means±s.d. of five different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. pGL₂ basal activity was measured in cells transfected with pGL₂ basal vector. The firefly luciferase data for each sample were normalized on the basis of the transfection efficiency measured by Renilla luciferase activity.

responsiveness were determined in transient transfection studies using the plasmids pEREmut-luc, pERE4 mut-luc and pERE1,2,3 mut-luc (see Materials and methods) (Fig. 5A).

Using the IRS-1 promoter mutated for deletion of all four ERE half sites, the CG-rich region, present in the full length of the IRS-1, was unable *per se* to yield E₂ responsiveness. On the contrary, only the ERE half site localized at the position nt -1500 to -1495, close to the Sp1 elements, was crucial in maintaining the E₂

Figure 3 E₂ up-regulates IRS-1 protein expression. Protein expression of IRS-1 in (A) CHO and (C) MCF-7 cells treated in the presence or absence of 10 and 100 pM and 1, 10 and 100 nM E₂ for 24 h. β-actin served as loading control. Representative results are shown. The histograms represent the means±s.d. of three separate experiments performed in (B) CHO and (D) MCF-7 cells, in which the band intensities were evaluated in terms of arbitrary densitometric units and expressed as the percentage of the control assumed as 100%. ●P<0.01 vs control.

Figure 4 E₂ responsiveness of different IRS-1 promoter constructs. Transcriptional activity of CHO cells co-transfected with pHEGO and with different IRS-1 promoter luciferase reporter constructs are shown. CHO cells were transiently transfected with pIRS-1A-luc, pIRS-1B-luc, pIRS-1C-luc 3'-deleted constructs and pIRS-1D-luc (see Materials and methods) together with pRL-TK. Cells were treated in the absence (C, control) or in the presence of 10 nM E₂ for 24 h. These results represent the means $\pm$ S.D. of five different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. pGL₂ basal activity was measured in cells transfected with pGL₂ basal vector. The firefly luciferase data for each sample were normalized on the basis of the transfection efficiency measured by Renilla luciferase activity.

transactivation of the IRS-1 promoter. Similar results were obtained by performing point mutations in ERE half sequences (data not shown). The important role of the fourth ERE half site together with the Sp1 region in conferring E₂ responsiveness was confirmed by a functional study performed with the following construct bearing: (i) the whole IRS-1 promoter, (ii) the fragment containing the first three ERE half sites and (iii) the fragment containing the fourth ERE half site close to the Sp1 elements (Fig. 5B). The results showed that pERE4/Sp1-luc, transfected in CHO cells, reproduced the E₂ responsiveness pattern similar to the full length of IRS-1 promoter (Fig. 5B).

On the basis of these findings, our attention was focused on the sequence ERE/Sp1 assumed to be a putative regulatory region target of E₂ action.

EMSA study

Nuclear extracts of CHO cells were incubated in the presence of ERE/Sp1-labeled oligonucleotide to prove if this region was able to bind ER α and/or Sp1 proteins. Nuclear proteins from CHO cells revealed the presence of a single band (Fig. 6, lane 1), which was inhibited by a 100-fold molar excess of the homologous ERE/Sp1 cold competitor (Fig. 6, lane 2), but remained substantially unchanged in the presence of the cold mutated competitor (Fig. 6, lane 3). The results showed an enhanced binding of the nuclear extracts, obtained from CHO cells over-expressing ER α , to the ERE/Sp1-labeled oligonucleotide (Fig. 6, lane 5) and particularly evident upon E₂ treatment (Fig. 6, lane 8). Both bands were abrogated by a 100-fold molar excess of the cold

competitor (Fig. 6, lanes 6 and 9). The specificity of the binding was demonstrated by immunodepletion induced by ER α and Sp1 antibodies (Fig. 6, lanes 10 and 11).

Using a labeled Sp1 oligonucleotide containing a GC box motif present in the Sp1-rich region of the IRS-1 mouse promoter (from nt -490 to nt -455), in a cell-free system, ER α protein *per se* was unable to bind

the Sp1 sequence (Fig. 7, lanes 1 and 2). However, the combined presence of ER α at a lower concentration and Sp1 proteins resulted in a clearly enhanced Sp1-DNA binding (Fig. 7, lanes 8 and 9) with respect to the one observed with Sp1 protein alone (Fig. 7, lanes 6 and 7). Sp1 protein was able to up-regulate its binding to the ERE/Sp1 sequence in a dose-related manner (Fig. 7, lanes 6 and 7). Such an up-regulatory effect produced by ER α was drastically attenuated in the presence of cold ERE oligonucleotide (Fig. 7, lanes 10 and 11). On performing a new set of experiments using the same ERE/Sp1-labeled oligonucleotide, as mentioned, it emerged that both Sp1 and ER α were able to bind this sequence (Fig. 8A, lanes 1 and 4). Both bindings were abrogated by a 100-fold molar excess of cold competitor (Fig. 8A, lanes 2 and 5) and restored in the presence of a mutated cold competitor (Fig. 8A, lanes 3 and 6). The specificity of the separate binding of the two proteins to the ERE/Sp1 sequence was demonstrated by the immunodepletion obtained in the presence of the two specific antibodies (Fig. 8A, lanes 8 and 9).

An increased amount of ER α enhanced Sp1 binding to the ERE/Sp1 sequence (Fig. 8B, lanes 6 and 7). In contrast, a progressive increase of Sp1 did not elicit any apparent influence on ER α binding (Fig. 8B, lanes 4 and 5). This up-regulatory effect was reversed by an excess of cold ERE oligonucleotide (Fig. 8B, lanes 8 and 9).

No cross-reaction was observed between the two specific antibodies (data not shown).

ER α and Sp1 are recruited to the ERE/Sp1 sequences of the IRS-1 promoter in CHO cells

The binding of ER α and Sp1 to the ERE/Sp1-containing sequence of the IRS-1 gene promoter was confirmed by ChIP assays. CHO cells were transiently transfected with pHEGO and treated or not treated with E₂ for 1 h. The chromatin was immunoprecipitated with anti-ER α anti-

Figure 5 Identification of IRS-1 promoter region responsive to E₂. (A) CHO cells were transiently co-transfected with pHEGO and with pIRS-1-luc or with pEREmut-luc, pERE4 mut-luc and pERE1,2,3 mut-luc, obtained by site-specific mutagenesis of IRS-1 promoter plasmid (see Materials and methods) together with pRL-TK. Cells were treated in the absence (C, control) or in the presence of different doses of E₂ for 24 h. (B) Transcriptional activity of CHO cells co-transfected with pHEGO and with pIRS-1-luc or pERE4/Sp-1-luc or pERE1,2,3-luc luciferase reporter constructs (see Materials and methods) together with pRL-TK are shown. Transfectants were treated in the absence (C, control) or in the presence of 100 nM E₂ for 24 h. The results represent the means $\pm$ S.D. of five different experiments assayed in triplicate transfections. pGL₂ basal activity was measured in cells transfected with pGL₂ basal vector. The firefly luciferase data for each sample were normalized on the basis of the transfection efficiency measured by Renilla luciferase activity.

Figure 6 Binding of nuclear extracts from CHO cells and CHO cells transfected with pHEGO (CHO/HEGO) to ERE/Sp1 oligonucleotide. Nuclear extracts from CHO (lanes 1–4) and CHO/HEGO cells (lanes 5–11) were incubated with a double stranded ERE/Sp1 sequence probe labeled with [γ^{32} P]ATP and subject to electrophoresis in a 4% polyacrylamide gel. CHO and CHO/HEGO nuclear extracts treated with 100 nM E₂ for 24 h incubated with probe are shown in lanes 4 and 8 respectively. Competition experiments were performed by adding as competitor a 100-fold molar excess of unlabeled ERE/Sp1 probe (lanes 2, 6 and 9) or a cold mutated (mut) competitor (lanes 3 and 7). The specificity of the binding was tested by adding to the reaction mixture an ER α antibody (α) (lane 10) or an Sp1 antibody (α) (lane 11). Lane 12 contains probe alone. The location of the ER α /Sp1/DNA (ER α /Sp1) complex is indicated by the arrow.

Figure 7 Binding of Sp1 to the Sp1 oligonucleotide in the presence of ERα. ³²P-labeled oligonucleotide containing the Sp1-binding site was incubated with: (i) 360 fmol (lane 1) or 540 fmol (lane 2) of ERα and (ii) 10 ng (lanes 3 and 6) or 15 ng (lane 7) of Sp1 and subjected to electrophoresis in a 4% polyacrylamide gel. ERα (360 fmol) was incubated in the presence of 10 ng (lane 8) or 15 ng (lane 9) Sp1 protein. A competition experiment was performed by adding as competitor a 100-fold molar excess of unlabeled probe (lane 4) or a cold mutated competitor (lane 5) or a cold ERE (lanes 10 and 11). Lane 12 contains probe alone. The location of the Sp1/DNA (Sp1) complex is indicated by the arrow.

body. Eluates from ERα IPs were re-immunoprecipitated with anti-Sp1 antibody (Re-ChIP) to confirm the co-existence of ERα/Sp1 complex on the promoter (Fig. 9). The recovered DNA, opportunely amplified by using specific primers mapping the ERE/Sp1-containing

sequence, showed an increased occupancy of this region by the two proteins under E₂ treatment (Fig. 9). No effect was observed when the amplified DNA region contained the other three non-functional ERE half sequences (data not shown).

Figure 8 ER-enhanced binding of Sp1 to the half ERE/Sp1-binding site. (A) 10 ng Sp1 or 360 fmol ER α proteins were incubated with a double stranded ERE/Sp1 sequence probe labeled with [γ - 32 P]ATP and subject to electrophoresis in a 4% polyacrylamide gel (lanes 1 and 4 respectively). The specificity of the binding was proved by incubating the reaction mixture containing Sp1 and ER α proteins with an anti-Sp1 or anti-ER α antibodies (lanes 8 and 9 respectively). Competition experiments were performed by adding as competitor a 100-fold molar excess of unlabeled probe (lanes 2 and 5) or a cold mutated (mut) competitor (lanes 3 and 6). Lane 10 contains probe alone. The location of the Sp1/DNA (Sp1) and ER α /DNA (ER α) complexes are indicated by the arrows. (B) 32 P-labeled oligo containing the half ERE/Sp1-binding site was incubated with 10 ng Sp1 and 360 fmol ER α (lane 1) and subjected to electrophoresis in a 4% polyacrylamide gel. Competition experiments were performed by adding as competitor a 100-fold molar excess of unlabeled probe (lanes 2) or a cold mutated (mut) competitor (lanes 3). Binding reaction contained 10 ng (lane 4) or 15 ng (lane 5) Sp1 protein in combination with 360 fmol ER α protein. Sp1 (10 ng) was incubated with 360 fmol (lane 6) or 540 fmol (lane 7) ER α , in the absence (lanes 6 and 7) or presence (lanes 8 and 9) of cold ERE. Lane 10 contains probe alone. The location of the Sp1/DNA (Sp1) and ER α /DNA (ER α) complexes are indicated by the arrows.

Discussion

Our previous findings have shown how treatment of CHO cells and estrogen-responsive positive breast cancer cell lines with E $_2$, for up to 24 h, revealed an up-regulatory effect of E $_2$ on the regulator region of the mouse IRS-1 gene (Mauro *et al.* 2001). We have here demonstrated that E $_2$ at concentrations ranging from 10 pM to 100 nM produces the same up-regulatory effect on IRS-1 protein content in both CHO and human breast cancer MCF-7 cells. This suggests that

there is a common regulatory mechanism controlling IRS-1 expression in the human and the mouse.

In order to investigate the molecular mechanism underlying the up-regulatory effect of E $_2$ on the mouse IRS-1 promoter we used the same cell type where it had been first functionally characterized. With this aim, we transiently co-transfected CHO cells with the mouse IRS-1 promoter and pHEGO and tested the response to different doses of E $_2$ ranging from 10 pM to 100 nM. The mouse IRS-1 gene, like other housekeeping genes, lacks the typical TATA and CAAT boxes (Araki *et al.*

Figure 9 Recruitment of ER α and Sp1 to the ERE/Sp1-containing IRS-1 promoter in CHO cells. CHO cells were transiently transfected with pHEGO and treated with 100 nM E₂ for 1 h (+) or left untreated (-). The cells were then cross-linked with formaldehyde, lysed and soluble, precleared chromatin was obtained as described in Materials and Methods. Chromatin (200 μ l) was immunoprecipitated with anti-ER α Ab (Santa Cruz Biotechnology) (ChIP:anti-ER) and eluates from this IP were re-immunoprecipitated with anti-Sp1 Ab (Santa Cruz Biotechnology) (Re-ChIP: anti-Sp1). The immune complexes were reverse cross-linked, and DNA was recovered by phenol/chloroform extraction and Ethanol precipitation. IRS-1 promoter regions containing ERE/Sp1 sequences were detected in the recovered DNA by PCR amplification with specific primers (Materials and Methods). To determine input DNA, the IRS-1 promoter fragment was amplified from 5 μ l purified soluble chromatin before immunoprecipitation. PCR products obtained at 35 cycles are shown. ChIP with non-immune IgG was used as negative control (N), M=marker. This experiment was repeated three times with similar results.

1995) and contains thirteen AP-1- and ten Sp1-binding sites and four ERE half sites.

It appears that in addition to binding to a classic ERE element, the ER may also modulate transcription indirectly by interaction with other DNA-binding proteins. Actually, ER interaction with AP-1-bound fos and jun proteins confers E₂ responsiveness to the ovalbumin (Gaub *et al.* 1990), *c-fos* (Weisz & Rosales 1990), collagenase (Webb *et al.* 1992) and IGF-I (Umayahara *et al.* 1994) genes. However, previous studies have demonstrated ligand- and cell-context specific differences in ER α /Ap1 and ER β /Ap1 action. For example, in HeLa cells both estrogens and anti-estrogens activated ER α /Ap1, but only anti-estrogens activated ER β /Ap1 (Paech *et al.* 1997). Thus, the latter observation led us to investigate whether the region of the IRS-1 promoter rich in AP-1-binding sites was responsive to E₂. Our results have shown that the AP-1-rich region failed to be up-regulated by E₂.

When we extended the molecular dissection downstream to implement the AP-1-rich region with the ERE half sites, the unresponsiveness to E₂ was still persistent. In contrast, the remaining downstream region of the IRS-1 promoter rich in Sp1-binding sites appears *per se* to be responsive to E₂ stimulation.

EMSA studies, performed in a cell-free system, using an oligonucleotide reproducing the Sp1 sequence present in the mouse IRS-1 promoter, revealed how ER α did not bind the Sp1 sequence but was able to enhance Sp1 binding to its own responsive element.

Sp1 was originally described as a *trans*-acting factor that bound to the GC box and activated transcription of the SV40 promoter (Dynan & Tjian 1983, Gidoni *et al.*

1984). However, it has been subsequently identified as a higher affinity consensus Sp1 site 5'-GGGGCGG GGC-3' and it has been also discovered that the sequences that varied from this consensus sequence displayed decreased affinities for Sp1 (Briggs *et al.* 1986). It is worth noting how the GC-rich region, when present in the full length of the IRS-1 mouse promoter mutated for deletion in all four ERE half sites, loses its intrinsic E₂ responsiveness. This led us to investigate the potential role of ERE half site as involved in IRS-1 E₂ responsiveness, taking into account the ability of ER α to bind as a monomer to the consensus ERE half site (Wood *et al.* 1998). Among the different ERE half sites tested, only the ERE at the position nt -1500 to -1495, appears to be crucial in conferring E₂ responsiveness to the whole promoter. The latter ERE half site was the closest one to the Sp1 sequence nt -1482 to -1477. Indeed, in CHO cells, only the construct bearing the ERE/Sp1 sequence has reproduced the same pattern of E₂ responsiveness as that given by the full length IRS-1 promoter. Because of this we reasonably postulated a functional interaction between ERE half sites and the Sp1-rich region downstream.

Results from the EMSA showed that the binding of the untreated nuclear extract to the labeled ERE/Sp1 oligonucleotide, bearing both ERE half site and Sp1 sequence (5'-GGTCAN₍₁₂₎CCGCCC-3'), resulted in a single band which was enhanced in the presence of ectopic ER α and drastically increased upon prolonged E₂ exposure. In the latter condition, a clear immunodepletion occurred in the presence of either anti-Sp1 or anti-ER α antibodies.

The ability of Sp1 and ER α to bind separately was demonstrated by two distinct bands which were abrogated in the presence of an excess of cold oligonucleotide and immunodepleted in the presence of an anti-ER α antibody in a cell-free system.

Progressively increased amounts of purified ER α protein enhanced Sp1 binding to the half ERE/Sp1-binding site in a dose-dependent manner, while increased amounts of Sp1 were unable to do so.

All these data have demonstrated that ER α enhances Sp1 binding and that both ER α and Sp1 can bind directly to the half ERE/Sp1-binding site. On the other hand, the binding of ER α and Sp1 to the ERE/Sp1-containing sequence of the IRS-1 gene promoter was confirmed by ChIP assay which showed an increased occupancy of this region by the two proteins under E₂ treatment. In contrast, this was not observed when, in the ChIP assay, we used the sequence containing the first three ERE half sites. On the basis of these findings, it emerges that the ERE/Sp1-binding site is crucially involved in mediating E₂ responsiveness to the IRS-1 gene. In contrast, the three non-functional ERE half sequences upstream of the ERE/Sp1 site are not involved in the process. This finding acquired relevance when we became aware of how the functional synergism between Sp1 and ER α , through the formation of the ER α /Sp1 complex, was responsible of the activation of other E₂-responsive genes. For instance, in the promoter region of such genes the half palindromic ERE sequence and Sp1 were separated by a number of nucleotides ranging from 10 to 23 nt, such as cyclin D1, bcl2, retinoic acid receptor α 1, IGF-binding protein 4, adenosine deaminase, DNA polymerase α , *c-fos*, cathepsin D, transcription factor-E2F1, creatine kinase B, human progesterone receptor A promoter and, recently, *cad* gene (Dubik & Shiu 1992, Wu-Peng *et al.* 1992, Krishnan *et al.* 1994, Rishi *et al.* 1995, Porter *et al.* 1996, 1997, Scholz *et al.* 1998, Wang *et al.* 1998, Petz & Nardulli 2000, Salvatori *et al.* 2000, Saville *et al.* 2000, Tanaka *et al.* 2000, Vyhldal *et al.* 2000, Li *et al.* 2001, Khan *et al.* 2003).

While models of DNA are typically drawn in a linear array, the packaging of DNA and proteins into the nucleus of a cell requires tremendous compaction. This compaction could facilitate interaction between *trans*-acting factors bound to more distant *cis* elements. For instance, both ER α and SP1 are known to directly associate with the Transcription Factor (TFII) component. In particular, Sp1 has been reported to recruit TFII/TFII-binding protein and mediate formation of the transcription preinitiation complex on the TATA-less promoter (Pugh & Tjian 1991).

On the other hand, ER α as is known, interacts with the TATA-binding protein (TBP) transcription factor IIb (TFIIb) and TBP-associated factor (TAF)_{II30} (Ing *et al.* 1992, Jacq *et al.* 1994, Sabbah *et al.* 1998)). Thus,

we can reasonably assume that the interaction of ER α and SP1, by recruiting TFII/TFII-binding protein, could foster the formation of a protein-protein network that helps to establish an active transcriptional complex. Furthermore, the E₂-dependent recruitment of coactivators such as CREB binding protein (CBP)/p300, which can function as a histone acetyltransferase (Ogryzko *et al.* 1996), could help remodel chromatin in different promoters and enhance formation of an interconnected protein-protein and protein-DNA network involved in activation of the IRS-1 gene.

Thus, the active complex ER α /E₂-Sp1 could trigger the interaction between *trans*-acting factors bound to more distant *cis* elements, like the GC downstream elements, potentiating the transcriptional machinery at the level of the whole GC-rich region of the IRS-1 promoter, which is a region reported to have positive active elements on IRS-1 promoter activity (Araki *et al.* 1995).

Thus, with the present findings, we have demonstrated the molecular mechanism through which E₂/ER α up-regulates mouse IRS-1 expression, thereby amplifying IGF-I/insulin signaling.

Since IRS-1 is sufficient to increase rRNA synthesis and cell size (Sun *et al.* 2003), its enhanced expression, upon prolonged E₂ exposure, may establish another intriguing link between E₂, cell growth and its mitogenic potentiality.

Acknowledgements

We thank Dr Domenico Sturino for the English revision of the manuscript and Dr Pasquale Cicirelli for technical assistance. This work was supported by A.I.R.C. grant 2003. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Ando S, Panno ML, Salerno M, Sisci D, Mauro L, Lanzino M & Surmacz E 1998 Role of IRS-1 signaling in insulin-induced modulation of estrogen receptors in breast cancer cells. *Biochemical and Biophysical Research Communication* **253** 315–319.
- Andrews NC & Faller DV 1991 A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Research* **19** 2499.
- Araki E, Haag BL, Matsuda K, Shichiri M & Kahn R 1995 Characterisation and regulation of the mouse insulin receptor substrate gene promoter. *Molecular Endocrinology* **9** 1367–1379.
- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JD & Struhl K 1988 Enzymatic manipulation of DNA and RNA. In *Current Protocols in Molecular Biology*, vol.1. pp 3.0.1–3.17.3 Hoboken, NJ: John Wiley & Sons Inc.
- Briggs MR, Kadonaga JT, Bell SP & Tjian R 1986 Purification and biochemical characterization of the promoter-specific transcription factor, Sp1. *Science* **234** 47–52.

- Clackson T, Gussow D & Jones PT 1991 General application of PCR to gene cloning and manipulation. In *PCR. A Practical Approach*, pp 185–192. Eds MJ McPherson, P Quirke & GR Taylor. New York: Oxford University Press.
- Dickson RB & Lippman ME 1987 Estrogenic regulation of growth factor secretion in human breast carcinoma. *Endocrine Reviews* **8** 29–43.
- Dickson RB & Lippman ME 1995 Growth factors in breast cancer. *Endocrine Reviews* **16** 559–589.
- Dubik D & Shiu R 1992 Mechanism of estrogen activation of c-myc oncogene expression. *Oncogene* **7** 1587–1594.
- Dynan WS & Tjian R 1983 The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. *Cell* **35** 79–87.
- Gaub MP, Bellard M, Scheuer I, Chambon P & Sassone-Corsi P 1990 Activation of the ovalbumin gene by the estrogen receptor involves the Fos-Jun complex. *Cell* **63** 1267–1276.
- Gidoni D, Dynan WS & Tjian R 1984 Multiple specific contacts between a mammalian transcription factor and its cognate promoters. *Nature* **312** 409–413.
- Guvakova MA & Surmacz E 1997 Overexpressed IGF-I receptors reduce estrogen growth requirements, enhance survival, and promote E-cadherin-mediated cell–cell adhesion in human breast cancer cells. *Experimental Cell Research* **231** 149–162.
- Ing NH, Beckman JM, Tsai SY, Tsai MJ & O'Malley BW 1992 Members of the steroid hormone receptor superfamily interact with TFIIB (S300-II). *Journal of Biological Chemistry* **267** 17617–17623.
- Jacq X, Brou C, Lutz Y, Davidson I, Chambon P & Tora L 1994 Human TAFII30 is present in a distinct TFIID complex and is required for transcriptional activation by the estrogen receptor. *Cell* **79** 107–117.
- Khan S, Abdelrahim M, Samudio I & Safe S 2003 Estrogen receptor/Sp1 complexes are required for induction of cad gene expression by 17 beta-estradiol in breast cancer cells. *Endocrinology* **144** 2325–2335.
- Krishnan V, Wang X & Safe S 1994 Estrogen receptor-Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells. *Journal of Biological Chemistry* **269** 15912–15917.
- Lee AV, Jackson JG, Gooch JL, Hilsenbeck SG, Coronado-Heinsohn E, Osborne CK & Yee D 1999 Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression *in vitro* and *in vivo*. *Molecular Endocrinology* **13** 787–796.
- Li C, Briggs MR, Ahlborn TE, Kraemer FB & Liu J 2001 Requirement of Sp1 and estrogen receptor alpha interaction in 17 beta-estradiol-mediated transcriptional activation of the low density lipoprotein receptor gene expression. *Endocrinology* **142** 1546–1553.
- Mauro L, Salerno M, Panno ML, Bellizzi D, Sisci D, Miglietta A, Surmacz E & Ando S 2001 Estradiol increases IRS-1 gene expression and insulin signaling in breast cancer cells. *Biochemical and Biophysical Research Communication* **288** 685–689.
- Molloy CA, May FEB & Westley BR 2000 Insulin receptor substrate-1 expression regulated by estrogen in the MCF-7 human breast cancer cell line. *Journal of Biological Chemistry* **275** 12565–12571.
- Morelli C, Garofalo C, Sisci D, del Roncon S, Cascio S, Tu X, Vecchione A, Sauter ER, Miller WH Jr & Surmacz E 2004 Nuclear insulin receptor substrate 1 interacts with estrogen receptor alpha at ERE promoters. *Oncogene* **23** 7517–7526.
- Ogryzko VV, Schiltz RL, Russanova V, Howard BH & Nakatani Y 1996 The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87** 953–959.
- Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson JA, Kushner PJ & Scanlan TS 1997 Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* **277** 1508–1510.
- Petz LN & Nardulli AM 2000 Sp1 binding sites and an estrogen response element half-site are involved in regulation of the human progesterone receptor A promoter. *Molecular Endocrinology* **14** 972–985.
- Porter W, Wang F, Wang W, Duan R & Safe S 1996 Role of estrogen receptor/Sp1 complexes in estrogen-induced heat shock protein 27 gene expression. *Molecular Endocrinology* **10** 1371–1378.
- Porter W, Saville B, Hoivik D & Safe S 1997 Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Molecular Endocrinology* **11** 1569–1580.
- Pugh BF & Tjian R 1991 Transcription from a TATA-less promoter requires a multisubunit TFIID complex. *Genes and Development* **5** 1935–1945.
- Quandt K, Frech K, Karas H, Wingender E & Werner T 1995 MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Research* **23** 4878–4884.
- Rishi A, Hhao ZM, Baumann R, Li XS, Sheikh S, Kimura S, Bashirelahi N & Fontana J 1995 Estradiol regulation of the human retinoic acid receptor gene in human breast carcinoma cells is mediated via an imperfect half-palindromic estrogen response element and Sp1 motifs. *Cancer Research* **55** 4999–5006.
- Sabbah M, Kang KI, Tora L & Redeuilh G 1998 Oestrogen receptor facilitates the formation of preinitiation complex assembly: involvement of the general transcription factor TFIIB. *Biochemical Journal* **336** 639–646.
- Salvatori L, Ravenna L, Felli MP, Cardillo MR, Russo MA, Frati L, Gulino A & Petrangeli E 2000 Identification of an estrogen-mediated deoxyribonucleic acid-binding independent transactivation pathway on the epidermal growth factor receptor gene promoter. *Endocrinology* **141** 2266–2274.
- Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA & Safe S 2000 Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. *Journal of Biological Chemistry* **275** 5379–5387.
- Scholz A, Truss M & Beato M 1998 Hormone-induced recruitment of Sp-1 mediates estrogen activation of the rabbit uteroglobin gene in endometrial epithelium. *Journal of Biological Chemistry* **273** 4360–4366.
- Sun H, Tu X, Prisco M, Wu A, Casaburi I & Baserga R 2003 Insulin-like growth factor receptor signaling and nuclear translocation of insulin receptor substrates 1 and 2. *Molecular Endocrinology* **17** 472–486.
- Surmacz E 2000 Function of the IGF-IR in breast cancer. *Journal of Mammary Gland Biology and Neoplasia* **5** 95–105.
- Tanaka N, Yonekura H, Yamagishi S, Fujimori H, Yamamoto Y & Yamamoto H 2000 The receptor for advanced glycation end products is induced by the glycation products themselves and tumor necrosis factor-alpha through nuclear factor-kappa B, and by 17 beta-estradiol through Sp-1 in human vascular endothelial cells. *Journal of Biological Chemistry* **275** 25781–25790.
- Umayahara Y, Kawamori H, Watada H, Imano E, Iwama N, Morishima T, Yamasaki Y, Kajimoto Y & Kamada T 1994 Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. *Journal of Biological Chemistry* **269** 16433–16442.
- Vyhlidal C, Samudio I, Kladde MP & Safe S 2000 Transcriptional activation of transforming growth factor alpha by estradiol: requirement for both a GC-rich site and an estrogen response element half-site. *Journal of Molecular Endocrinology* **24** 329–338.
- Wang F, Hoivik D, Pollenz R & Safe S 1998 Functional and physical interactions between the estrogen receptor Sp1 and nuclear aryl hydrocarbon receptor complexes. *Nucleic Acid Research* **26** 3044–3052.
- Webb P, Lopez GN, Greene GL, Baxter JD & Kushner PJ 1992 The limits of the cellular capacity to mediate an estrogen response. *Molecular Endocrinology* **6** 157–167.

- Webb P, Nguyen P, Valentine C, Lopez GN, Kwok GR, McNerney E, Katzenellenbogen BS, Enmark E, Gustafsson JA, Nilsson S & Kushner PJ 1999 The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. *Molecular Endocrinology* **13** 1672–1685.
- Weisz A & Rosales R 1990 Identification of an estrogen response element upstream of the human *c-fos* gene that binds the estrogen receptor and the AP-1 transcription factor. *Nucleic Acids Research* **18** 5097–5106.
- Wood JR, Greene GL & Nardulli AM 1998 Estrogen response elements function as allosteric modulators of estrogen receptor conformation. *Molecular and Cellular Endocrinology* **18** 1927–1934.
- Wu-Peng X, Pugliese T, Dickerman H & Pentecost B 1992 Delineation of sites mediating estrogen regulation of the rat creatine kinase B gene. *Molecular Endocrinology* **6** 231–240.
- Yee D & Lee AV 2000 Crosstalk between the insulin-like growth factor and estrogens in breast cancer. *Journal of Mammary Gland Biology and Neoplasia* **5** 1–5.

Received in final form 21 October 2005

Accepted 26 October 2005

Made available online as an Accepted Preprint 18 November 2005