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Immediate early gene X-1 (*IEX*-1), a hydroxytamoxifen regulated gene with increased stimulation in MCF-7 derived resistant breast cancer cells

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Abstract

The efficacy of tamoxifen in breast cancer treatment only lasts a few years and the tumor eventually recurs. We performed selective subtractive hybridization to isolate mRNAs that were differentially expressed in MCF-7 derived cells, in which resistance had been induced through long-term culture in the presence of hydroxytamoxifen (OHT). Among the 15 mRNAs found to be overexpressed, we focused on Immediate early gene X-1 (*IEX*-1) mRNA because of the recognized contribution of its expression to apoptosis or cell cycle progression, depending on the cell type and culture conditions.

We observed that *IEX*-1 expression was stimulated by OHT, that the degree of increase was greater in resistant cells (four-fold versus 1.5-fold) and that this OHT regulation was estrogen receptor dependent. A detailed study of the *IEX*-1 promoter indicated that it involved NF-κB. Our cells were not cross-resistant to faslodex, a pure antiestrogen, which moreover was inefficient in regulating *IEX*-1 expression.

Altogether, our data suggest that the greater *IEX*-1 expression in OHT resistant cells is related to their ability to grow in the presence of OHT. Knowledge on the capacity of OHT to stimulate gene expression and its NF-κB dependence should contribute to a better understanding of tamoxifen pharmacology and allow new drug strategies to be designed that would delay antiestrogen resistance acquisition.
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Keywords: Acquired antiestrogen resistance; MCF-7 breast cancer cells; Faslodex; Hydroxytamoxifen; *IEX*-1; NF-κB; Regulation of gene expression

1. Introduction

Tamoxifen is the main adjuvant treatment for breast cancer patients with tumors expressing estradiol receptor alpha (ER). However, its efficacy in controlling remission of metastatic cancers or increasing survival only lasts a few years and the patient inevitably relapses. Tumor cells either acquire tamoxifen resistance or the initial tumor is heterogeneous and a few primarily resistant cells are selected by the tamoxifen treatment and a new tumor emerges despite antiestrogen therapy.

Since primary resistance is closely associated with ER negativity, it was long considered that acquired resistance might be due to modifications in ER expression. This is not, however, a common event and not all resistant tumors contain a lower amount of ER or express a mutated ER or a function-lacking ER. Besides, the resistant tumor is generally still sensitive to alternative endocrine therapies [1]. Other studies have focused on alterations further along the ER pathway, but there is presently no clear-cut evidence of any single mechanism leading to resistance. For instance, decreased levels of N-CoR (nuclear receptor corepressor) [2] and high AIB1 (or SRC-3, estrogen receptor coactivator) [3] were shown to be correlated with the acquisition of resistance to tamoxifen. On the other hand, another study provided no support for the hypothesis that inappropriate expression of nuclear receptor interacting proteins (TIF-1, SUG-1, RIP140 and SMRT) is a mechanism for resistance to tamoxifen [4]. Furthermore, increased AP-1 DNA binding and/or increased activity of the AP-1 activating enzyme, c-Jun NH2-terminal kinase, has often been observed in acquired tamoxifen resistance [5–7]. Lastly, the NF-κB complex, which is known to be repressed by ER in an E2-dependent manner [8,9], was described to have its level and activity increased in MCF-7 derived cells (MCF7/LCC9) rendered resistant to faslodex and cross-resistant to tamoxifen [10]. Alternative growth-signaling pathways were also explored. In particular, clinical reports have shown that the EGF receptor is overexpressed in many human breast tumors and that overexpression is associated with a lack of

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response to endocrine therapy [11]. In in vitro studies and in agreement with clinical observations, cells that become capable of growing in the presence of faslodex (FASMCF cells) display enhanced EGF receptor signaling [12]. Nevertheless, in an earlier work with faslodex resistant MCF-7 derived cells, no altered expression of receptors in the ErbB-family was detected [13].

Following a work on silencing of a few genes in hydroxytamoxifen (OHT) resistant cells [14], a wider screening of gene expression modifications was performed here, involving selective subtractive hybridization (SSH), to isolate mRNAs differentially expressed in resistant cells. The cell model was the MVLN cell line, a clonal MCF-7 derived cellular model developed in our laboratory to screen potential endocrine disruptors [15], and $MVLN^{OHT}$ cells in which resistance had been induced through long-term culture in the presence of OHT [14]. The growth of $MVLN^{OHT}$ cells was found stimulated by OHT, a phenotype observed in clinical responses although its incidence is only 20% [16]. Fifteen mRNAs were found to be overexpressed. We focused on one of these mRNAs, that of Immediate early response gene X-1 (*IEX*-1) because of its recognized contribution to apoptosis or cell cycle progression, depending on the cell type and culture conditions [17–19].

We showed for the first time that *IEX*-1 expression was stimulated by OHT in MCF-7 and MCF-7 derived cells and that the stimulation was more pronounced in OHT resistant cells than in parent cells. OHT stimulated *IEX*-1 expression was ER dependent and the NF-κB binding site was the main *IEX*-1 promoter binding site involved in this regulation. Our observation supports the assumption that a rise in the expression of *IEX*-1 might contribute to the ability of OHT resistant cells to grow in the presence of OHT.

2. Materials and methods

2.1. Materials

Materials for cell culture came from Life Technologies (Cergy-Pontoise, France). OHT and faslodex (formerly ICI 182,780) came from Zeneca. 17β-Estradiol (E2), 4′,6-diamidino-2-phenylindole (DAPI), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and trichostatine A (TSA) were purchased from Sigma Chimie (Saint Quentin Fallavier, France). Restriction endonucleases and DNA Random Labelling NEBlot kits from New England Biolabs, PCR-Select cDNA Subtraction kits and pNF-κB Vector from Clontech were purchased from Ozyme (Montigny le Bretonneux, France). The PolyATtract mRNA isolation system III used for isolating poly A^+ RNA and the pGEM-T vector system and competent JM 109 cells used for cloning PCR products were from Promega (Charbonnières, France). QIAprep Spin Plasmid Miniprep Test kits, QIAquick PCR purification kits and RNeasy RNA extraction kits were from Qiagen (Courtabœuf, France). The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit was from PE Applied Biosystems (Paris, France). Nylon Hybond-N^+ membrane was from Amersham (Les Ulis, France). A UV-Stratalinker 1800 used to cross-link RNA on Nylon membranes and cloned Pfu DNA polymerase were from Stratagene (La Jolla, CA, USA). Ultrahyb hybridization buffer was from Ambion (CliniSciences, Montrouge, France). A Fujix BAS1000 PhosphorImager was used to analyze Northern blots. FuGene 6 transfection reagent was from Roche Diagnostics (Meylan, France). Luciferin was synthesized by G. Auzou (Inserm, Montpellier, France) according to Bowie [20].

2.2. Cell lines and culture conditions

Breast cancer MCF-7 cells and the MVLN cells derived from MCF-7 cells by stable transfection with a pVit-tk-luc plasmid [15] were cultured in DMEM with phenol red, supplemented with 5% FCS (FCS medium). Medium was replaced every other day. $MVLN^{OHT}$ and MCF-7^{OHT} cells were obtained by culturing parent cells in the presence of 2×10^{-7} M OHT in DMEM without phenol red supplemented with 3% of a steroid-free, dextran-coated charcoal-treated fetal calf serum (DCC medium) for 6 months. At the end of treatment, over 30 different clones were isolated from $MVLN^{OHT}$ cells [14] and some of them (cl 7, cl 8, cl 20, cl 27 and cl 32) were used in the present study. MCF-7 cells, MVLN cells and MVLN clones have been haplotyped with a combination of nine CA repeat microsatellite markers from the Genethon collection, respectively localized on chromosomes 1, 6 and 17 [21].

2.3. Cell growth assay

For each population assayed, cells were first cultured in DCC medium for 7 days. They were then harvested and 2×10^4 cells per well were seeded in 24-well tissue culture cluster plates in the same medium. One day later, the medium was replaced by fresh medium containing E2 (10^{-9} M), OHT (10^{-7} M), faslodex (10^{-7} M) or vehicle alone (0.1% ethanol). These media were renewed every 2 days. The cells of three wells from each culture condition were arrested when about 80% confluency was reached in the most favorable condition (in the presence of E2) by fixing cells with 200 μl absolute ethanol. Wells were assayed for their DNA content by measuring its fluorescence in a SpectraMAXgeminiXS (Molecular Devices) in the presence of DAPI [22].

2.4. Short hormonal treatments, RNA preparation and selective subtractive hybridization (SSH)

SSH was performed as indicated by the supplier with the PCR-Select Clontech subtraction kit to isolate mRNAs that were more expressed in resistant $MVLN^{OHT}$ cells growing in OHT-containing medium than in sensitive MVLN cells cultivated in the same medium. The bulk resistant cell culture was used as a source of resistant cells rather than isolated

resistant clones to obtain the various overexpressed mRNAs in resistant cells even if they were not overexpressed in all of them. Since resistant cells grow in the presence of OHT, whereas sensitive cell growth is slowed down in the same medium, a simple subtraction of sensitive cell mRNAs from resistant cell mRNAs could generate multiple unwanted mRNAs, i.e. those reflecting only the growth status of resistant cells. We therefore avoided selecting these mRNAs by subtracting a combination of mRNAs from sensitive cells cultivated in the presence of OHT and mRNAs from the same cells growing in the presence of E2.

Cells were first subjected to a short hormonal treatment: resistant and sensitive cells were cultured for 3 days in DCC medium and then for 4 days in DCC medium containing either 10^{-7} M OHT (for sensitive and resistant cells) or 10^{-9} M E2 (for sensitive cells only). At the end of treatment, total RNA was extracted with a Qiagen kit and RNA quality was controlled by measuring the 28S:18S RNA ratio (>1.5). Poly A^+ RNA was purified with the Promega kit. The cDNA synthesized from 2 μg of poly A^+ RNA from resistant cells (cultured in OHT-containing medium) was used as the SSH "tester" and the "driver" contained a mixture of cDNAs synthesized from 1.5 μg poly A^+ RNA from OHT sensitive cells cultured in the presence of OHT and the same amount from sensitive cells cultured in the presence of E2, for the above described reason. The tester/driver ratio was 1/30.

2.5. Cloning and sequencing of the subtraction product obtained by SSH

The final SSH PCR mixture, enriched for differentially expressed cDNAs, was purified with a PCR Purification Kit and ligated overnight at 16 °C in the pGEM-T vector (as advised by Clontech). JM109 competent cells were transformed with 10 μl of the ligation mixture and the clones obtained were subsequently sequenced in both directions after mini-preparation with a Qiagen Miniprep Kit. Sequencing was performed using oligonucleotide primers in the pGEM-T vector sequence. Homology searches were performed using the BLAST program to identify genes [23].

2.6. Reverse Northern blot analysis of clones generated by SSH

To screen cDNA fragments cloned in pGEM-T vector for modified expression, we immobilized their PCR-amplified products on two identical Nylon membranes using a dot-blot apparatus and hybridized the membranes to $^{[32]}$P-labeled cDNAs from either OHT sensitive MVLN or resistant $MVLN^{OHT}$ cells that had grown for 3 days in DCC medium and then for 4 days in DCC medium containing 10^{-7} M OHT, according to Poirier et al. [24]. Various quantities of β-actin PCR product were spotted on membranes to standardize signals. The results were analyzed with a PhosphorImager.

2.7. Northern blot analysis of IEX-1 expression

One of the clones generated by SSH and found to be differentially expressed by reverse Northern blotting was coding *IEX*-1. *IEX*-1 expression was further analyzed by Northern blotting to confirm differences of mRNA levels and quantify it in different situations. $MVLN^{OHT}$, MVLN, MCF-7^{OHT} and MCF-7 cells were subjected to various treatments at the concentration and for the times indicated in the figure legends. At the end of treatment in T75 culture flasks, confluence was 80% and RNA was extracted. Approximately 100 μg total RNA were obtained from one flask. Twenty microgram of total RNA in an ethidium bromide-containing solution were electrophoretically separated on a 1% agarose denaturing gel and transferred to Nylon membrane. The RNA transferred onto a given membrane was estimated by measuring the ethidium bromide fluorescence with NIH image software. It was then UV cross-linked to the membrane. The membrane was hybridized at 42 °C overnight in Ultrahyb buffer with a random labelled $^{[32]}$P-labeled *IEX*-1 cDNA probe obtained from the corresponding insert in pGEM-T issued from the SSH. After stringency washes, filters were exposed to the PhosphorImager screen to evaluate *IEX*-1 expression and then autoradiographed. After dehybridization, the membrane was again hybridized with $^{[32]}$P-labeled 18S rRNA cDNA probe to standardize *IEX*-1 expression. The 18S cDNA probe was obtained by random primer labeling of a template generated by PCR with the following oligonucleotid e primers from Genosys Biotechnologies (Montigny Le Bretonneux, France): 5′-CTTCCGGGAAACCAAAGTCT-3′ (upper) and 5′-GGCCTCACTAAACCATCCAA-3′ (lower). The data are presented as mean ± S.D. and were analyzed by the Student's *t*-test when only two groups were compared. For more than two group comparisons, a Kruskal Wallis non-parametric test was performed in addition with Fisher post-ANOVA test for each individual comparison. Differences were considered significant at $P < 0.05$.

2.8. Cloning of IEX-1 promoter into a plasmidic vector and transient transfections

We analyzed the 1200 bp sequence of *IEX*-1 promoter, issued from the continuous 1,796,938 bp genomic sequence of the HLA class I region located at 6p21.3 [25], with MatInspector software [26]; it contained some already described binding sites in the proximal promoter part [27–29] and two half EREs in −760 to 756 and −527 to 523 positions upstream of the initiation site. To obtain the −776+21 *IEX*-1 promoter part as a PCR product with Pfu DNA polymerase and facilitate the cloning experiments, we used *IEX*-1 promoter primers containing a restriction enzyme site in their 5′-part: 5′-AGTCGACAGATCTTGCGTGCATA-TAACTGGGTCAG-3′ (upper) and 5′-CGTCGACAAGCT-TGAGCGGAGTGTAAGGCCAAGT-3′ (lower) with a *Bgl*II and a *Hind*III restriction site, respectively. Thanks

to the inserted restriction sites, we obtained pIEX-Luc by substituting the *IEX*-1 promoter part with the Vit-tk part of pVit-tk-Luc [15] built from pPoly III vector [30]. Transient transfections were performed in MCF-7^{OHT} and MCF-7 cells with 300 ng plasmid per well in 12-well tissue culture plates with the FuGene 6 reagent.

2.9. Luciferase assay in transiently transfected cells

Lysates of transiently transfected cells were prepared as recommended by Promega Corporation. Briefly, cells were washed twice with 1 ml of PBS and lysed with 0.4 ml of lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mm EDTA, 10% glycerol, 1% Triton X-100) for 10 min. Cell lysate (100 μl) was transferred to wells of a 96-well plate and luminescence was detected after injection of 100 μl of luciferase detection buffer (20 mM Tricine, pH 7.8, 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.2 mM EDTA, 0.53 mM ATP, 0.27 mM coenzyme A, 0.48 mM luciferin). The results were normalized using β-galactosidase activity as internal standard (on 20 μl of cell lysate).

3. Results

3.1. Development of OHT resistant MVLN cells and selective subtractive hybridization (SSH)

The growth assay for MVLN^{OHT} cells (MVLN cells that had been cultivated for 6 months in the presence of OHT), performed by measuring end point DNA content after about a week of growth in culture medium, and that of untreated parent cells revealed that MVLN^{OHT} cell growth was greatly stimulated when cells were grown in the presence of OHT (Fig. 1). Whereas, parent MCF-7 cell growth is known to be completely arrested in estrogen-free medium and OHT-containing medium, untreated parent MVLN cell growth was slightly stimulated in the presence of OHT (Fig. 1). Resistance had therefore been induced through long-term culture in the presence of OHT. There was no cross-resistance to faslodex, the other antiestrogen tested, which did not stimulate growth of parent or treated cells.

An SSH was then performed to isolate mRNAs that were more expressed in resistant MVLN^{OHT} cells growing in OHT-containing medium than in sensitive MVLN cells cultivated in the same medium. We considered that the use of this clonal MVLN cell line, instead of the more heterogeneous parent MCF-7 cells, could a priori give us greater confidence in the SSH objectivity. The last PCR amplification of the SSH produced cDNAs migrating as a few discrete bands on 1% agarose gel (result not shown). We therefore expected to obtain a small number of overexpressed products in resistant MVLN^{OHT} cells in comparison to sensitive cells. Two hundred clones picked from the subtracted library and ligated in pGEM-T vector appeared to be composed of only 20 different species, which were then analyzed by reverse Northern blotting. The results of a typical reverse Northern blot are presented in Fig. 2A and B. Fifteen mRNAs showed at least a two-fold difference between resistant MVLN^{OHT} cells and sensitive parent cells and corresponded to known sequences (Fig. 2C).

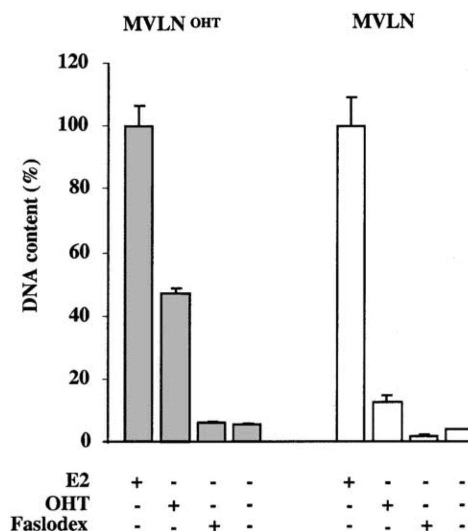


Fig. 1. Cellular growth of normal MVLN cells and MVLN cells that had previously grown for 6 months in the presence of 10^{-7} M OHT. Both cell types were seeded in 24-well plates, as indicated in Section 2.1 and grown in DCC medium alone or containing 10^{-9} M E2, 10^{-7} M OHT or 10^{-7} M faslodex. For both cell types, cells from each culture condition were arrested when about 80% confluency was reached in the presence of E2 and the well DNA content measured. Data are expressed relative to results obtained with cells grown in E2-containing medium and are means ± standard deviation for three experiments.

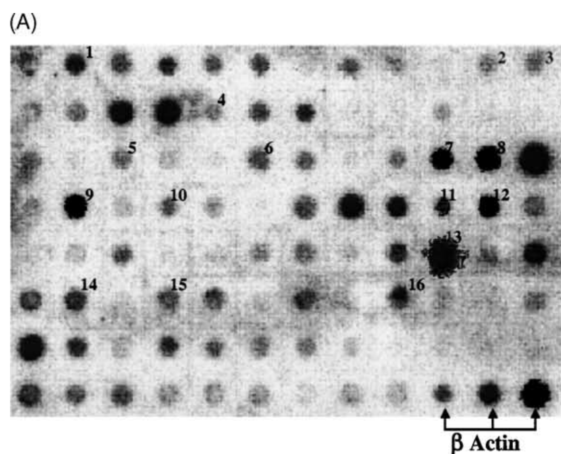
3.2. Selection of a differentially expressed gene, Immediate early gene X-1 gene (IEX-1) (also known as IER3)

Among the 15 overexpressed mRNAs, *IEX*-1 mRNA was selected because of its high stimulation level observed in reverse Northern blot (Fig. 2C) and above all because its expression is known to be linked with cell growth regulation. It was, indeed, long associated with cell growth in fibroblasts, pancreatic carcinoma cells and keratinocytes [27,31–33], but was then connected to the apoptosis process in T47D and Hela cells [19,34]. Depending on culture conditions, a positive or negative action of *IEX*-1 on 293 cell and keratinocyte growth was finally highlighted [17,18]. The *IEX*-1 cDNA fragment obtained in the SSH corresponded to fragment 924–1270 in the IER3 mRNA sequence.

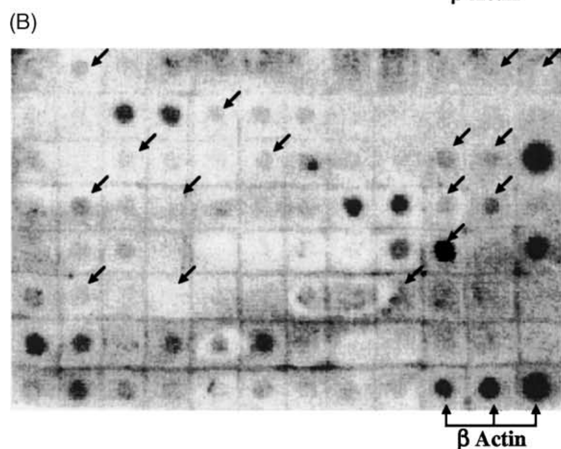
3.3. OHT stimulates IEX-1 expression in OHT resistant MVLN^{OHT} cells more than in parent MVLN cells

To verify *IEX*-1 differential expression, Northern blotting of mRNA from resistant MVLN^{OHT} and parent MVLN cells subjected to short hormonal treatments was performed. Fig. 3 shows that OHT treatment of resistant MVLN^{OHT}

(A)



(B)



(C)

UniGene Symbol	UniGene Number	Fold increase	Approved Gene Name [aliases]	#
PSMB4	Hs.89545	3	*Proteasome (prosome, macropain) subunit, beta type, 4 [HsN3, PROS26]*	7
RGS16	Hs.183601	9	*Regulator of G-protein signalling 16 [A28-RGS14, RGS-r]*	
SLC5A6	Hs.321579	10	*Solute carrier family 5 (sodium-dependent vitamin transporter), member 6 [SMVT]*	3
EEF1B2	Hs.421608	27	*Eukaryotic translation elongation factor 1 beta 2*	2
HNRPAB	Hs.81361	2.0	*Heterogeneous nuclear ribonucleoprotein A/B [ABBP1]*	13
MTCH1	Hs.279939	3.5	*non available (Mitochondrial carrier homolog 1 / CGI-64)*	4, 5
DDR1	Hs.75562	*	*Discoidin domain receptor family, member 1 [RTK6]*	
IER3	Hs.76095	8	*Immediate early response 3 [IEX-1, DIF-2, PRG1]*	10, 14, 15, 16
EDF1	Hs.174050	8.5	*Endothelial differentiation-related factor*	9
RPS24	Hs.180450	6.5	*1*	8
EEF1G	Hs.256184	*	*Ribosomal protein S24*	
			Eukaryotic translation elongation factor 1	
CD9	Hs.1244	2	*gamma*	
KRT8	Hs.242463	9	*CD9 antigen (p24) [BA2]*	
RPL6	Hs.409045	3.5	*Keratin 8*	1, 6, 11,
			Ribosomal protein L6 [TAXREB107]	12
XBP1	Hs.149923	3		
			X-box binding protein 1	

Fig. 2. Reverse Northern blot analysis of SSH. Two identical membranes were spotted with 93 PCR-amplified cDNAs issued from the SSH pGEM-T ligation product and then probed with: (A) ^{32}P-labeled cDNA from MVLN^{OHT} cells or (B) parent MVLN cells. β-Actin PCR product (1, 3, 10 ng) was spotted on membranes to standardize signals. The results were analyzed with a PhosphorImager. Spots selected for their differential expression were numbered in A (and pointed at in B) and stemmed from the following genes: *RPL*6 (Spot # 1, 6, 11, 12); *EEF1B*2 (Spot # 2); *SLC5A*6 (Spot # 3); *MTCHI* (Spot # 4, 5); *PSMB*4 (Spot # 7); *RPS*24 (Spot # 8); *EDF*1 (Spot # 9); *IEX*-1 *or IER*3 (Spot # 10, 14–16) and *HRNPAB* (Spot # 13). (C) List of genes whose cDNAs were present in the SSH final product and whose differential expression was confirmed by two independent reverse Northern blots. Some of them (*) were only detected in resistant cells.

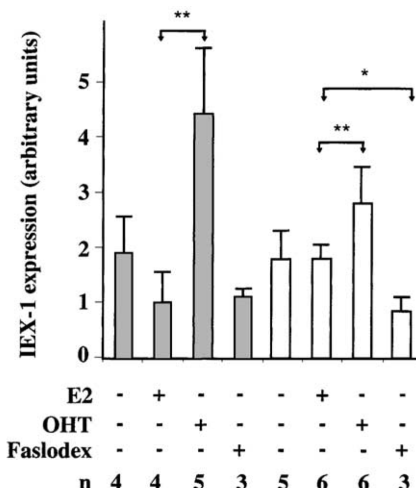


Fig. 3. Northern blot analysis of *IEX*-1. MVLN^{OHT} (shaded bars) and MVLN cells (open bars) had grown in DCC medium alone or containing 10^{-9} M E2, 10^{-7} M OHT or 10^{-7} M faslodex for 4 days. After transferring RNA onto a nylon membrane, the membrane was hybridized with a ^{32}P-labeled *IEX*-1 cDNA probe. ^{32}P-labeling signals were quantified with a PhosphorImager and standardized relative to 18S rRNA detected with ^{32}P-labeled 18S rRNA cDNA probe. Mean standardized values ± standard deviation for *n* (3–6) experiments were expressed relative to that obtained with MVLN^{OHT} cells grown in E2-containing medium. * and ** correspond to $P < 0.05$ and <0.01, respectively, in the Fisher post-test analysis. The Student's *t*-test *P*-value was <0.001 when OHT-induction levels of *IEX*-1 expression were compared in resistant and parent cells.

cells, increased *IEX*-1 expression four-fold over the level observed in the presence of E2. Faslodex, a pure antiestrogen, had no stimulating effect. There was no significant difference between E2 treatment and no treatment. In sensitive MVLN cells, the OHT-induced increase in *IEX*-1 expression was 1.5-fold. The increase was smaller than in resistant cells ($P < 0.001$ in the Student's *t*-test analysis). *IEX*-1 expression levels were identical in the presence and in the absence of E2, as in resistant cells, and it collapsed below that level in the presence of faslodex.

3.4. Reproducible regulation of IEX-1 expression by OHT in clones isolated from resistant MVLN^{OHT} cells and in parent MCF-7 cells

To confirm the regulation of *IEX*-1 expression in various related cells, we first analyzed five clones isolated from resistant MVLN^{OHT} cells (selected in a previous work for their differing progesterone receptor expression level [14]). Growth was stimulated by OHT in all clones studied,

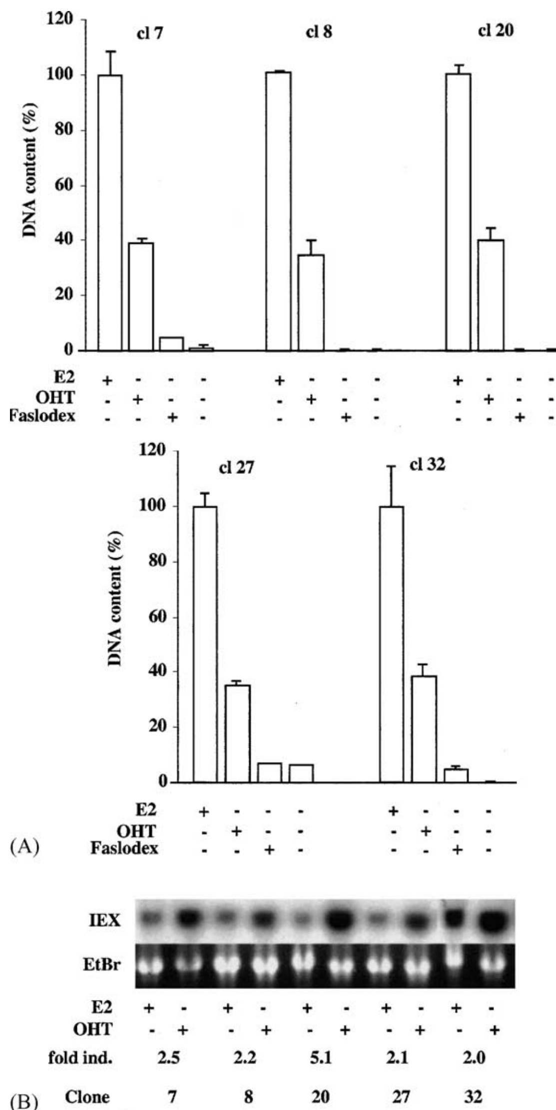


Fig. 4. (A) Cellular growth of five clones (cl 7, cl 8, cl 20, cl 27 and cl 32) isolated from $MVLN^{OHT}$ cells. All cell types were seeded in 24-well plates and grown in DCC medium alone or containing 10^{-9} M E2, 10^{-7} M OHT or 10^{-7} M faslodex. Cells from each culture condition were arrested when about 80% confluency was reached in the presence of E2 and the well DNA contents were measured. Data are expressed relative to that obtained with cells grown in E2-containing medium and are means ± standard deviation for three experiments. (B) *IEX*-1 expression of clones grown for 4 days in DCC medium containing 10^{-9} M E2 or 10^{-7} M OHT before their RNA was extracted and Northern blot performed. *IEX*-1 expression was quantified after standardization relative to EtBr stains of 18S rRNA.

whereas faslodex had no effect (Fig. 4A). When *IEX*-1 expression was analyzed by Northern blot in the five clones (Fig. 4B), we noted that the OHT stimulation levels (versus E2) ranged from 2 to 5. In all clones, stimulation was therefore greater than in sensitive parent MVLN cells.

In parent MCF-7 cells (Fig. 5), *IEX*-1 expression was again stimulated by OHT and the stimulation was more pronounced (five-fold) in 6 month OHT treated $MCF\text{-}7^{OHT}$ cells, first described in a previous paper [35]).

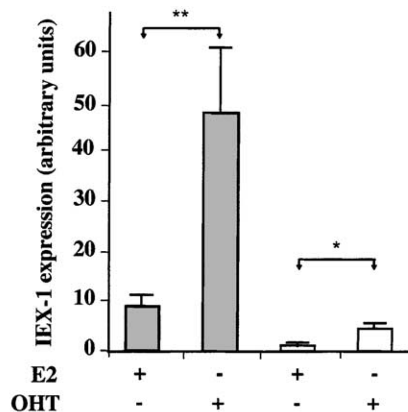


Fig. 5. *IEX*-1 expression of $MCF\text{-}7^{OHT}$ cells (shaded bars) and normal MCF-7 (open bars) when grown for 4 days in DCC medium containing 10^{-9} M E2 or 10^{-7} M OHT before RNA extraction and Northern blot analysis as described in Fig. 3. Mean standardized values ± standard deviation for three experiments were expressed relative to that obtained with parent MCF-7 cells grown in E2-containing medium. * and ** correspond to $P < 0.05$ and <0.01, respectively, in the Student's *t*-test analysis of *IEX*-1 expression.

3.5. Characterization of IEX-1 regulation of expression OHT stimulates IEX-1 expression via the estrogen receptor

In the above results, *IEX*-1 expression was stimulated by OHT and not by faslodex, the other antiestrogen. We, therefore, wondered whether ER was involved in the phenomenon. Fig. 6, in which $MVLN^{OHT}$ cells were used, shows that OHT stimulation of *IEX*-1 expression was prevented by a 10-fold higher concentration of E2, indicating that *IEX*-1 expression was stimulated via an estrogen receptor-dependent pathway.

3.6. Phorbol ester stimulates IEX-1 expression

IEX-1 expression can be mediated through multisignal transduction pathways, including the phorbol ester TPA [36]. We tested whether TPA would stimulate *IEX*-1 expression

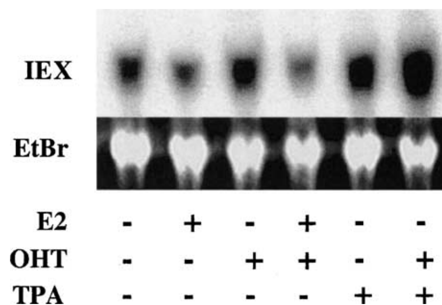


Fig. 6. Effect of E2 and TPA on OHT stimulation of *IEX*-1 expression. $MVLN^{OHT}$ cells were treated for 4 days with 10^{-6} M E2, 10^{-7} M OHT and with both 10^{-6} M E2 and 10^{-7} M OHT. When indicated, 5×10^{-8} M TPA was added 2 h before RNA extraction. A typical Northern blot of *IEX*-1 is represented with the corresponding EtBr stains of 18S rRNA.

in our cell model as it does in various other cell systems and whether the stimulation level was similar to that obtained with OHT. As shown in Fig. 6, a high stimulation level was observed in the presence of TPA. The effect of TPA was greater than that of OHT and the two effects were additive.

3.7. Time dependence of IEX-1 stimulation by OHT

It was important to determine whether *IEX*-1 could be detected after a shorter OHT treatment time than the 4 days used to treat cells prior to SSH. We analyzed the kinetics of MCF-7^{OHT} cells because this cell type displayed the greatest OHT stimulation. Fig. 7A shows that the *IEX*-1 rise was detectable after as little as 6 h OHT stimulation, with a subsequent steady increase until at least 72 h stimulation.

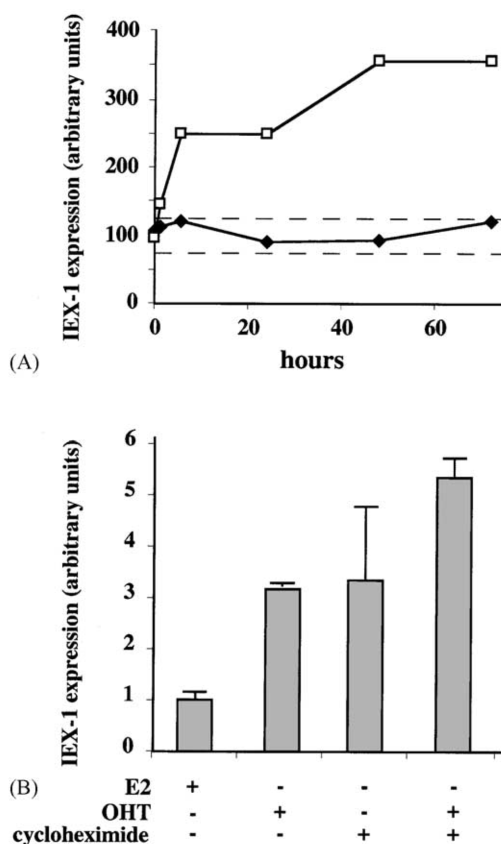


Fig. 7. (A) Time dependence of OHT stimulation of *IEX*-1 expresssion. MCF-7^{OHT} cells were grown for the time indicated in DCC medium containing 10^{-9} M E2 (◆) or 10^{-7} M OHT (□) before RNA extraction and Northern blot analysis. Values standardized relative to 18S rRNA were expressed as a percent of the mean value obtained at zero treatment time (dotted lines frame the mean ± standard deviation for six experiments). (B) *IEX*-1 expression in the presence of cycloheximide. MCF-7^{OHT} cells were treated for 6 h with 10^{-9} M E2, 10^{-7} M OHT or cycloheximide (10 μg/ml) and with both cycloheximide and OHT before RNA extraction and Northern blot analysis. Mean standardized values ± standard deviation for three experiments were expressed relative to that obtained with cells grown in E2-containing medium.

3.8. OHT stimulation of IEX-1 expression does not require newly synthesized proteins

IEX-1 is an immediate-early gene, rapidly triggered by various external stimuli and, in squamous carcinoma cells, its expression was shown to be unaffected by inhibition of protein synthesis [36]. To investigate whether the pattern would be the same for OHT stimulation of its expression, we performed a 6 h treatment of MCF-7^{OHT} cells with cycloheximide in the presence or absence of OHT. Fig. 7B shows that cycloheximide did not prevent OHT stimulated *IEX*-1 expression. Alone, cycloheximide stimulated *IEX*-1 expression, as already reported [32,36] and as it does for the expressions of other genes, supposedly by inhibiting the synthesis of mRNA decay or turnover factors. A slight increase was observed when, besides cycloheximide, cells were treated with OHT. OHT may therefore act directly on *IEX*-1 expression without synthesis of intermediate proteins.

3.9. IEX-1 gene promoter

To identify the promoter part that sustained OHT stimulation of *IEX*-1 expression, we first cloned the −776+21 *IEX*-1 intact promoter in a luciferase expressing reporter system (Fig. 8A), performed transient transfections in MCF-7^{OHT} and MCF-7 cells and checked that responses to various stimuli were coherent with the trends observed in Northern blot experiments. We then designed a number of deletion mutants fused to the luciferase reporter gene (Fig. 8B).

3.10. Conditions of IEX-1 promoter activity measurements

Since the *IEX*-1 promoter is weak, we took advantage of the presence of an SP1/CAAT site in its proximal part. This site, which is known to bind SP1 and NF-Y transcription factors, is often reported to be stimulated by TSA, a histone deacetylase inhibitor. As shown in Fig. 9A, we observed two–three-fold stimulation of wild-type promoter activity by 250 nM TSA. Cellular toxicity appeared at a higher TSA concentration. For all tested hormone conditions, qualitatively comparable results were obtained in the presence or absence of TSA (not shown). A minimal part of the promoter containing the SP1/CAAT site was, therefore, common to all the deletion mutant constructions and the following experiments were thus performed in the presence of TSA. All experiments were also confirmed in the absence of TSA.

3.11. Wild-type pIEX-Luc reporter gene activity reflects the regulation of cellular IEX-1 revealed by Northern blot experiments

Regulation of the wild-type pIEX-Luc reporter gene transiently transfected in MCF-7^{OHT} and MCF-7 cells under various effector conditions (Fig. 9B) suitably reflected the regulation of cellular *IEX*-1 revealed by Northern blot experiments (Figs. 3, 5 and 6). In particular, we observed an

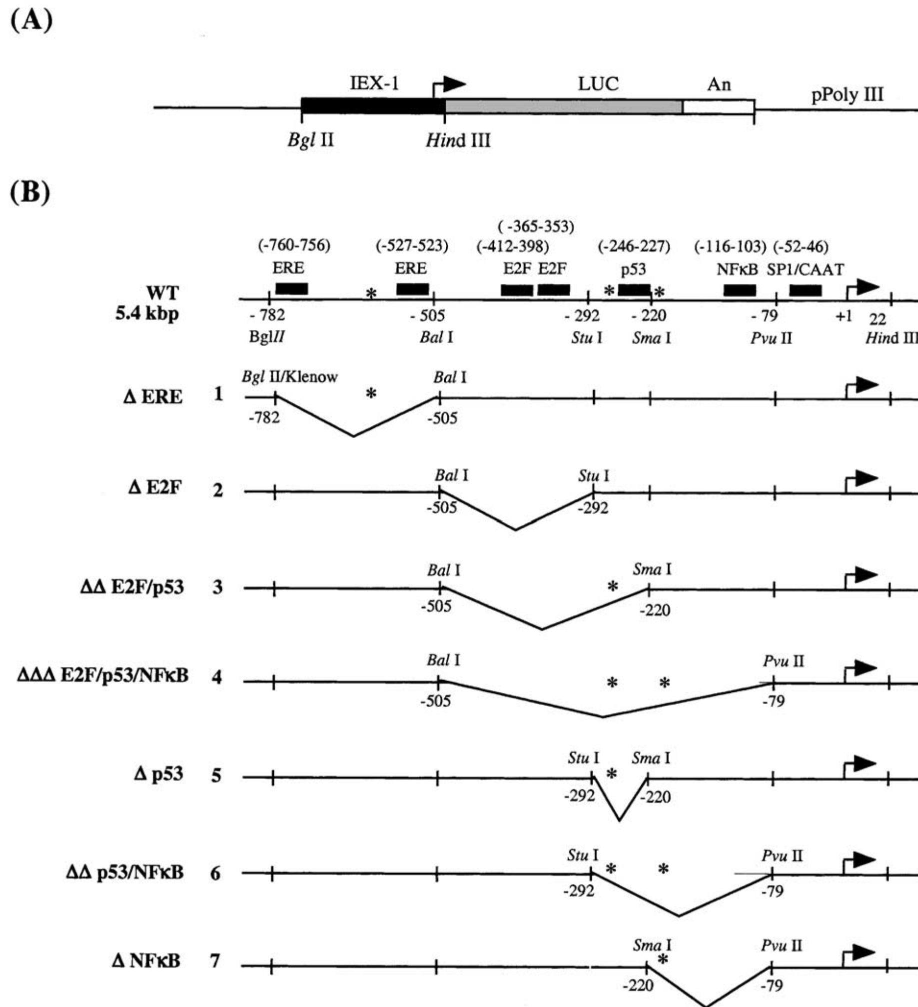


Fig. 8. DNA plasmids to study *IEX*-1 regulation. (A) IEX-Luc plasmid. Intact *IEX*-1 promoter part (−776 to +21) was inserted upstream of the luciferase reporter gene in the pPoly III vector to give pIEX-Luc. (B) Wild-type *IEX*-1 promoter with the various responsive elements described in the literature and deletion mutants obtained with single site restriction enzyme digestions giving blunt ends in pIEX-Luc. In position −782, the blunt end was obtained by successively using *Bgl*II and Klenow enzymes. For all mutants but one, deletions were confirmed (*) by using one of the *Pfl*MI (at −618), *Bam*HI (at −281) or *Nar*I (at −166) restriction enzymes which gave one and two restriction fragments in deletion mutants and wild-type promoter, respectively.

absence of effect of E2, increased expression in the presence of OHT and higher overall expression in MCF-7^{OHT} cells. We, therefore, studied the role of several transcriptional effector binding sites in the *IEX*-1 promoter through an analysis of the activity of the wild-type and truncated promoters described in Fig. 8B.

3.12. OHT-driven IEX-1 regulation is not mediated through the two ERE half sites

The absence of the two half EREs in the *IEX*-1 promoter (Fig. 8B, construction 1) did not provoke a loss of upregulation by OHT (Fig. 10A), suggesting that the OHT-induced increase in *IEX*-1 expression was mediated in another way. This was confirmed by the fact that the construction containing only the two half EREs upstream of the minimal promoter (Fig. 8B, construction 4) did not respond to OHT treatment or to other tested effectors (Fig. 10B).

3.13. p53 represses IEX-1 transcription

The above data suggested that OHT regulation was mediated through more proximal sites located between half EREs and the SP1/CAAT site like, for example, binding sites for p53 or NF-κB which are known to be functional in the *IEX*-1 promoter [29,37]. In our cell model, transfecting a plasmid with a mutant promoter lacking the p53 binding site (Fig. 8B, construction 5) gave rise to a higher general transcription level (Fig. 10C), suggesting a repressing action of p53 or of a p53-mediated effect. The mutant promoter controlled expression was not as clearly upregulated by OHT and TPA as the wild-type controlled expression. A

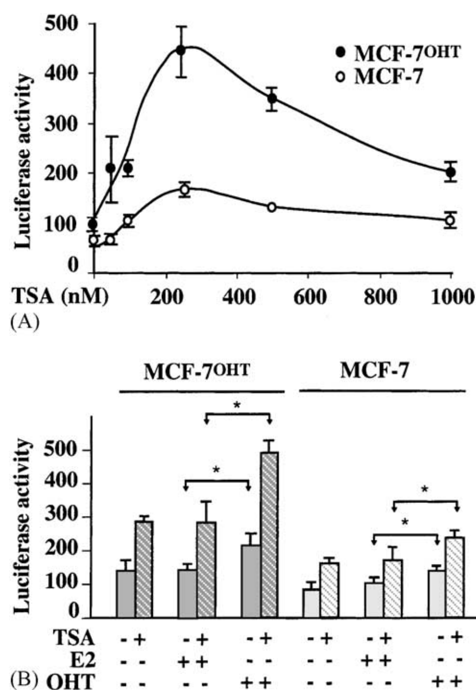


Fig. 9. Expression of luciferase reporter gene under control of the wild-type *IEX*-1 promoter in MCF-7^{OHT} cells (dark symbols) and MCF-7 cells (light symbols). (A) TSA concentration dependence of *IEX*-1 promoter activity. Both cell lines transiently transfected with pIEX-Luc and treated for 48 h with 10^{-7} M OHT and for the last 9 h with TSA at various concentrations. (B) Comparative luciferase expression in both cell lines transiently transfected with pIEX-Luc and treated for 48 h under various hormone conditions (10^{-9} M E2 or 10^{-7} M OHT) and in the presence (hatched bars) or absence (plain bars) of 250 nM TSA. Luciferase activity ± standard deviation for three experiments was expressed as arbitrary units of luciferase reporter activity after correction for β-Gal activity in the same extract as described in Section 2. * corresponds to $P < 0.05$ in the Fisher post-test analysis of luciferase activity.

significant effect was only noted in both cell types when the two effectors, OHT and TPA, were present, and in MCF-7^{OHT} cells when TPA alone was present. These results nevertheless suggested that a site mediating the OHT-induced increase in *IEX*-1 expression might be located elsewhere.

3.14. OHT regulation of IEX-1 is mainly mediated through the NF-κB pathway

This was shown in experiments using the other deleted mutant constructions. First, all plasmids with deleted mutant promoters lacking the NF-κB binding site (Fig 8B, constructions 4, 6 and 7) were not responsive to OHT treatment, as shown with the construction 4 deleted mutant (Fig. 10B). Incidently, it should be noted that reporter gene expression in response to TPA was lost in these mutants lacking the NF-κB site and was, therefore, mediated through this site, in agreement with the known NF-κB factor dependence of TPA activation [38]. Secondly, all plasmids with deleted mutant promoters containing the NF-κB binding site (Fig. 8B, wild-type *IEX*-1 construction and constructions 1, 2, 3 and

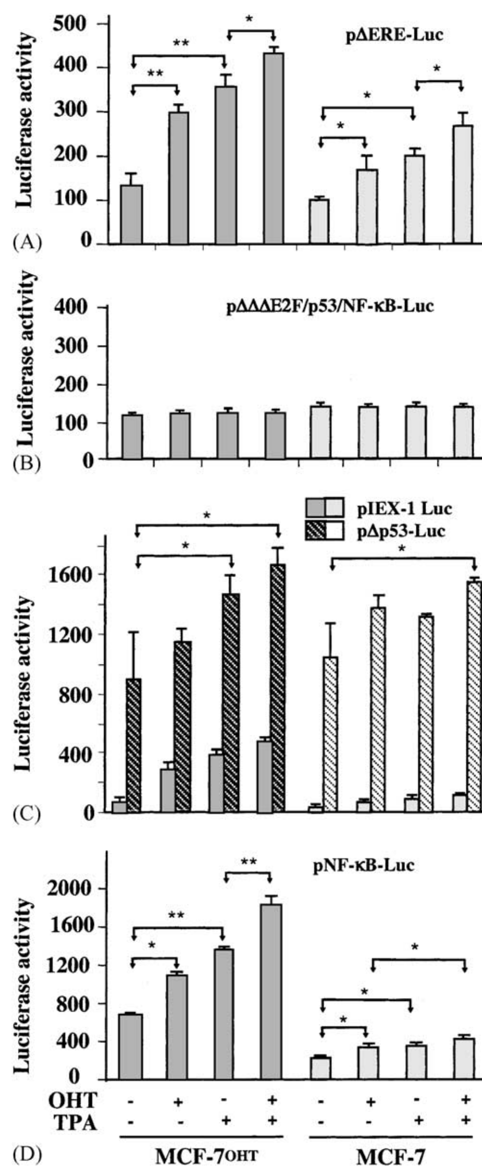


Fig. 10. Expression of the luciferase reporter gene under control of mutant *IEX*-1 promoters (Fig. 8) and of the NF-κB consensus sequence in MCF-7^{OHT} cells (dark symbols) and MCF-7 cells (light symbols). (A–D) Expression of luciferase in both cell lines was measured after treatment of transiently transfected cells with 10^{-7} M OHT for 48 h and/or with 50 nM TPA for 9 h. The reporter gene was under control of: (A) a mutant *IEX*-1 promoter (construction 1) in which the fragment containing putative half-EREs (region: −782 to 505) was deleted, (B) a mutant *IEX*-1 promoter (construction 4) in which the fragment containing half-EREs was located upstream of the minimal promoter part (after deletion of region: −505 to 79), (C) either the wild-type pIEX-Luc or a mutant promoter containing construction 5 in which the fragment including the p53 binding site (region: −292 to 220) was deleted, and (D) a promoter containing four tandem copies of the NF-κB consensus sequence. Luciferase activity ± standard deviation for three experiments was expressed as arbitrary units of luciferase reporter activity after correction with the β-Gal activity in the same extract as described in Section 2. * and ** correspond to $P < 0.05$ and <0.01, respectively, in the Fisher post-test analysis of luciferase activity.

5) were responsive to OHT and TPA treatment, as shown for the wild-type construction (Fig 9C) or construction 1 (Fig. 10A). Finally, the implication of the NF-κB site was further confirmed using the pNF-κB-Luc vector in which luciferase reporter expression is under the specific control of four tandem copies of the NF-κB consensus sequence fused to a TATA-like promoter. As shown in Fig. 10D, this typical NF-κB controlled luciferase expression was qualitatively regulated like that under control of the *IEX*-1 promoter, in MCF-7^{OHT} cells as in parent MCF-7 cells, suggesting that the NF-κB site was involved in OHT regulation of *IEX*-1 expression. Importantly, greater overall NF-κB controlled luciferase expression was observed in MCF-7^{OHT} cells than in parent MCF-7 cells, suggesting that the reproducibly higher transcriptional activity in MCF-7^{OHT} cells (Fig. 9B and C, Fig. 10A) was due to a greater NF-κB activity.

4. Discussion

4.1. Resistant cells

In a quest to determine what differentiates resistant breast cancer cells and cells whose growth is sensitive to antiestrogen treatment, we performed selective subtractive hybridization (SSH) between OHT resistant and sensitive cells. The resistant cells used were MVLN^{OHT} cells that were raised in the lab from MVLN cells, an MCF-7 derived cell line, in which resistance was induced through long-term culture in the presence of OHT [14]. OHT resistant MVLN^{OHT} cells, together with five derivative clones, behaved like several OHT resistant cells described in the literature [39–44]. Their growth was stimulated by OHT and still E2 dependent. There was moreover no cross-resistance between OHT and faslodex, a pure antiestrogen compound. This result is interesting in the light of the fact that no cross-resistance between tamoxifen and faslodex was previously observed in 70% of patients who responded initially to tamoxifen and subsequently failed [1]. Evidence of tamoxifen cell growth stimulation at relapse of advanced breast cancer was obtained in clinical trials, where the frequency of response to tamoxifen withdrawal was 10–30% [16,45].

4.2. SSH

Fifteen genes were found to be overexpressed in resistant MVLN^{OHT} cells grown in the presence of OHT. The properties of some of them are worthy noting: (a) *IEX*-1 and *DDR*1 genes are adjacent on chromosome 6 in position 6p21.3, only separated by 140 kbp, whereas a third gene (*CGI*-64) is closely located in 6p21.1. (b) EEF1B2 and EEF1G are elongation factors responsible for the delivery of aminoacyl tRNAs to ribosomes. An increase in their mRNA levels has been observed in tumors and cultured cells. (c) The transcription factor XBP-1 expression was described to be stimulated in faslodex resistant MCF-7 cells in a SAGE experiment [10]. Emphasis was placed upon one of the 15 differentially expressed genes, i.e. *IEX*-1, an immediate early gene (15) (also called *DIF*-2 [46], *PRG*-1 [47] and homologous to murine *gly*96 [27]), because of its recognized contribution to apoptosis or cell cycle progression, depending on the cell type and culture conditions [18]. Its expression can be mediated through multisignal transduction pathways. It was indeed shown to be increased by growth factors in fibroblasts [27] and by various stimuli such as ionizing radiation, the mitogenic compounds TPA or okadaic acid, the tumor necrosis factor-α in epithelial cells [36], and Vitamin D3 in keratinocytes [33]. It is a target gene for the tumor suppressor p53 [29,48]—its expression was shown to be repressed [27,31–33,36] or induced by p53 [49]—and for NF-κB [29,48] and SP1 [29,48]. Studies focused on the NF-κB dependence of *IEX*-1 expression revealed upregulation of *IEX*-1 during breast tumor cell growth inhibition [34,50] or highlighted a positive or negative action for IEX-1 on growth of various cell types (293 cells, keratinocytes, HeLa cells), depending on the growth conditions [17–19].

4.3. IEX-1 expression

We showed that OHT stimulated *IEX*-1 expression in MVLN^{OHT} and in MCF-7^{OHT} cells. OHT stimulation of *IEX*-1 expression was ER dependent and cycloheximide experiments suggested that it did not require newly synthesized proteins. We then performed a detailed analysis of *IEX*-1 promoter regulation. We showed that a $-776 + 21$ sequence of the *IEX*-1 promoter was sufficient to recover *IEX*-1 expression regulation in our cell model, especially regulation by OHT. Firstly, we showed that no regulation was sustained by OHT through the ERE half sites located upstream of the *IEX*-1 promoter. We showed a marked increase in overall *IEX*-1 expression when the p53 binding site was removed, suggesting a p53 mediated inhibiting effect, as also described in HaCaT keratinocytes [51]. With this Δp53 mutant, there was however less pronounced regulation by OHT or TPA, suggesting a saturated expression rate of *IEX*-1. We could have expected p53 activation of *IEX*-1 expression since it was described that p53 activation of *IEX*-1 expression is inhibited by *c-Myc* [37]. As, indeed, *c-Myc* expression is estrogen-dependent in MCF-7 cells [52,53], inhibition of *c-Myc* expression by OHT could have been the way OHT would activate p53 and therefore stimulate *IEX*-1 expression. We then showed that the NF-κB binding site was the main binding site involved in OHT regulation of *IEX*-1 expression.

4.4. NF-κB regulation of IEX-1 expression

The NF-κB function is known to be repressed by ER bound to estradiol in various systems [8,9]. On the other hand, very dissimilar results have been published on the effect of antiestrogens on NF-κB activity—they can have no effect [8,44,54], the same effect as E2 [55], or an opposing

effect [56]. In our cell model, OHT, compared to DCC medium, had a stimulatory effect on *IEX*-1 expression while E2 had no effect. We could hypothesize that the similar levels of *IEX*-1 expression obtained in the presence and absence of E2 might reflect a residual level of estrogenic activity after serum charcoal-treatment, sufficient to inhibit NF-κB controlled expression to a basal level. It was, moreover, shown that E2 liganded ER inhibited the NF-κB function by decreasing the CBP/HAT activity required for NF-κB transcriptional activity [8]. Very similarly, the involvement of direct HAT inhibition and also HDAC2 recruitment to the p65-CBP/HAT complex was described as a dexamethasone liganded GR mechanism for the repression of NF-κB (p65) activity in A549 cells [57]. Interestingly, repression was obtained for much lower dexamethasone concentrations than that needed for dexamethasone stimulated transcriptional activities. If ER acts as GR, the balance between HAT and HDAC would be altered by residual estrogen activity and this alteration would lead to an inhibited *IEX*-1 expression in DCC medium, through repressed NF-κB activity. OHT displacement of the ER-bound ligand or more likely an OHT-bound ER would then allow *IEX*-1 expression, e.g. by trapping repressing factors. It was, indeed, an OHT specific effect since faslodex was inefficient in stimulating *IEX*-1 expression. Faslodex is, moreover, known to down-regulate ER.

4.5. IEX-1 expression and resistance

We observed more pronounced OHT stimulation of *IEX*-1 expression in 6 month OHT treated MVLN and MCF-7 cells as compared to parent cells and greater NF-κB controlled expression in MCF-7^{OHT}, which could possibly have been due to higher NF-κB expression or activity in these long-term OHT treated cells. Activation of NF-κB, thus suppressing the apoptotic potential of anticancer drugs, is indeed a main pathway involved in chemotherapy induced resistance [58,59]. Besides, constitutive activation of NF-κB was observed in the progression of breast cancer to hormone-independent growth, which is believed to be the ultimate step and most invasive form of breast cancer [60]. Importantly, the NF-κB pathway was also shown to be activated in faslodex resistant cells, i.e. MCF7/LCC9 cells [10]. Note that two other genes identified in the SSH, i.e. *RSG*16 and *XBP*1 (Fig. 2C), contain an NF-κB binding site in their promoter and that their overexpression in our 6 month OHT treated cells might also reflect higher NF-κB activity. XBP-1, a transcription factor binding and activating cAMP responsive elements (CRE) [61], was likewise observed overexpressed in the above-mentionned MCF7/LCC9 cells within which increased CRE activity was moreover detected. In our resistant cell model, as suggested for MCF7/LCC9 cells, survival from antiestrogen exposure might rely on increased activity of signaling pathways like NF-κB and cAMP pathways. In addition, the ERK pathway might synergize with the NF-κB pathway in its anti-apoptotic potential. In a previous work, we indeed showed that prolonged OHT treatment increases the level of phosphorylated ERKs in MCF-7 cells [35] and *IEX*-1 was very recently shown to be one of the numerous ERK substrates and, in turn, to be an ERK activator [62]. Upon phosphorylation, *IEX*-1 acquires the ability to inhibit cell death induced by various stimuli [62]. The greater increase in the antiapoptotic phosphorylated *IEX*-1 could partially explain the faster growth of resistant cells. On the other hand, during short OHT treatment of sensitive cells, *IEX*-1 expression was associated with cell growth arrest as described in other breast cancer cells in which up-regulation of *IEX*-1 is partly responsible for cell growth inhibition [50].

In conclusion, we showed for the first time that *IEX*-1 expression was stimulated by OHT in MCF-7 and MCF-7 derived cells in an NF-κB site dependent manner. This might reflect abolition of E2-dependent downregulation of the NF-κB pathway that would occur when ER is bound to OHT. In addition, *IEX*-1 expression was more stimulated by OHT in 6 month OHT treated cells than in parent cells, possibly through a rise in NF-κB expression or activity. An increase in the expression of *IEX*-1, whose phosphorylation by ERKs leads to cell death inhibition, might be involved in the ability of OHT resistant cells to grow in the presence of OHT. Knowledge on the capacity of OHT to stimulate gene expression and its NF-κB dependence should contribute to a better understanding of tamoxifen pharmacology and allow new drug stategies to be designed that would delay antiestrogen resistance acquisition.

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