Hydroxytamoxifen Induces a Rapid and Irreversible Inactivation of an Estrogenic Response in an MCF-7-derived Cell Line

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ABSTRACT

The MVLN cell line was established in our laboratory from MCF-7 cells by stable transfection with the luciferase gene under the control of an estrogen-responsive element from the Xenopus vitellogenin A2 gene. This cell line allowed us to visualize the induction by hydroxytamoxifen of a heterogeneity in the cell population with regard to the expression of the luciferase gene. Treated cells lost their estradiol-inducible luciferase activity, progressively and irreversibly; the luciferase expression of 80% of the cells was irreversibly inactivated by a 12-day hydroxytamoxifen treatment. We showed that this inactivation process was specific for an estrogenic response and was mediated by the estrogen receptor. Tamoxifen itself gave rise to such an inactivation, whereas other compounds belonging to the triphenylethylenic family but differently substituted on the ethylenic carbon and the ICI 164,384 compound were not as efficient. This irreversible inactivation was accompanied by a sharp decrease in the luciferase mRNA level; however, the estrogen receptor function and the cellular transcriptional machinery were not affected by the treatment. Although this antiestrogen treatment neither affected the estrogen-dependent cell growth nor irreversibly inhibited the expression of the natural pS2 gene, these results highly suggest that long-term antiestrogen therapy may lead to some heterogeneity in tumor cells throughout the course of patient treatment.

INTRODUCTION

Tamoxifen (a nonsteroidal estrogen partial agonist/antagonist) was first evaluated 30 years ago for the palliative treatment of advanced breast cancer (1, 2). Today, long-term tamoxifen therapy remains the endocrine treatment of choice for selected patients with all stages of breast cancer (3). Moreover, long-term treatment with tamoxifen is receiving additional attention because it is currently evaluated for preventive treatment in healthy women at risk for breast cancer (4). However, all of the side effects of such prolonged treatment are not yet known, and this has given rise to controversies.

Although tamoxifen curative treatment is efficient in many patients, one-half are initially resistant to this therapy, and all patients eventually develop acquired tamoxifen resistance leading to tumor progression. It has been implied in the past that this progression to insensitivity arises as a result of a mutation event followed by cell selection (5), but tumors are generally constituted of a heterogeneous population of cells that have a wide range of phenotypes (6, 7). This initial cellular heterogeneity of the tumor may be involved in the development of resistance, leading to tumor progression. But the appearance of cellular heterogeneity could also be a consequence of the antiestrogenic treatment, leading to the further selection of resistant cells. The mechanisms responsible for such a diversity are not yet known, but evidence is now emerging of a phenotypic or epigenetic mechanism involving stable alterations in the program of gene expression across a large proportion of the cell population, which may not simply represent selection of the most aggressive phenotype (8). Thus, Daly and Darbre (9, 10) have shown that long-term growth of the ZR-75-1 human breast cancer cell line in the absence of estrogens results, for many clones, in a loss of estrogen-stimulated growth by a mechanism involving up-regulation of the basal growth rate in the absence of steroid. The high frequency of appearance of such clones indicates phenotypic changes across a wide proportion of the cell population rather than the appearance of spontaneous mutations.

We investigated whether a prolonged antiestrogenic treatment of cancer cells with hydroxytamoxifen (a metabolite of tamoxifen with high affinity for estrogen receptor) could lead to a cellular heterogeneity of an estrogenic response. We used a cell line derived from MCF-7 cells that expresses the luciferase gene under the control of a single ERE (11). The luciferase gene, stably integrated into MCF-7 cells, allows the rapid and direct visualization of the estrogenic response without cell destruction and, hence, the precise analysis of the cellular heterogeneity toward this response. Although the literature has often described the effect of a prolonged agonist deprivation, our work was focused on the effects of prolonged antiestrogenic treatment because: (a) it is closer to the therapeutic situation; and (b) these effects need thorough investigation, since they are currently evaluated for preventive therapy. Besides, antiestrogens were shown to have their own effects, in the absence of estradiol, on such responses as growth factor expression (12), and the genotoxicity of tamoxifen may be implicated in the appearance of mutations leading to irreversible phenotypic cell changes (13, 14).

In this study, we showed that when MVLN cells were treated with hydroxytamoxifen, they progressively and irreversibly lost their estradiol-inducible luciferase activity, e.g., a 12-day hydroxytamoxifen treatment irreversibly inactivated the luciferase expression of 80% of the cells. The use of this reporter gene allowed us to visualize and isolate clones of selected luciferase activities. The growth rate and the luciferase mRNA expression of individual inactivated clones were analyzed. To our knowledge, such a rapid effect of hydroxytamoxifen, affecting a large cellular population, has not yet been reported. The understanding of such an inactivating effect may have implications for future tamoxifen therapy.

MATERIALS AND METHODS

Materials

Materials for cell culture and the Moloney murine leukemia virus reverse transcriptase came from GIBCO-BRL (Cergy Pontoise, France). Luciferin, estradiol, and 4',6-diamidino-2-phenylindole were purchased from Sigma Chemical Co. (St. Louis, MO). Tamoxifen Z, 4-hydroxy-tamoxifen Z, and ICI 164,384 were a gift from Dr. A. E. Wakeling (Imperial Chemical Industries, Macclesfield, England). Toremifene and nafoxidine were from Farmos (Turku, Finland) and Upjohn Co. (Kalamazoo, MI), respectively. LN1643 and LN2839 were from Laroch Navarron (Puteaux, France). Clomiphene was from Merrel.

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2 The abbreviations used are: ERE, estrogen responsive element; GAPDH, glyceraldehyde phosphate dehydrogenase; Vit, vitellogenin; tk, thymidine kinase; DMEM, Dulbecco’s modified Eagle’s medium; FCS medium, DMEM with phenol red supplemented with 3% fetal calf serum; DCC medium, DMEM without phenol red supplemented with 3% of a steroid-free, dextran-coated charcoal-treated FCS; RT-PCR, reverse transcription-polymerase chain reaction; cDNA, complementary DNA; TRE, 12-O-tetradecanoylphorbol 13-acetate responsive element.
E2-REGULATED GENE INACTIVATION BY HYDROXYTAMOXIFEN

Douglas Research Laboratories (Cincinnati, OH). RNazol B reagent was from Bioprobe (Montreuil, France). The Taq DNA polymerase and the RNase inhibitor RNAsin were from Promega Corp. (Madison, WI). A single photon counting camera (ARGUS-100) from Hamamatsu Photonics (Hamamatsu, Japan) was used to detect luciferase activity in intact cells, while detection of luciferase activity on cell-free extracts was done using an LKB Wallac 1251 luminometer (Sundyberg, Sweden). Oligonucleotide primers, from Eurogentec (Seraing, Belgium), had the following sequences: (a) oligonucleotides that hybridize with the coding sequence of the luciferase gene, 5'-CTAGCTA- CACGACCTATTTGATTA-3' (upper) and 5'-ATATCTTGTGATTAAT- TAAAGA CT-3' (lower). The fragment generated by these oligonucleotides was 312 base pairs long; and (b) oligonucleotides that hybridize with the coding sequence of the GAPDH gene, 5'-TCCGACGCGGACCATG-3' (upper) and 5'-GAGAAGTATAACCATGATTTG-3' (lower). The fragment generated by these oligonucleotides was 170 base pairs long.

Cell Lines, Cell Culture Conditions, and Clone Isolation

The obtention of stable transfected cells (called MVLN cells) has been described elsewhere (11). These cells were established by transfecting MCF-7 cells with the pVit-tk-Luc plasmid. In this reporter gene, the fragment −331/-87 of the 5' flanking region of Xenopus vitellogenin A2 gene, which contains a palindromic ERE, is inserted in front of the Herpes simplex virus promoter for tk as previously reported for pVit-tk-CAT (15). This regulatory part controls the firefly luciferase structural gene. Cells were cultured either in FCS medium or DCC medium. Medium was replaced every other day.

In order to obtain separate clones in tissue culture flasks, cells were dispersed at a 1 cell/cm² density. They were grown for about 1 month in FCS medium until clones were large enough to be visible through the flask bottom without magnification. When isolated clones were needed, they were scraped, and cells were grown after dispersion in culture flasks.

Cell Treatments

Before MVLN cells were treated with antiestrogens, they were grown in DCC medium for 2 days. Antiestrogens in 0.1% ethanol (final concentration) were then added to the medium. Medium was replaced every other day, and cells were grown for various periods of time. At the end of antiestrogen treatments, they were rinsed twice with DCC medium alone, trypsinized, and seeded either in DCC medium supplemented with estradiol (at the indicated concentration) or in FCS medium. Culture medium was then replaced every other day. Luciferase activity was assayed in cellular homogenates after various treatment or stimulation times.

Luciferase Assay in Cellular Homogenates

Cells were rinsed twice in luminescence buffer (15 mM potassium phosphate, 8 mM MgCl₂, pH 7.4), suspended in 1 ml of luminescence buffer containing 2 mM ATP, and kept frozen until the luciferase assay was performed as described elsewhere (11) in the presence of 0.2 mM luciferin, 0.2 mM CoA, and 0.5% Triton X-100. The protein assay was performed on 0.1 ml of cellular homogenate according to Lowry's method (16). The experimental values were expressed as arbitrary luminescence units per mg of protein.

Luciferase Assay in Intact Cells and Selection of Cell Clones on the Basis of their Luminous or Nonluminous Phenotype

A single photon counting camera enabled us: (a) to obtain images of cells grown as separate clones (containing about 200 cells); (b) to obtain images of luminiscence clones (after the culture medium was replaced by a fresh one containing 0.3 mM sterile luciferin); and (c) to locate these luminiscence clones by superimposition of these images. When their luminiscence was over 2-fold the luminiscence background of the camera, their phenotype was termed “luminous”; if not, it was termed “nonluminous.” Luminous (L) and nonluminous (NL) clones obtained from cells treated with 200 nM hydroxytamoxifen for 12 days were individually harvested for further analysis.

Transient Transfection Experiments

Transient transfections of half-confluent MCF-7 or MVLN cells dispatched in 6-well tissue-culture plates were performed with various amounts of the pVit-tk-Luc plasmid (0.2 to 1 µg/well), according to the calcium phosphate procedure described by Sambrook et al. (17). When MCF-7 cells were transfected, 1 µg of pCH 110 plasmid (expressing β-galactosidase) was added as internal control.

Cell Growth Experiments

Doubling Time. Cells grown in FCS medium were seeded in 24-well tissue-culture plates (15,000 cells/well). One day later, the medium was replaced with fresh medium containing 1 nM estradiol. Cells were grown in triplicate wells for 1 to 7 days. The well DNA contents were determined according to a method using the fluorescence enhancement of 4',6-diamidino-2-phenylindole complexed with DNA (18). Doubling time was determined by semi logarithmic analysis of DNA content.

Proliferation Sensitivity to Estrogen and Antiestrogens. Cells were grown in DMEM without phenol red and supplemented with 1% steroid-free serum for 7 days before they were seeded in 24-well tissue-culture plates (about 100,000 cells/well). One day later, the medium was replaced with fresh medium containing 1 nM estradiol or 100 nM hydroxytamoxifen or no (anti)-hormone. For each type of cells, the growth experiments were stopped when about 80% of confluence was reached in the estradiol-treated wells (from 4 to 8 days). The well DNA contents were determined as described above.

Northern Blot Analysis of pS2

MCF-7 cells were seeded in 100-mm dishes in DCC medium. One day later, medium was replaced with DCC medium with or without 200 nM hydroxytamoxifen. Cells were then grown for 12 or 20 days in their respective medium. At that time, medium was replaced with medium containing 1 nM estradiol, and cells were grown for various times. At the end of each incubation, total RNA was isolated according to the method of Chomczynski and Sacchi (19) using the RNazol B reagent. Ten µg of total RNA were separated electrophoretically on a 1% agarose denaturing gel and transferred to a nylon membrane. The membrane was hybridized overnight with 32P-labeled pS2 and 36B4 probes (20) at 42°C in 50% formamide. After stringency washes, filters were exposed to Amersham Hyper films MP. Autoradiograms were scanned densitometrically to determine the relative amount of pS2 mRNA to that of 36B4, whose mRNA synthesis is hormone independent.

RT-PCR Analysis

RT. The method we used was derived from that described by Dukas et al. (21). Total RNA was extracted from NL2, NL6, NL10, L12, L18, and L21 clones as described above and was retrotranscribed into cDNAs. Reverse transcriptase reactions were carried out using 1 µg of total RNA in 25 µl of 20 mM Tris-HCl buffer (pH 8.4) containing 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂, 3 µM oligo (dT)₉, 500 µM deoxynucleotide triphosphates, 20 units RNasin, and 200 units of Moloney murine leukemia virus reverse transcriptase. The cDNA synthesis was performed for 1 h at 37°C. The reaction mixture was then heated at 98°C for 5 min to dissociate cDNA/RNA hybrids and to inactivate the enzyme. Control reaction without the enzyme was performed to ensure that the mix was not contaminated with genomic DNA.

PCR. The PCR was performed using two pairs of oligonucleotide primers, one specific for the luciferase coding sequence and one specific for the GAPDH gene used as an internal standard (GAPDH level is not influenced by steroid treatment). For each pair of primers, the PCR was performed in separate tubes.

RESULTS

Irreversible Inhibition of the Luciferase Gene Expression by Long-lasting Antiestrogenic Treatments. As shown in Fig. 1, after 1 day of hydroxytamoxifen treatment, the basal luciferase activity of the MVLN cells was about 20,000 arbitrary units/mg of protein. Upon a subsequent 2-day estradiol stimulation, the luciferase activity was raised to 200,000 units, which is close to the maximal inducible luciferase activity of these cells. However, when the cells were treated with the antiestrogen for a longer period of time, a drastic decrease in
The fact that clones expressing a nonluminous phenotype had not been reversed during their 1-month culture in FCS medium (medium sufficient to produce more than 50% of the maximal luciferase activity in MVLN cells) confirmed the irreversible character of the phenomenon. The intrinsic luciferase activity of luminous and nonluminous clones was measured in cell homogenates and compared to MVLN cell luciferase activity in the same conditions. Table 1 shows that the activity of clones selected for their luminous phenotype was as great as that of MVLN cells. The average luminescence value of clones belonging to the nonluminous phenotype was 50- to 70-fold lower than that of the luminous phenotype. With such a luminescence value, these clones were not detectable in intact cells. When they had been grown for several months in FCS medium, their maximal value of luminescence did not rise. In one instance, a luminous clone which had an intermediate value of luminescence was found to be a mixture of nonluminous and luminous cells. When luminous clones were treated again with hydroxytamoxifen, they underwent the same inactivation as the MVLN cells (data not shown).

Different Efficiencies of Various Antiestrogens. Fig. 2 shows that, with the pure steroidal antagonist ICI 164,384, an irreversible inhibition of inducible luciferase activity was obtained, but the inhibition level was lower. The inhibition induced by antihormones was further analyzed by treating cells with hydroxytamoxifen, ICI 164,384, and tamoxifen at various concentrations for either 12 or 20 days (Fig. 4). Cells were then stimulated by 1 μM estradiol for 2 days. The inhibition of luciferase stimulation with the three antihormones was greater after 20 days of pretreatment. For both treatment times, the inhibition reached a plateau at 10 nM hydroxytamoxifen or ICI 164,384. Nevertheless, the inhibition level was much greater with hydroxytamoxifen than with ICI 164,384, although their affinities for the estradiol-induced luciferase activity were observed, following a quasi first-order process (half-inactivation time, 8 days).

We then checked whether the inhibition of luciferase activity observed after the prolonged antiestrogenic treatment was reversible. Reversion of inhibition was attempted by prolonged exposure of 12-day hydroxytamoxifen-treated cells to medium containing 1 nM estradiol (Fig. 2). Luciferase activity was measured in cell homogenates at various times of the reversion treatment. The activity obtained with hydroxytamoxifen-treated cells did not reach the activity of control cells, even after a long period of stimulation. This suggested that the extinction of luciferase expression was irreversible over this period of time.

Irreversible Inhibition Is Due to a “Full” Inactivation of Individual Cells. We next investigated whether the partial level of reversion of luciferase activity was due to a decreased activity of all the cells or whether only a portion of the cells were switched off by the treatment. The irreversible phenotype of inhibited cells (described in Fig. 2) allowed us to treat MVLN cells with hydroxytamoxifen for various times before to disperse them in order to obtain separate clones after 1 month of culture in FCS medium. At that time, the recording of luminescence activity in intact cells showed that some clones were luminous, whereas others had no detectable luciferase activity and were, therefore, nonluminous (note that their size was similar to that of luminous clones). The number of luminous clones decreased as a function of hydroxytamoxifen treatment time (Fig. 3). We noticed a slight decrease in the percentage of luminous clones when control cells had grown in DCC medium alone and no decrease when they had grown for up to 4 months in FCS medium.

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the estradiol receptor are comparable (22). In the case of tamoxifen treatment (whose affinity for the estradiol receptor is 200 times less than that of hydroxytamoxifen), the maximal inhibition was reached at 1 μM, and its level was close to that observed with hydroxytamoxifen treatment. We may, therefore, consider that the efficiencies of hydroxytamoxifen and tamoxifen are similar, taking into account their different affinities.

Inhibition of the luciferase stimulation was also tested by cell treatment with other compounds belonging to the triphenylethylene family, but differently substituted on the ethylenic carbon, at a concentration of 0.1 μM for hydroxylated compounds and 1 μM for nonhydroxylated compounds. After the treatment, the luciferase activity was stimulated with 1 nM estradiol for 2 days (Fig. 5). None of the compounds tested was able to induce an irreversible inhibition of the luciferase expression at the level obtained with hydroxytamoxifen.

Irreversible Inhibition Is Prevented by Estradiol. When MVLN cells were treated for 12 days with 200 nM hydroxytamoxifen in the presence of 100 nM estradiol, only a slight decrease in luciferase activity was observed (Table 2). These results, as well as those obtained previously in Fig. 4, suggested that the process of irreversible inactivation observed with the antiestrogen alone was mediated by the estrogen receptor.

Efficient Expression of a Transiently Transfected Vit-tk-Luc Plasmid. The estrogen receptor functionality of the antiestrogen-treated cells was investigated. MCF-7 cells (from which MVLN cells derive), treated or not with 200 nM hydroxytamoxifen for 12 days, were transiently transfected with pVit-tk-Luc (identical to the endogenous plasmid integrated in MVLN cells). Fig. 6a shows that the level of normalized luciferase activity and the induction factor were identical in hydroxytamoxifen-treated and untreated control cells. In the case of MVLN cell transfection experiments (Fig. 6b), a long-lasting treatment by hydroxytamoxifen was necessary in order to inhibit the high endogenous luciferase activity (control). In MVLN cells previously treated either for 30 or 50 days with hydroxytamoxifen and, therefore, nonluminous, the transient transfection with the Vit-tk-Luc plasmid produced a luciferase expression to a level greatly enhanced compared to the luminescence of nontransfected cells, and the induction factor by estradiol (compared to hydroxytamoxifen) was similar to that of transfected MCF-7 cells (although low in these experiments, the induction factor was similar in treated and untreated cells). In experiments not shown here, we also observed that transfections of treated or untreated cells with a plasmid allowing the expression of the estrogen receptor had no effect on the basal or estradiol-induced luciferase activity. In conclusion, MVLN cells treated for 30 days and more with hydroxytamoxifen efficiently transcribed a newly transiently transfected p-Vit-tk-Luc plasmid.

Very Low Level of the Luciferase mRNA in Nonluminous Clones. To ensure that the absence of luciferase activity in nonluminous clones was not due to an alteration of the luciferase enzymatic activity, the luciferase mRNA level was compared in luminous and nonluminous clones by RT-PCR assay. The mRNA level was unambiguously much higher in luminous clones than in nonluminous clones for the same amount of GAPDH expression (Fig. 7a). In Fig. 7b, we show a quantification of the PCR product obtained by amplification of increasing amounts of cDNAs from clones L21 and NL6.

Table 2  Irreversible inactivation is prevented by estradiol

<table>
<thead>
<tr>
<th>Day</th>
<th>Luciferase activity (%)</th>
</tr>
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<tbody>
<tr>
<td>Day -4</td>
<td>Day 0</td>
</tr>
<tr>
<td>DCC</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>OH-Tam</td>
<td>17 ± 0.1</td>
</tr>
<tr>
<td>OH-Tam + E2</td>
<td>88 ± 4</td>
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Fig. 4. Influence of the antiestrogen concentration on the inducible luciferase activity of the MVLN cells. MVLN cells grown in DCC medium were treated with hydroxytamoxifen, ICI 164,384, or tamoxifen at concentrations ranging from 1 nM to 1 μM. At the end of treatment of 12 days (A) or of 20 days (B), cells were rinsed with DCC medium alone and transferred into a new flask. They were then stimulated by growing for 2 days in FCS medium supplemented with 1 μM estradiol. At the end of stimulation treatments, luciferase activity was assayed and expressed as a percentage of luciferase activity of MVLN cells treated as above, except that antihormone was omitted. Results are expressed as the mean of three determinations; bars, SD.
The increases in the resulting PCR products for the GAPDH cDNA and for the luciferase cDNA ensure that the amplification process was still in an exponential phase (up to 2 μl of reverse transcriptase mixture).

**Growth Properties of Isolated Clones.** Luminous and nonluminous clones, isolated by the procedure described above, were tested for their growing rates. Table 1 shows that the doubling time of these clones tested in the presence of 1 nm estradiol was not significantly different from that of MVLN cells. Moreover, Fig. 8 shows that the growth rates of all the clones tested were raised by estradiol and inhibited by hydroxytamoxifen to the same extent as in MVLN cells.

**Inhibition of pS2 Expression by Hydroxytamoxifen Treatment.**

- The mRNA expression of the natural estrogen-inducible pS2 gene was analyzed by Northern blot experiments after a 12- or 20-day treatment by 200 nm hydroxytamoxifen. Fig. 9 shows that the inhibition occurring after hydroxytamoxifen treatment was only transient and that the mRNA expression was fully recovered after 7 days of stimulation with 1 nm estradiol.

**DISCUSSION**

The problem of hormone resistance presents a major impediment to the long-term effectiveness of tamoxifen treatment of breast cancers (3). The development of such a resistance may imply either the initial heterogeneity of the tumor or heterogeneity resulting from the antiestrogenic treatment by itself. We began this study by investigating whether a prolonged treatment of cancer cells with hydroxytamoxifen could lead to a cellular heterogeneity of an estrogenic response.

MVLN cells are MCF-7 cells stably transfected with the firefly luciferase gene under the control of an ERE and derived from one cell. It was previously shown that the response of the chimeric gene carried by these cells paralleled natural estrogenic responses such as cell growth and progesterone receptor induction in MCF-7 cells (23). The methodology we used (detection of the reporter bioluminescent gene without cell destruction) allowed us to analyze a great number of clones (>200) and made possible the observation that the luciferase expression of more than 80% of the MVLN cells was irreversibly turned off by a 12-day treatment with hydroxytamoxifen. It should be stressed that the irreversible inactivation of the cells was not due to the persistence of hydroxytamoxifen because clones, isolated from hydroxytamoxifen-treated cells, derived from one treated cell and had been, indeed, grown for 1 month in the absence of hydroxytamoxifen until their cell number had reached about 200. Moreover, the nonluminous clones have kept their phenotype for at least 3 months.

The inactivation process is thought to be mediated by the estrogen receptor because it was prevented by estradiol. Moreover, a 12-day...
hydroxytamoxifen treatment of a stably transfected cell line derived from MCF-7 cells and containing the (TRE)$_3$-tk-Luc gene did not inactivate the response to 12-O-tetradecanoylphorbol 13-acetate. This last result showed that the inactivation process was specific of the estrogen-regulated response carried by the Vit-tk-Luc plasmid and was not mediated only by the "tk" part of the promoter.

The magnitude of inactivation was dependent on the structure of the antiestrogen used. Considering that the tamoxifen affinity for the estrogen receptor is 200 times weaker than that of hydroxytamoxifen, we can state that this compound was as efficient as its hydroxylated derivative (Fig. 4). Striking differences exist among the triphenylethylenic compounds. Thus, compounds substituted with a heteroatom on the double-bond, or close to it, are much less efficient (Fig. 5), as is the case even with the hydroxylated LN 2839, whose affinity for the estrogen receptor is high. The steroidal ICI 164,384 compound was less potent than hydroxytamoxifen, although their affinities for the estrogen receptor are close; the level of inducible luciferase activity after ICI 164,384 treatment was 4 times higher than after hydroxytamoxifen treatment (Fig. 4). However, the phenotypic stability of nonluminous clones obtained by an ICI 164,384 treatment suggested that ICI 164,384 and hydroxytamoxifen-induced inactivation processes were of the same nature (data not shown). It is not yet firmly established whether ICI 164,384 prevents the estradiol receptor from binding to DNA by impairing the receptor dimerization or by another mechanism (24, 25). Distinct mechanisms of action could account for the observed differences in efficiency. The understanding of the inactivation process would help to elucidate that point.

Various hypotheses account for the inactivation process. The presence of low but detectable luciferase activity (Table 1) and mRNA level (data not shown) in nonluminous clones, as well as the sequencing of the luciferase gene after genomic PCR amplification, rule out the possibility of the loss of the luciferase gene during the inactivation process. Several studies show evidence favoring a gene modification hypothesis as opposed to those implicating the estrogen receptor. The measurement of the estradiol receptor by enzyme-linked immunosorbent assay showed that hydroxytamoxifen treatment raised the amount of receptor from 200 to 300–700 fmol/mg protein (data not shown). A detailed study by Gyling and Leclercq (26) and Leclercq et al. (27) already showed that hydroxytamoxifen treatment of MCF-7 cells led to the accumulation of the estradiol receptor, although part of it did not bind estradiol. As hydroxytamoxifen treatment seems to alter the receptor processing in a complex fashion, we rather chose to investigate its functionality: (a) the expression of a transiently transfected Vit-tk-Luc plasmid into the hydroxytamoxifen-treated MCF-7 parent cell line was as efficient as when transfected into untreated cells. Moreover, it was also efficiently expressed when transfected into MVLN cells made barely luminous by hydroxytamoxifen treatment; (b) cotransfection of a plasmid expressing the estrogen receptor together with the Vit-tk-Luc plasmid did not lead to an increased expression yield (data not shown); and (c) growth rates of nonluminous clones were still raised by estradiol and lowered by hydroxytamoxifen, and $p$S2 gene expression was only transiently inhibited in hydroxytamoxifen-treated MCF-7 cells and fully recovered after 7 days of estradiol stimulation.

The low level of luciferase mRNA, on the other hand, suggested that hydroxytamoxifen treatment lowered the level of transcription of the gene. Moreover, when the cell line containing the (TRE)$_3$-tk-Luc gene was treated with hydroxytamoxifen, the luciferase activity was not inhibited, indicating that the mRNA stability or posttranslational processing were not affected by such a treatment (mRNAs transcribed from Vit-tk-Luc or (TRE)$_3$-tk-Luc are identical).

Modification of the luciferase gene itself could involve epigenetic mechanisms, such as methylation or chromatin remodeling, and mutations induced by the antiestrogenic treatment. Concerning epigenetic mechanisms, it has been shown that a long-term deprivation of steroids as well as a short estradiol treatment could affect the methylation status of hormone-regulated promoters. After long-term steroid deprivation of S115 cells, the degree of methylation of mouse mammary tumor virus-long terminal repeat was correlated with the loss of expression of the mouse mammary tumor virus genes (28, 29). The $de$ novo methylation of CpGs was considered to be responsible for the irreversible step toward androgen insensitivity. Moreover, short-term estradiol treatment of immature chickens triggered the demethylation of mCpGs in the coding and noncoding region of the vitellogenin gene (30). This process paralleled the gene activation and the interaction of several proteins (NHP-1 and MDBP-2) with DNA (31). It was postulated that the demethylation process of CpGs of the MDBP-2 binding site might be a consequence of a change in chromatin structure. The question now arises as to whether the hydroxytamoxifen treatment altered the chromatin structure and the methylation state of the luciferase gene under the control of the ERE from the *Xenopus* vitellogenin A2.

The fast and irreversible inactivation process induced by hydroxytamoxifen treatment led us to think that it might arise from a mutation. It is, indeed, known that tamoxifen is a potent genotoxic in the liver and the uterus and is able to form adducts with DNA (13, 14). On the other hand, it has been established that DNA repair is more efficient in an actively transcribed gene (32), and it is, therefore, possible that a gene repressed by a genotoxic antiestrogen might be efficiently mutated. Nevertheless, we need to explain why no irre-

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versible loss of the estrogen regulation of the pS2 gene was induced by hydroxytamoxifen. Our hypothesis is that multiregulated genes may be protected from rapid inactivation by the transcription factor occupation of their promoters.

In conclusion, the luciferase gene integrated in the MVLN cell line is a sensitive probe for testing effects induced by long-term treatments with antiestrogens. We showed indeed that a prolonged antiestrogen treatment could lead to a phenotypic alteration across a large proportion of the cells. We further observed that the inactivation process described takes place while cell growth is still hormone regulated. A longer antiestrogen treatment might affect multiregulated genes, in particular, those involved in cell growth. In addition, the irreversible inactivation of the Vit-tk-luc gene reveals striking differences among the antiestrogens tested that should be further investigated. Chimeric genes under the control of more complex promoters will be tested for their potentiality to irreversible inactivation.

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Hydroxytamoxifen Induces a Rapid and Irreversible Inactivation of an Estrogenic Response in an MCF-7-derived Cell Line

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