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Comparison of Estrogen Receptor DNA Binding in Untreated and Acquired Antiestrogen-resistant Human Breast Tumors[1]

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ABSTRACT

Preliminary studies have suggested that measuring the ability of immunoreactive 67-kDa estrogen receptor (ER) to bind DNA and form *in vitro* complexes with its cognate estrogen response element (ERE) might serve to identify breast tumors most likely to respond to antiestrogens like tamoxifen. Data from two different surveys of untreated primary breast tumors confirmed that only 67% (74 of 111) of ER-positive tumors express a receptor capable of forming ER-ERE complexes by gel-shift assay, with tumors of lower ER content having significantly reduced ER DNA-binding frequency (56%) relative to those of higher ER content (82%; $P = 0.007$). In contrast to these untreated tumors, a panel of 41 receptor-positive breast tumors excised after acquiring clinical resistance to tamoxifen during either primary ($n = 26$) or adjuvant therapy ($n = 15$) showed a significantly greater ER DNA-binding frequency, with nearly 90% capable of forming ER-ERE complexes ($P < 0.02$). To assess experimentally whether ER DNA-binding function is altered during the development of antiestrogen resistance, nude mouse MCF-7 tumor xenografts were analyzed before and after the acquisition of *in vivo* resistance to either tamoxifen or a pure steroidal antiestrogen, ICI 182,780. Tamoxifen-resistant MCF-7 tumors retained full expression of 67-kDa DNA-binding ER, and despite a markedly reduced ER content in the ICI 182,780-treated tumors, the expressed ER in these antiestrogen-resistant tumors exhibited full ability to form ER-ERE complexes. These findings indicate that breast tumors with acquired antiestrogen resistance continue to express ER of normal size and DNA-binding ability and suggest that the failure of antiestrogens to arrest tumor growth during emergence of clinical resistance results from an altered gene-regulatory mechanism(s) other than ER-ERE complex formation.

INTRODUCTION

Human breast carcinomas that express ER[4] are more likely to respond to endocrine therapy with tamoxifen (1). However, 30–70% of ER-positive primary tumors (according to their coexpression of the estrogen-inducible PgR) exhibit *de novo* resistance to endocrine therapy (2). This could be explained if a significant proportion of endocrine-resistant ER-positive primary tumors produced a ligand-binding and immunoreactive isoform of ER that was transcriptionally incapable of mediating growth arrest when bound by a tamoxifen-like antagonist.

Biochemical and antibody-based assays that quantitate ER in human tumors recognize specific regions within the ligand-binding domain of the receptor protein. It is believed that ER-mediated gene regulation requires the binding of ligand-occupied receptor dimers to a defined hormone-responsive DNA sequence called the ERE, usually located in an upstream regulatory region of the target gene. Defects within the zinc-finger DNA-binding domain of the ER can result in an immunoreactive receptor capable of binding ligand but unable to complex with ERE-containing DNA. Thus, mutation, transcriptional splicing error, or a posttranslational ER modification affecting the receptor's DNA-binding domain can each result in a nonfunctional receptor in an apparently ER-positive tumor. Electrophoretic mobility shift (gel-shift) assay measures the ability of ER to bind a DNA probe containing the cognate ERE sequence and has been used to detect immunoreactive DNA-binding ER from human breast tumors. Both truncated DNA-binding ER isoforms and intact (67-kDa) immunoreactive ER that have lost the ability to bind DNA have been identified from a small collection ($n = 51$) of unselected primary breast tumor samples (3, 4). In particular, this initial survey found that ≥30% of ER-positive breast tumors express a 67-kDa ER isoform incapable of binding DNA and forming ER-ERE complexes *in vitro*, suggesting that the gel-shift assay might identify ER-expressing breast tumors unable to respond to antiestrogen therapy (4).

Loss of ER from originally ER-positive primary tumors by the time of their first relapse and treatment with tamoxifen may account for acquired resistance in a proportion of cases (5, 6). However, we recently reported that primary breast tumors that become resistant after an initial objective clinical response to tamoxifen usually continue to express ER at the time of relapse, although a significant proportion (~50%) failed to express the estrogen-regulated proteins PgR and pS2 (7). These results are consistent with clinical studies indicating that more than half of tumors with acquired tamoxifen resistance fail to respond to second-line endocrine therapy (8). It remains unclear whether or not the lack of second-line endocrine responsiveness results from an acquired alteration in tumor ER function, such as loss of ER DNA binding, which might also account for an absence of PgR or pS2 expression in these tumors. Therefore, the present study was undertaken to determine whether acquired antiestrogen resistance is associated with the inability of ER to form ER-ERE complexes *in vitro* along with absent PgR and pS2 tumor expression. We studied 41 ER-positive tumors excised from breast cancer patients after their development of clinical resistance to tamoxifen. For comparison, an additional 60 untreated tumors were also analyzed to expand upon our preliminary survey, resulting in a total of 111 unselected ER-positive primary breast tumors, many of which would be expected to exhibit *de novo* tamoxifen resistance. Lastly, results from these human tumor analyses were compared with an experimental study of ER-positive human breast tumors excised from a nude mouse MCF-7 tumor xenograft model before and after acquired antiestrogen resistance to assess independently in this model whether ER DNA-binding function is altered by the *in vivo* development of resistance to either tamoxifen or the pure steroidal antiestrogen ICI 182,780.

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[4] The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; PgR, progesterone receptor; E2, estradiol; EIA, enzyme immunoassay; CHO, Chinese hamster ovary.

MATERIALS AND METHODS

Human Tumors for ER Analysis. Primary untreated ER-positive breast carcinomas (n = 60) were derived from United Kingdom (Royal Marsden Hospital), United States (San Francisco) and German (Heidelberg) breast tumor banks. Tamoxifen-resistant ER-positive tumors (n = 41) were derived from two different previously reported sample collections at the Royal Marsden Hospital (7): (*a*) 26 primary breast tumors from patients initially responsive to tamoxifen (complete and partial objective clinical responses) and surgically excised only after the development of acquired tamoxifen resistance; and (*b*) 15 tumor samples excised from patients relapsing with local-regional disease after primary treatment with surgery and ≥2 years of adjuvant tamoxifen, with relapses occurring while on tamoxifen.

Tumor extracts for ER analysis were prepared from 100–200 mg of cryopreserved (−80°C) samples of tumor that were pulverized in a tissue dismembrator (Braun Medical Ltd.). Frozen tumor powder was added to 1.5 ml of ice-cold extraction buffer containing appropriate protease inhibitors [20 mM Tris (pH 7.5), 10 mM DTT, 20% glycerol, 0.4 M KCl, 5 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 μg/ml pepstatin] and solubilized by polytron homogenization. The resulting homogenate was centrifuged at 10,000 rpm for 15 min at 4°C, and the clear supernatant was removed and stored at −80°C until assayed. Total immunoreactive ER was assayed in the cytosol by ER EIA (Abbott Laboratories), and protein concentration was quantified by the Bradford method.

MCF-7 Xenograft Model. Xenografts were established in athymic nude mice as described previously (9). Estrogen supplementation was provided in the form of a 0.25-mg E2 pellet (Innovative Research, Rockville, MD) placed s.c. in the interscapular region of the mice. The effects of tamoxifen and ICI 182,780 on the growth of established tumors were studied after the tumors had reached a size of 8–10 mm (3–5 weeks). At this time, the animals were randomly allocated into three treatment groups: (*a*) continued estrogen supplementation; (*b*) removal of the E2 pellet plus treatment with 500 μg of tamoxifen citrate (Zeneca Pharmaceuticals, Wilmington, DE) in peanut oil (injected s.c. each day from Monday to Friday); and (*c*) removal of the E2 pellet and treatment with 5 mg of ICI 182,780 (Zeneca Pharmaceuticals, Macclesfield, United Kingdom) in castor oil (s.c. injections once a week). Tumor growth was assessed, and tumor volumes were measured twice a week as described previously (9).

Tumors were harvested during the antiestrogen-sensitive growth phase (*i.e.*, 2–4 weeks after treatment initiation), when tumor volumes were regressing on either tamoxifen or ICI 182,780 (10). With continued tamoxifen treatment, tumors developed resistance and regrew after a median of 104 days, whereas with ICI 182,780, tumor progression did not occur until after a median of 200 days. A total of nine tamoxifen-sensitive, nine ICI 182,780-sensitive, seven tamoxifen-resistant, and six ICI 182,780-resistant tumors were harvested. Tumor homogenates were prepared as described above for analysis of ER content by EIA, Western blot, and for subsequent gel-shift analysis of ER DNA binding.

Gel-Shift and Western Blot Assays for ER. Tumor samples were assayed for ER DNA binding (ER-ERE complex formation) by incubation with 2 μg of poly(deoxyinosinic-deoxycytidylic acid) (Boehringer Mannheim) in 100 mM KCl, 10 mM Tris (pH 7.5), 2 mM DTT, and 5% (v/v) glycerol with or without 0.1 μg of anti-ER antibody (either D547 or D75) at room temperature for 10 min, as described previously (3, 4). The D547 and D75 monoclonal anti-ER antibodies (Abbott Laboratories) recognize widely separated epitopes in the D and E domains of the ER, respectively (11). DNA binding was initiated by further incubation for 10 min at room temperature with 10 fmol of ^{32}P-5′ end-labeled duplexed ERE oligomers (35-mer sense strand based on the *Xenopus* vitellogenin A2 consensus ERE sequence, 5′-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3′), and the final reaction volume of 20 μl was incubated at 20°C for 20 min. DNA-bound protein complexes were then electrophorectically separated on a 4.2% loosely cross-linked native polyacrylamide gel and visualized by autoradiography. Positive controls included ER-transfected CHO cells (3, 4). Sensitivity of the gel-shift assay was 0.25 fmol of ER, and positivity was defined as autoradiographic evidence of antibody-supershifted (immunoreactive) ER-ERE complexes, as described previously (3, 4).

To measure total immunoreactive 67-kDa ER, sample extracts were denatured by boiling in 1% SDS and 5% β-mercaptoethanol and electrophorectically separated using a 10% SDS-polyacrylamide gel. Proteins were transferred onto a nylon membrane using a transblotter, and after membranes were blocked with 2% BSA for 1 h, they were incubated with anti-ER antibody. Detection of 67-kDa ER was performed as described previously (3, 4).

PgR and pS2 Expression. The expression of PgR was determined from tumor cytosol by EIA (Abbott Laboratories), with a positive value being regarded as ≥15 fmol/mg protein. Tumor expression of pS2 was measured immunohistochemically using the BC-6 monoclonal antibody (gift from Prof. P. Chambon; Strasbourg, France) as described previously (12), with a positive value being assigned if ≥10% cells demonstrated cytoplasmic staining.

RESULTS

Of the 60 untreated ER-positive primary breast carcinomas analyzed in this survey, 41 (68%) contained ER capable of forming immunoreactive ER-ERE complexes by *in vitro* gel-shift assay. This frequency of ER DNA binding is similar to that found in our limited initial survey, and together, the data from both surveys demonstrate that 74 of 111 (67%) ER-positive primary tumors express DNA-binding ER (Table 1). Tumors with an ER content of 10–99 fmol/mg protein showed a significantly lower frequency of ER DNA-binding capability than tumors with an ER content of ≥100 fmol/mg protein (56 *versus* 82%; $\chi^2 = 7.2$; $P = 0.007$). Fig. 1 illustrates representative gel-shift results from six tumors expressing ER incapable of producing ER-ERE complexes (Fig. 1*A*) and six others in which comparably loaded ER protein produces easily detectable immunoreactive ER-ERE complexes (Fig. 1*B*). Negative results with human tumors were verified using additional anti-ER monoclonal antibodies (*e.g.*, H222, which recognizes an epitope in the ligand-binding domain) to rule out loss of epitopes as a possible reason for an absent supershifted band. Supershifting the ER-ERE complexes with anti-ER antibody serves the critical purpose of distinguishing DNA-bound ER from other potential ERE-complexing members of the nuclear hormone receptor superfamily. Using control forms of recombinant ER and cell-extracted ER (from CHO ER cells), both D547 and D75 seemed to supershift 100% of the total retarded ER-ERE complex (Fig. 1). Therefore, any residual complex comigrating with unsupershifted ER-ERE in human tumor extracts is likely to represent non-ER protein (*e.g.*, COUP and H-2RIIBP) found in cell and tumor extracts that can also bind ERE (3, 13).

Unlike the 67% ER DNA-binding frequency observed with the collection of 111 untreated ER-positive tumors, the 41 tamoxifen-resistant ER-positive tumors exhibited a significantly greater ER DNA-binding frequency, with nearly 90% showing ER-ERE complex formation ($\chi^2 = 5.7$; $P < 0.02$). For the 26 ER-positive tumors relapsing after objective clinical responses to tamoxifen, mean ER content was 81 fmol/mg (range, 14–247 fmol/mg) with full-length (67-kDa) immunoreactive receptor documented by Western blotting and normal ER DNA-binding function observed in 21 (81%) of these samples (Table 2). Thus, although in some tumor extracts more than one supershifted ER-ERE band was detected (Fig. 1*B*), Western blotting of these same extracts only revealed the full-length immunoreactive 67-kDa ER. For the 15 ER-positive tumors relapsing at ≥2

Table 1 *Frequency of DNA-binding ER in untreated primary tumors in relation to absolute ER level*

Compiled results are from previous reports (3, 4) together with samples collected from London, Heidelberg, and San Francisco in the present survey, as described in "Materials and Methods."

ER content (fmol/mg)	DNA Binding ER-ERE		
	Prior reports	Present survey	Total
(10–99)	13/25 (52%)	24/41 (59%)	37/66 (56%)
(>100)	20/26 (77%)	17/19 (90%)	37/45 (82%)
			74/111 (67%)

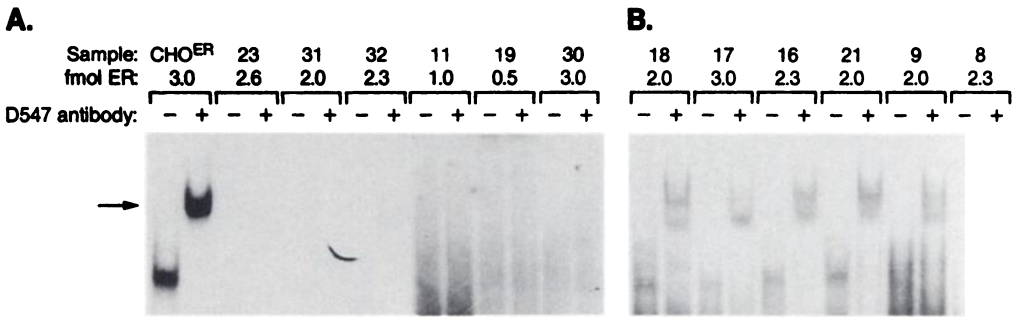

Fig. 1. Gel-shift mobility assays of (*A*) six tumor samples with undetectable immunoreactive (supershifted) DNA-binding ER and (*B*) six tumor samples with readily detectable immunoreactive DNA-binding ER. Control ER extract was derived from CHO cells transfected with ER. Comparable amounts of ER (in femtomoles) were loaded in each lane as shown. In *B*, extracts from six tumors bound the radiolabeled ERE probe, and the resulting ER-ERE complex was supershifted by the anti-ER antibody D547 (*arrow*, supershifted complexes). In contrast, three of the six tumors in *A* showed a nonspecific band that was not supershifted by any of several anti-ER antibodies, including D547. The free (unbound) probe that runs at the bottom of the gel is not shown.

Table 2 *Detection of immunoreactive DNA-binding ER (ER-ERE) in human breast tumors with acquired resistance to tamoxifen categorized by ER content (ER-EIA quantification) and phenotype (expression of PgR/pS2)*

Equal volumes of ER-containing tumor extracts were loaded for gel-shift (ER-ERE complex formation) assays.

Tumor no.	ER-EIA (fmol/mg)	PgR-EIA (fmol/mg)	pS2 (% cells)	Immunoreactive ER-ERE
ER positive, PgR or pS2 positive				
74	247	1	80	+
30	237	0	70	−
75	177	60	90	+
36	175	106	0	+
51	139	7	90	+
54	127	8	40	+
31	97	83	60	−
84	91	48	80	+
29	91	64	0	−
4	85	168	10	+
21	83	74	50	+
17	75	0	80	+
5	69	23	0	−
8	59	14	10	+
81	41	5	80	+
38	14	3	90	+
				(12/16 positive)
ER positive, PgR and pS2 negative				
57	203	3	0	+
41	178	2	0	+
40	97	0	0	+
22	82	0	0	+
48	79	13	0	+
18	79	2	0	+
82	76	0	0	+
86	63	1	0	+
49	21	1	0	+
28	21	0	0	−
				(9/10 positive)

years of adjuvant tamoxifen therapy, mean ER content was 112 fmol/mg (range, 13–369 fmol/mg), and normal ER DNA binding was observed in all of these samples (Table 3). ER-ERE complex formation did not correlate with PgR or pS2 expression in either set of tamoxifen-resistant tumors (Tables 2 and 3); it was notable that among the 19 ER-positive and tamoxifen-resistant tumors failing to express either PgR or pS2, all but 1 demonstrated normal ER DNA binding.

ER content and DNA binding were also studied in xenografted MCF-7 tumors demonstrating acquired antiestrogen resistance. Among the nude mouse tumors initially growth-inhibited (tamoxifen-sensitive phase) and subsequently growth-stimulated (tamoxifen-resistant phase) by tamoxifen, there was no significant difference in ER content (measured by EIA) when compared with control tumors in E2-treated animals (Fig. 2). Both tamoxifen-sensitive and tamoxifen-resistant MCF-7 tumors contained the 67-kDa ER capable of forming immunoreactive ER-ERE complexes (Fig. 3). By comparison, MCF-7 tumors initially growth-inhibited by the pure steroidal antiestrogen ICI 182,780 contained lower levels of ER than the E2-treated controls (mean, 43 ± 23 *versus* 186 ± 23 fmol/mg), and tumors that developed acquired resistance to ICI 182,780 expressed even lower ER levels (mean, 17 ± 3 fmol/mg protein). Despite the reduced ER content of these ICI 182,780-treated tumors, the expressed receptor retained its normal 67-kDa size and demonstrated DNA binding.

DISCUSSION

In a previous report, we described several types of breast tumor ER isoforms observed on the basis of their *in vitro* ability to bind DNA and form immunoreactive ER-ERE complexes (4). Truncated DNA-binding isoforms may result from partial proteolytic cleavage of ER protein or alternative ER mRNA splicing (3, 14). The latter mechanism may produce a truncated ER isoform (*e.g.*, exon 5 variant) unable to bind ligand but capable of constitutively transactivating an estrogen-responsive gene like PgR (15). However, at the RNA level, such truncated ER variants do not seem to be expressed at sufficient levels in untreated and treated breast tumors to account for the emergence of most forms of tamoxifen resistance (16, 17). In contrast, our preliminary survey suggested that at least 30% of unselected ER-positive primary breast tumors contain a form of full-length (67-kDa) ER that is unable to bind DNA (4). This type of dysfunctional ER could arise by posttranslational modification of the protein's

Table 3 *Detection of immunoreactive DNA-binding ER (ER-ERE) in human breast tumors that relapsed during adjuvant tamoxifen therapy categorized by ER content (ER-EIA quantification) and phenotype (expression of PgR/pS2)*

Tumor no.	ER-EIA (fmol/mg)	PgR-EIA (fmol/mg)	pS2 (% cells positive)	Immunoreactive ER-ERE
ER positive, PgR or pS2 positive				
59	369	17	60	+
73	230	500	0	+
52	154	463	0	+
12	60	2	50	+
55	37	25	0	+
45	13	26	0	+
				(6/6 positive)
ER positive, PgR and pS2 negative				
76	223	0	0	+
71	134	0	0	+
80	128	2	0	+
33	115	0	0	+
14	68	0	0	+
77	61	0	0	+
60	49	7	0	+
78	26	0	0	+
56	25	1	0	+
				(9/9 positive)

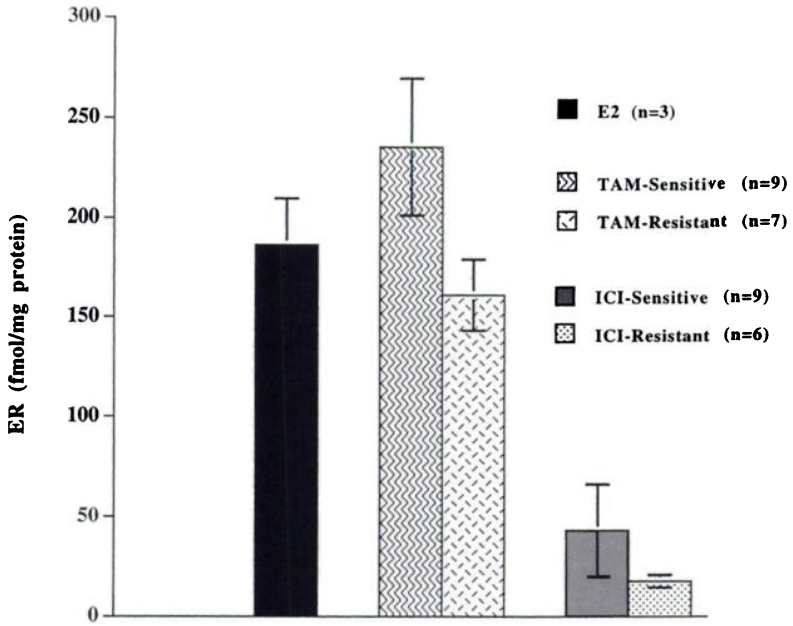

Fig. 2. Mean ± SE ER protein levels determined by EIA in MCF-7 xenografts treated with either E2, tamoxifen (*TAM*), or the pure antiestrogen ICI 182,780. Tumors treated with either antiestrogen were harvested in either the sensitive or resistant phase of *in vivo* growth.

DNA-binding domain (*e.g.*, intracellular oxidation of thiol groups determining the zinc-finger structures) and could potentially account for a significant proportion of ER-positive tumors exhibiting resistance to receptor-binding antagonists such as tamoxifen (4). The data presented in Table 1 confirm and extend our earlier survey results and indicate that only two-thirds (74 of 111) of primary untreated ER-positive breast tumors contain an immunoreactive receptor capable of binding DNA. Using a slightly different technique from the gel-shift assay procedure used here to survey primary breast tumors (n = 79), another group of investigators has reported a 30% discordance between the DNA-binding and ligand-binding detection of tumor ER and has similarly concluded that assessment of both ER content and ER DNA-binding function might improve the predictive specificity of patients most likely to benefit from endocrine therapy (18).

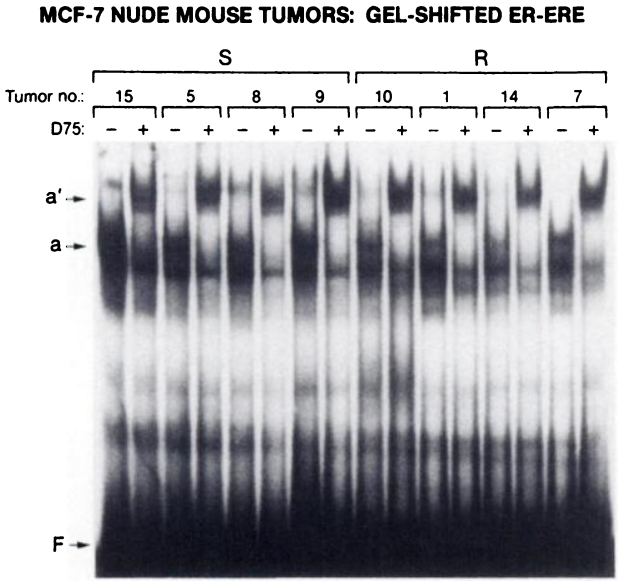

Fig. 3. Gel-shift mobility assay of four tamoxifen-sensitive (*S*) and four tamoxifen-resistant (*R*) MCF-7 xenografts grown in oophorectomized athymic (nude) mice. MCF-7 xenografts were initially established with E2 and then treated with tamoxifen by daily s.c. injection. Tumors were harvested in either the sensitive phase when tumor volume was regressing (2–4 weeks after initiation of therapy) or when acquired tamoxifen-resistant growth developed after a period of 4–6 months of tamoxifen therapy. All tumor extracts bound to the labeled oligonucleotide probe (*a*), and this band was further supershifted by the anti-ER monoclonal antibody D75 (*a'*). Free labeled probe (*F*) runs at the bottom of the gel.

ER levels can fall with tamoxifen therapy (19–21), and clonal selection of low ER-expressing metastases from a primary breast tumor with heterogeneous ER expression represents a potential contributing mechanism for acquired tamoxifen resistance (5, 6, 22). Using an immunohistochemical ER assay unaffected by concurrent administration of tamoxifen, we have shown that breast tumor ER expression, although occasionally reduced in level, usually continues with the acquisition of tamoxifen resistance after an initial clinical response (7). However, up to 50% of tamoxifen-resistant tumors may fail to coexpress PgR or pS2. Thus, given the observed correlation in primary untreated tumors between lower tumor ER and reduced ability to form ER-ERE complexes (Table 1), another feasible hypothesis for the development of tamoxifen resistance and loss of PgR and pS2 expression would be expression of a 67-kDa ER isoform incapable of binding to DNA. The present study shows that in contrast to the 67% frequency of DNA-binding ER in untreated ER-positive tumors, breast tumors selected for having developed clinical resistance to tamoxifen demonstrate a significantly greater DNA-binding ER frequency, with nearly 90% of these ER-positive tumors capable of forming ER-ERE complexes ($P < 0.02$).

These ER-positive tumors had acquired their resistance to tamoxifen after an initial objective clinical response (n = 26) or after at least 2 years of tamoxifen adjuvant therapy (n = 15). Thus, these subsets of tamoxifen-resistant tumors represent relapses of primary breast tumors that most likely originally contained functionally intact ER mechanisms accounting for their sensitivity to tamoxifen at the time this therapy was initiated. If the hypothesis is true that acquisition of tamoxifen resistance occurs independent of treatment-related variations in tumor ER, then the ~90% frequency of DNA-binding ER observed in these relapsing tumors probably reflects preservation of functioning ER as originally expressed in the pretreated tumors and suggests that gel-shift detection of DNA-binding ER in primary tumors might predict for initial endocrine responsiveness. Taken together with our previous finding of intact ER mRNA in these samples (16), the data indicate that most of the ER expressed in tumors with acquired clinical resistance to tamoxifen has normal protein and transcript size as well as DNA-binding function. Furthermore, in

tumors with absent supershifted ER-ERE, this was confirmed by more than one ER monoclonal antibody to rule out epitope loss in the D/E region yielding a false negative result.

Prior studies have established an association between ER DNA binding and PgR expression in ER-positive primary untreated breast tumors (3, 18). For the tamoxifen-resistant tumors analyzed in this study, ER DNA binding did not correlate with PgR or pS2 expression. As reported earlier, nearly half (19 of 41) of tumors with acquired tamoxifen resistance failed to express either PgR or pS2 (7); notably, all but one of these demonstrated normal ER-ERE complex formation. Furthermore, 10% (4 of 41) of the tamoxifen-resistant tumors demonstrated expression of either PgR or pS2 without detectable formation of ER-ERE complexes. These latter cases represented a subset of low ER-expressing tumors (5–31 fmol/mg) for which the detection of ER-ERE complex formation can be technically more demanding, raising the possibility of false negative gel-shift results (4). Alternatively, these four cases might represent tumors in which the expressed ER is truly unable to bind DNA and in which PgR or pS2 expression is constitutive and transcriptionally independent of ER-ERE complex formation. Because the responsiveness of the 41 tamoxifen-resistant tumors to second-line endocrine therapy was unknown, the predictive ability of either ER-ERE complex formation or PgR/pS2 expression in this setting could not be determined. However, the anticipated response rate to second-line endocrine therapy of patients exhibiting prior responsiveness to tamoxifen might be >50% (8). Thus, given the lack of correlation between ER DNA binding and PgR/pS2 expression in these tumors with acquired tamoxifen resistance, a prospective clinical study would be of interest to determine whether either ER-ERE complex formation or PgR/pS2 expression at the time of relapse is predictive of subsequent tumor response to second-line endocrine therapy.

The association of ER DNA binding, PgR/pS2 expression, and antiestrogen resistance was tested experimentally in a well-characterized MCF-7 human xenograft model. Previous studies with this model have shown that *in vivo* acquisition of tamoxifen resistance is not associated with any significant change in ER, PgR, or pS2 expression in s.c. implanted MCF-7 tumors (9, 23, 24). We not only verified that resistance to tamoxifen is unrelated to any significant change in ER content as compared to either E2-stimulated or tamoxifen-sensitive MCF-7 tumors (Fig. 2), but we also demonstrated expression of normal DNA-binding ER during each of the treatment responses (Fig. 3). ICI 182,780, a pure steroidal antiestrogen distinguished by its ability to inhibit ER dimerization and down-regulate ER and PgR/pS2 expression, is also associated with eventual development of resistance and tumor progression in the MCF-7 model, but after about twice as long a treatment interval as with tamoxifen (10). In the present study, ICI 182,780 not only reduced ER expression in both sensitive and resistant MCF-7 tumors, but the remaining ER expressed in these tumors seemed fully capable of binding DNA (Fig. 2). Thus, in this model system as well as with the human tumor samples, acquisition of antiestrogen resistance after an initial clinical response to therapy is associated with retention of ER DNA-binding ability, independent of alterations in the level of tumor ER and PgR/pS2 expression.

In conclusion, it has been suggested that assessment of ER DNA binding (by *in vitro* formation of ER-ERE complexes) might provide improved predictive information about the endocrine responsiveness of primary breast tumors. Whereas *de novo* resistance to tamoxifen is usually associated with lack of ER expression, it is also recognized that at least 30% of ER-positive tumors may be resistant to primary endocrine therapy. Based on this and earlier studies, it may be proposed that only those ~70% of untreated ER-positive tumors that contain DNA-binding ER are capable of responding to an antiestrogen such as tamoxifen, and this suggestion deserves a prospective clinical analysis. In turn, when breast tumors that have shown an initial endocrine response become clinically resistant to tamoxifen, they seem to retain their DNA-binding ER, indicating that acquired tamoxifen resistance results in an an altered gene-regulatory mechanism(s) not affecting ER-ERE complex formation. Such tamoxifen-activated regulatory mechanisms that depend on ER but not on ER DNA binding have been identified and are currently under study in models of acquired antiestrogen resistance (25).

REFERENCES

1. McGuire, W. L. Hormone receptors: their role in predicting prognosis and response to endocrine therapy. Semin. Oncol., *5:* 428–433, 1978.
2. McGuire, W. L. Steroid hormone receptors in breast cancer treatment strategy. Recent Prog. Horm. Res., *36:* 135–156, 1980.
3. Scott, G. K., Kushner, P., Vigne, J-L., and Benz, C. C. Truncated forms of DNA-binding estrogen receptor. J. Clin. Invest., *88:* 700–706, 1991.
4. Montgomery, P. A., Scott, G. K., Luce, M. C., Kaufmann, M., and Benz, C. C. Human breast tumors containing non-DNA-binding immunoreactive (67-kDa) estrogen receptor. Breast Cancer Res. Treat., *26:* 181–189, 1993.
5. Spataro, V., Price, K., Goldhirsch, A., Cavalli, F., Simoncini, E., and Castiglioni, M. Sequential estrogen receptor determinations from primary breast cancer and at relapse: prognostic and therapeutic relevance. Ann. Oncol., *3:* 733–740, 1992.
6. Kuukasjärvi, T., Kononen, J., Helin, H., Holli, K., and Isola, J. Loss of estrogen receptor in recurrent breast cancer is associated with poor response to endocrine therapy. J. Clin. Oncol., *14:* 2584–2589, 1996.
7. Johnston, S. R. D., Saccani-Jotti, G., Smith, I. E., Salter, J., Newby, J., Coppen, M., Ebbs, S. R., and Dowsett, M. Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. Cancer Res., *55:* 3331–3338, 1995.
8. Howell, A., DeFriend, D., and Anderson, E. Mechanisms of response and resistance to endocrine therapy for breast cancer and the development of new treatments. Endocr. Rel. Cancer, *43:* 5–21, 1993.
9. Osborne, C. K., Hobbs, K., and Clark, G. M. Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res., *45:* 584–590, 1985.
10. Osborne, C. K., Coronado-Heinsohn, E. B., Hilsenbeck, S. G., McCue, B. L., Wakeling, A. E., McClelland, R. A., Manning, D. L., and Nicholson, R. I. Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. J. Natl. Cancer Inst., *87:* 746–750, 1995.
11. Greene, G. L., Sobel, N. B., King, W. J., and Jensen, E. V. Immunochemical studies of estrogen receptors. J. Steroid Biochem., *20:* 51–56, 1984.
12. Detre, S., King, N., Salter, J., MacLennan, K., McKinna, J. A., and Dowsett, M. Immunohistochemical and biochemical analysis of the estrogen-regulated protein pS2, and its relation with estrogen receptor and progesterone receptor in breast cancer. J. Clin. Pathol. (Lond.), *47:* 240–244, 1994.
13. Hamada, K., Gleason, S. L., Levi, B-Z., Hirschfeld, S., Appella, E., and Ozato, K. H-2RIIBP, a member of the nuclear hormone receptor superfamily that binds to both the regulatory element of major histocompatibility class I genes and the estrogen response element. Proc. Natl. Acad. Sci. USA, *86:* 8989–8293, 1989.
14. Osborne, C. K., Elledge, R. M., and Fuqua, S. A. W. Estrogen receptors in breast cancer therapy. Sci. Med., *3:* 32–41, 1996.
15. Fuqua, S. A. W., Fitzgerald, S. D., Chamness, G. C., Tandon, A. K., McDonnell, D. P., Nawaz, Z., O'Malley, B. W., and McGuire, W. L. Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res., *51:* 105–109, 1991.
16. Daffada, A. A. I., Johnston, S. R. D., Smith, I. E., Detre, S., King, N., and Dowsett, M. Exon 5 deletion variant estrogen receptor messenger RNA expression in relation to tamoxifen resistance and progesterone receptor/pS2 status in human breast cancer. Cancer Res., *55:* 288–293, 1995.
17. Dowsett, M., Daffada, A. A. I., Chan, C. M. W., and Johnston, S. R. D. Estrogen receptor mutants and variants in breast cancer. Eur. J. Cancer, in press, 1997.
18. Foster, B. D., Cavener, D. R., and Parl, F. F. Binding analysis of the estrogen receptor to its specific DNA target site in human breast cancer tissue. Cancer Res., *51:* 3405–3410, 1991.
19. Taylor, R. E., Powles, T. J., Humphreys, J., Bettelheim, R., Dowsett, M., Casey, A. J., Neville, A. M., and Coombes, R. C. Effects of endocrine therapy on steroid receptor content of breast cancer. Br. J. Cancer, *45:* 80–85, 1982.
20. Hull, D. F., Clark, G. M., Osborne, C. K., Chamness, G. C., Knight, W. A., and McGuire, W. L. Multiple estrogen receptor assays in human breast cancer. Cancer Res., *43:* 413–416, 1983.
21. Encarnacion, C. A., Ciocca, D. R., McGuire, W. L., Clark, G. M., Fuqua, S. A., and Osborne, C. K. Measurement of steroid hormone receptors in breast cancer patients on tamoxifen. Breast Cancer Res. Treat., *26:* 237–246, 1993.
22. Osborne, C. K. Heterogeneity in hormone receptor status in primary and metastatic breast cancer. Semin. Oncol., *12:* 317–326, 1985.
23. Gottardis, M., and Jordan, V. C. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res., *48:* 5183–5187, 1988.
24. Gottardis, M. M., Jiang, S-Y., Jeng, M-H., and Jordan, V. C. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res., *49:* 4090–4093, 1989.
25. Webb, P., Lopez, G. N., Uht, R. M., and Kushner, P. J. Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol. Endocrinol., *9:* 443–456, 1995.

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