T47D<sub>CO</sub> Cells, Genetically Unstable and Containing Estrogen Receptor Mutations, Are a Model for the Progression of Breast Cancers to Hormone Resistance


Departments of Medicine (M. L. G., N. L. K., D. F. G., W. M. W., L. L. W., K. B. H.), Obstetrics and Gynecology (K. K. L.), and Pathology (L. A. M., K. B. H.), University of Colorado Health Sciences Center, Denver, Colorado 80262

ABSTRACT

We postulate that one mechanism for the progression of breast cancers to hormone resistance involves the acquisition of mutant estrogen receptors (ERs) by genetically unstable cell subpopulations. The T47D human breast cancer cell line may be a model for such progression, having sublines that are ER positive and estrogen responsive, ER positive and estrogen resistant, or ER negative. Also, T47D cells can be either hyperdiploid (HD) or hypertetraploid (HT) or persistently alternate between these states. T47D<sub>CO</sub> cells are a HD and ER-positive, but estrogen-resistant, subline of T47D cells that undergoes spontaneous tetraploidization. Such a stable variant, designated T47D<sub>A</sub>, is 85% HT (Cancer Res., 49: 3943, 1989). We now show that single-cell clones derived from the mixed HD/HT T47D<sub>CO</sub> can be either HD or HT, and can be either estrogen responsive or estrogen resistant, for growth and for progesterone receptor regulation. To begin the study of ER in this model system of T47D<sub>CO</sub> and their derivatives, we have generated complementary DNA libraries from the parental HD T47D<sub>CO</sub> cells and have isolated three ER complementary DNA mutants. These include two frame-shift/termination mutants that would encode ERs truncated in the DNA-binding domain and in the hormone-binding domain and a third mutant with a large in-frame deletion spanning the hinge region and a part of the hormone-binding domain. If expressed, these mutant ERs would lack hormone-binding capacity and would be undetected by the anti-ER antibodies currently in clinical use. Genetic instability, when associated with mutant ERs in subpopulations of breast tumor cells, may provide the selective pressure leading to hormone resistance. T47D<sub>CO</sub> cells and their clonal derivatives provide a model for the systematic study of ER mutations and other mechanisms of hormone resistance in Stage IV breast cancer.

INTRODUCTION

Faithful expression of genetic information is lost in tumor cells due to the formation of spontaneous cell variants (1). In breast cancer, this evolution is marked by progression of tumors from hormone-dependent, through hormone-responsive, to hormone-resistant states. Resistant tumors fail to respond to the growth inhibitory signals of antiestrogens (2) and, by inference, to the growth stimulatory signals of estrogens. Based on recent studies, it has been suggested that in MCF-7 human breast cancer cells, activation of H-ras oncogenes partially overrides the growth stimulatory signals of estrogens in vitro, and their tumor-promoting activity in athymic mice (3). However, when driven by a homologous promoter, ras oncogenes do not sustain MCF-7 cell growth in vivo in the absence of estrogens (4). Furthermore, neither the presence nor the level of expression of H-ras, abl, erb B1, fms, fos, myb, or neu gene products is different in estrogen-dependent and -resistant tumors (4). Since estrogen-inducible growth factors also fail to replicate the growth-promoting properties of estrogens (5, 6), it appears that acquisition of estrogen resistance in breast cancers involves mechanisms other than, or in addition to, activation of protooncogenes or autonomy of growth factor production.

What other factors could trigger this switch to hormone resistance? A necessary factor in the estrogen response pathway is the ER (7). ER content, as assessed by ligand binding or immunoassay, has been measured routinely in primary and metastatic breast cancers (7), but ERs have only recently begun to be analyzed in detail at the molecular level (8–10). While a restriction fragment length polymorphism has been described in the ER gene of primary breast tumors (8), and a DNA polymorphism has been described in exon 1 of the ER gene in a subset of patients with breast tumors (9), no clear association has been made between ER defects and estrogen resistance (10).

In related receptors, however, glucocorticoid, androgen, and vitamin D<sub>3</sub> resistance syndromes are associated with inheritance or acquisition of mutant forms of the cognate receptors (11–15). Although hereditary estrogen resistance appears to be incompatible with life, acquired estrogen resistance involving mutant ERs may exist. Indirect evidence in support of such a mechanism comes from clinical data. Malfunctioning receptors may explain why 40–50% of ER-positive metastatic breast tumors fail to respond to antiestrogen therapy (7). Tumors that are ER negative are most likely to be resistant (2, 7). In resistant tumors, lack of hormone-binding activity could be due to total loss of receptor protein or to mutations in ERs that lead to either nonfunctional or constitutively active receptor forms. Such mutant receptors may not be measured by the commonly used ligand binding or immunoassays. It is interesting, therefore, that variant ER mRNAs have been detected in human breast cancer biopsy specimens (16, 17). Finally, based on the experience with ER and other members of the steroid receptor superfamily in transfection systems (18–22), mutant ERs may exist that are not only insensitive to the regulatory effects of estrogens but that promote growth through constitutive activation of estrogen-responsive genes. Together, these data suggest that mutant ERs may play a role in the etiology of resistance.

To search for evidence that mutant ERs play a role in hormone resistance, we turned to the T47D human breast cancer cell line as a model system (23, 24). These cells are
enigmatic. Different sublines have been described as ER positive and estrogen responsive (25, 26), as ER positive and estrogen resistant (27, 28), and as ER negative (25). Some sublines are genetically unstable (26, 29, 30), as measured by changing DNA and estrogen responsive (25, 26), as ER positive and estrogen are in transition from the estrogen-responsive, ER-positive state positive, estrogen-resistant HD T47DCO stocks (27).

Based on these observations, we postulated that T47D cells are in transition from the estrogen-responsive, ER-positive state to the estrogen-resistant, ER-negative state and serve as a model for the study of this process. We postulated further that a primary genetic event in the progression of breast cancers to hormone resistance may be the acquisition of dominant mutant ERs by genetically unstable subpopulations of cells. To test these ideas, we have generated a series of clonal cell lines from the mixed HD/HT T47DV cells and analyzed the chromosomal instability and estrogen responsiveness of the subpopulations derived from this variant stock. Also, as a first step in the analysis of the ER in T47DCO, T47DV, and its clonal derivatives, we have generated two cDNA libraries from the parental ER-positive, estrogen-resistant T47DCO subline and sequenced several ER cDNAs. In addition to wild-type ER, three mutant ER cDNAs have been identified. The results suggest that genetic instability and mutant ERs may both play a significant role in a breast cancer cell line modeling hormone-resistant Stage IV breast cancer.

MATERIALS AND METHODS

T47D Cloning by Limiting Dilution. One confluent T-175 flask of Mycoplasma-free T47DV stocks was harvested 41 passages after a HT population had spontaneously emerged from the HD T47DCO parental cell line (29). By this time the stock had stabilized, with 15% of the cells remaining HD. To obtain a single-cell suspension, cells were pelleted, then resuspended, triturated, and incubated ~3 min in 10 ml of 0.05% trypsin and 0.50 mM EDTA prepared in phosphate-buffered saline. Cells were diluted in antibiotic-free CGM containing 5% fetal calf serum (27) to obtain suspensions of 50, 10, or 5 cells/ml. Each well of three 96-well plates was plated with 0.1 ml of each cell dilution together with 0.1 ml of cell-free T47D-conditioned CGM. After 7 and 14 days, wells were inspected and those containing a single colony were scored. At confluence, cells in 54 wells that had been scored twice as a single colony were transferred to 24-well plates (passage 1) and grown in CGM. Doubling times varied considerably among the subclones. Some of the subclones succumbed to senescence, but 33 thrived and have been characterized (Table 1).

Growth and FCM Assays for PR and DNA. The simultaneous FCM assay was described in detail elsewhere (31). Briefly, 4 x 10^6 T47D cells/T-175 flask were seeded and grown to confluence. Cells were pretreated for 60 min with 0.1 μM synthetic progesterone 225020 (Roussel-UCLAF, Paris, France) to uncover or stabilize immunoreactive epitopes (31), then harvested, and counted to estimate growth. Three to 4 million cells were fixed by treatment with 0.5% paraformaldehyde and then permeabilized with 0.1% Triton X-100. Duplicate sets were washed and then incubated for 60 min at 4°C with a mixture of two anti-PR monoclonal antibodies: 10 μg/ml of AB-52 and 12 μg/ml of B-30 (31, 32). Nonspecific binding was determined in parallel sets of cells incubated with 20 μg/ml of mouse monoclonal IgG1 (Coulter Immunology, Hialeah, FL). The cells were washed and incubated for 30 min at 4°C with 40 μg/ml of fluorescein isothiocyanate-conjugated, affinity-purified, human-absorbed goat anti-mouse F(ab')2 (Boehringer-Mannheim, Indianapolis, IN). DNA was then labeled at 37°C by simultaneous treatment with RNase A and propidium iodide. Cells were passed through a nylon mesh and 10,000 were analyzed on a Coulter Epics 752 flow cytometer using modified Coulter Easy 2 Software (31) and the LogMean data analysis program. PR as measured by this FCM immunoassay correlates highly with total cellular receptors (cytosol + nuclear fractions) measured by radioligand assay. PR-specific binding is expressed as mean fluorescence/cell; standard curves allow conversion of fluorescence units to fmol/mg DNA. Hormone Effects on Growth and PR in Clonal Sublines. To screen the 33 T47DVclonal sublines for the effects of hormones on cell growth and PR, parallel sets of T-175 flasks were seeded with cells and incubated with CGM (27). Twenty-four h later cells were refed with CGM or switched to medium containing 5% DCC-stripped (27) serum with no additions or containing 10 nM estradiol or 1 μM tamoxifen; in some experiments estradiol and tamoxifen were added together. Cells were fed every 2 days, harvested 8 days after plating, counted to assess growth, and assayed in duplicate for PR and DNA by flow cytometry. Significant differences (P < 0.01, Student’s t test) between the DCC-stripped serum and other sets are marked by an asterisk in Figs. 4 and 5. Within the duplicate flasks, the four PR determinations routinely differed by <5%.

In more detailed growth experiments, 1 x 10^6 cells were plated in duplicate T175 flasks containing CGM. Twenty-four h later cells were refed with CGM or switched to medium containing the hormone conditions described above. Cells were fed every 2 days and duplicate flasks from each set were harvested after approximately 4, 7, and 11 days. Cells were counted and assayed in duplicate for DNA and PR content by the simultaneous FCM assay.

Table 1 Initial ploidy status, PR content, and proliferation rate of 33 subclones of T47D.

<table>
<thead>
<tr>
<th>Subclone</th>
<th>Initial ploidy*</th>
<th>Initial specific PR*</th>
<th>Proliferation rate*</th>
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<tbody>
<tr>
<td>V12</td>
<td>HD</td>
<td>83°</td>
<td>++</td>
</tr>
<tr>
<td>V25</td>
<td>HD</td>
<td>63°</td>
<td>+++</td>
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<tr>
<td>V26</td>
<td>HD</td>
<td>78°</td>
<td>+++</td>
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<td>V28</td>
<td>HD</td>
<td>18°</td>
<td>+</td>
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<tr>
<td>V29</td>
<td>HD</td>
<td>ND°</td>
<td>++</td>
</tr>
<tr>
<td>V36</td>
<td>HD</td>
<td>55°</td>
<td>+++</td>
</tr>
<tr>
<td>V37</td>
<td>HD</td>
<td>95°</td>
<td>++++</td>
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<td>V39</td>
<td>HD</td>
<td>42°</td>
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<tr>
<td>V44</td>
<td>HD</td>
<td>48°</td>
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<td>V50</td>
<td>HD</td>
<td>52°</td>
<td>++</td>
</tr>
<tr>
<td>V51</td>
<td>HD</td>
<td>14°</td>
<td>+</td>
</tr>
<tr>
<td>V18</td>
<td>HD-HT</td>
<td>51°</td>
<td>++</td>
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<tr>
<td>V21</td>
<td>HD-HT</td>
<td>104°</td>
<td>++++</td>
</tr>
<tr>
<td>V22</td>
<td>HD-HT</td>
<td>166°</td>
<td>++++</td>
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<td>V45</td>
<td>HD-HT</td>
<td>164°</td>
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<td>HT</td>
<td>163°</td>
<td>+</td>
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<td>223°</td>
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<td>++</td>
</tr>
<tr>
<td>V53°</td>
<td>HT</td>
<td>175°</td>
<td>++</td>
</tr>
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* Passages 3-5.
* Passages 3-6.
* Estimated from passage number achieved by 12 weeks and 20 weeks after subcloning. +, >48 h doubling time; ++++, <24 h doubling time.
* Expressed in fluorescence units (Fl. U.); linearly related to cytoplasmic + nuclear PR expressed as fmol/mg DNA; range, 14-223 Fl. U. = 710-8100 fmol/mg DNA.
* ND, not done.
* Spontaneously senescent after several passages.

M. L. Graham, P. B. Jewett, and K. B. Horwitz, manuscript in preparation.
cDNA Isolation and Sequencing of Mutant ER cDNAs. Two cDNA libraries were made. For library I, total poly(A)+ mRNA, isolated from ER-positive, estrogen-resistant T47Dco cells, was used to direct cDNA synthesis using two human ER-specific oligonucleotides as primers. One 24-mer, 5'-CCC ACC TTC ACC CCT GCC CTC CCC-3' was complementary to base pairs 1052 to 1075 of the ER message (21) in the hinge region D; the second, a 30-mer, 5'-GGG GGA AAG AGA GCT GTT ACA AAG ATT TAG-3' was complementary to base pairs 2230 to 2259 in the 3' UTR (33-35). The cDNA was synthesized and cloned into bacteriophage SWA1-4 (36) by Clontech (Palo Alto, CA).

In library II, cytoplasmic poly(A)+ mRNA was the starting material. Double-stranded cDNA was synthesized using the two ER oligonucleotides as primers. EcoRI/NorI linker-adaptors were ligated to blunt-ended cDNAs. The products were phosphorylated, and fragments larger than 300 base pairs were gel purified, electroeluted, and ligated to λgt10 cut with EcoRI and dephosphorylated. Ligated recombinants were packaged into phage using in vitro packaging extracts (Invitrogen, San Diego, CA) and were amplified in Escherichia coli C6000Hfl. The two cDNA libraries consisted of 4 × 10^5 and 3 × 10^6 independent recombinants prior to the amplification step.

Approximately 5 × 10^4 phage from each library were screened for ER sequences by plaque hybridization using a 2.1-kilobase human ER nick-translated cDNA probe encompassing the protein-coding region, which was kindly supplied by Andrew Baker (35). Seven independently isolated recombinant phage from library I and five from library II were randomly picked and purified to homogeneity by three rounds of screening. Bacteriophage were prepared from plate lysates and phage DNA was purified, using Lambda Sorb (Promega, Madison, WI) according to the manufacturer's instructions.

For library I, pGEM-1 containing the cDNA was prepared by recircularization after release from SWA1-4 by restriction digestion with Spel (36). For library II, the cDNA insert was isolated with EcoRI and recloned into pGEM7Zf(+) (Promega). Double-stranded plasmid DNA containing the cDNA insert was alkali denatured (37) and both strands were sequenced (Sequenase, United States Biochemical Corp.) using SP6 and T7 promoter primers and overlapping synthetic ER oligonucleotide primers. Sequences were compared against those reported in the Genetic Sequence Data Bank (GenBank, Beckman, release 59).

RESULTS

Genetic Instability. When they were developed from the pleural effusion of a postmenopausal Stage IV breast cancer patient, T47D cells contained ER and PR and had a modal chromosome number of 66 (23, 24). Soon thereafter we described a subline that had little cytoplasmic ER and abnormal nuclear ER but had much higher PR levels than the original cells (27). This subline, named T47Dco, was unresponsive to the effects of estrogens and tamoxifen but was responsive to progestins. The PRs, although normal by all usual criteria, were not dependent on estrogens for induction (27). T47Dco cells are HD (Fig. 1, Panel 1) with a modal chromosome number of 62. They contain two copies of chromosome 6 (29), which carries the ER gene (38). In current stocks, PR levels are 5600 fmol/mg DNA and ER levels are ~130 fmol/mg DNA. T47Dco cells remain resistant to the effects of estradiol and tamoxifen on growth and PR (not shown).

By combined PR/DNA flow cytometry we have monitored the gradual emergence of a stable HT population from one of the HD T47Dco stocks. The HT population has a growth advantage over the HD population and after 50 passages, HT cells represent 85% of the cells in this mixed stock, designated T47Dv (Fig. 1, Panel 2). The HT cells of T47Dv have a modal chromosome number of 120 and 4–5 copies of chromosome 6. PR levels are approximately 7200 fmol/mg DNA in the HD subpopulations and 8400 fmol/mg DNA in the HT subpopulations by quantitative FCM, which allows assignment of PR levels to subpopulations of cells having different DNA content. Total cellular ER levels are ~80 fmol/mg DNA by ligand binding (29). T47Dv cells show a small, but significant, inhibition of growth and PR in stripped serum, which is reversed by estradiol (not shown).

To obtain pure HD and HT cell populations from the T47Dv stock, subclones were generated by limiting dilution. Thirty-three clones were propagated to passage 5 and analyzed for DNA ploidy phenotype, doubling time, and PR content. Panels 3–5 of Fig. 1 show three examples of the 33 clones. Eleven clones were purely HD (Panel 3), and 18 were purely HT (Panel 5). Surprisingly, 4 clones (Panel 4) had a mixed phenotype at the earliest time when DNA evaluation was possible (passage 3). The phenotypes of the 33 subclones are summarized in Table 1. They differ in DNA ploidy, stability of ploidy, doubling times, and PR content, as well as sensitivity to estrogens (see below). The data underscore the cellular heterogeneity of the parental T47Dv and T47Dco lines.

The emergence of the mixed HD/HT clones (Fig. 1, Panel 4) is explained by repeat analyses of the phenotypically pure stocks such as those of Panels 3 and 5. We find that, with continued passage, instability persists in some of the subcloned cell lines. Of the original 11 HD clones at passage 5, two formed HT subpopulations by passage 10, and of the 4 mixed clones, 2 lost their HT subpopulations by passage 10. Among the 18 HT clones, many were stable over time. However, two patterns of instability emerged. Five HT lines underwent spontaneous cell death after a variable time of growth; this was not seen in any of the HD clones. Most interesting was line V24, which appeared to revert from pure HT to HD between passages 5 and 10.

These data suggest that genetic instability produces not just a single structural chromosomal abnormality; instability is a continuous process characterized by repeated bouts of chromosomal endoreduplication, reversion, and death of subpopulations. This dynamic process lends to expansion and contraction of selected cell populations and a relentless remodeling of the tumor.

Heterogenous Estrogen Responses. Fig. 2 compares the growth of four representative T47Dv subclones (V27, V24, V43, and V12) after 7 days in serum-containing medium, or in medium containing DCC-stripped serum with no additions, with 10 nM estradiol or with 1 μM tamoxifen. V27 is an estrogen-responsive HT clone. Stripping the serum removes a factor(s) that stimulates growth, which can be replaced by estradiol. The cells are further inhibited by tamoxifen (Fig. 2A). Some clones, like predominantly HD V24, remain responsive to the growth inhibitory effects of tamoxifen but are no longer stimulated to grow by estradiol, either at the low levels found in 5% serum or at 10 nM (Fig. 2B). Other subclones, such as V43, another HT clone, are growth inhibited by both estradiol and tamoxifen (Fig. 2C). This may represent a continuum of the previous phenotype in which estradiol had no growth-stimulating effects. Finally, V12 is representative of an estrogen-resistant HD clone (Fig. 2D). Neither stripping the serum nor hormone additions have significant effects on growth. If tamoxifen has any effect, it is a paradoxical one to augment growth.

There was no correlation between ploidy status and estrogen or tamoxifen effects on growth, suggesting that specific genetic changes, rather than global ploidy changes, confer hormone resistance. Also, PR regulation and growth responses to estro-
gens were often dissociated. In the subclones of Fig. 2, PR were induced by tamoxifen in V27 and suppressed by both hormones in V24, V43, and V12 (not shown, but see Fig. 3).

PR regulation by estrogen is also heterogenous in the T47Dv subclones. Fig. 3 shows four contrasting examples of PR responses. A few clones, including the HD V51, retain a classic response (39), with PR induction by estradiol and suppression by tamoxifen (Fig. 3A and see HT clone V4 in Fig. 5). As with growth, the PRs in many clones are suppressed by tamoxifen but are resistant to induction by estradiol. The predominantly HD clone, V21, is an example (Fig. 3B). Others are suppressed by both tamoxifen and 10 nM estradiol as shown by the HT clone V4 in Fig. 5). While growth of V27 can be regulated by estradiol (Fig. 2A), its PRs respond paradoxically with estradiol inhibiting, and tamoxifen stimulating, PR levels (Fig. 3D). Three other clones demonstrate this paradoxical increase in PR levels with tamoxifen.

After screening the majority of 33 T47Dv subclones for regulation of growth and PR by estradiol and tamoxifen, more detailed time-course experiments were done on selected subclones. Subclone V36 is typically resistant to the growth (Fig. 4A) or PR regulatory (Fig. 4B) effects of estrogens. Subclone V4, on the other hand, appeared to require estradiol for growth (Fig. 5A) and PR (Fig. 5B), although tamoxifen did not reverse the growth or PR stimulatory effects of estradiol. Despite the fact that it is HT, V4 grows slower than the estrogen-resistant HD line V36. Aggressiveness and tetraploidy may be related in breast tumors (see "Discussion"), but clearly the HT state does not necessarily confer growth advantage. Thus, many factors must explain why HT tumors have the poorest clinical prognosis (40). We did find that augmented growth can occur in conditions that are normally expected to be inhibitory. Subpopulations of breast cancer cells that are resistant to tamoxifen, or even stimulated to grow by tamoxifen, may explain why metastatic tumors in patients undergoing tamoxifen treatment eventually progress, often after an initial period of growth suppression.

Natural Estrogen Receptor Mutants. We postulated that the heterogenous estrogen responses of the clonal cell lines may be explained by the presence of mutant ER. As the first phase of an analysis of ER in T47Dco and its derivatives, we prepared two cDNA libraries from total or cytoplasmic poly(A)+ mRNA...
of the parental T47Dco cells (Fig. 1, Panel 1). Library I, made from total poly(A)+ mRNA, yielded two clones having wild-type ER sequences (not shown) and one clone of ~0.9 kilobases that encodes part of ER exon 2 and all of exon 3 (Fig. 6). At the precise junction between exons 3 and 4, the ER sequence homology ends and the adjacent sequence is not found in ER cDNA (33, 35), in the reported ER intron sequences (41), or in the DNA database. Compared to wild-type ER, the region immediately upstream of the exon 3/intron 3 junction has two inserted T-residues. This leads to a disruption of the protein-reading frame by generating a TGA termination codon. There are also two point mutation substitutions just distal to the insertions. Together, these mutations may be responsible for the abnormal splicing reaction observed. This cDNA would encode an ER protein truncated at aa 250 in the DBD, just beyond the last cysteine (aa 245) of the second DNA-binding finger (Fig. 7, Top). The putative protein would lack the nuclear localization signal and hormone-binding domains of ER (34, 42).

Library I also yielded a 2-kilobase clone that appears to be an RNA-processing intermediate or splicing error and contains ~1 kilobases of intron 5 linked upstream of exon 6 and three clones with an insertion in exon 5 (not shown). The insert in the three clones contains at least two blocks of direct repeats of ~130 nucleotides terminating in A residues that are 70–85% homologous to the human alu family (43, 44).

To ensure that the clones isolated from the first library did not have an overrepresentation of unprocessed nuclear mRNAs, a second library was made from cytoplasmic mRNA. In addition to clones consistent with wild-type ER, library II has yielded two other mutant cDNAs that would encode proteins with potentially important biological activity. One clone has a point deletion in the hormone-binding domain just upstream of the end of exon 5 (Fig. 8). This leads to a frame-shift and a translation termination 7 codons later (Fig. 7, Bottom). This mutant cDNA would encode an ER protein truncated in the middle of the HBD at aa 417, with a unique 7 aa COOH-terminal end. The putative protein would be unable to bind hormone or the anti-ER antibody H222 (34, 45).

Library II also yielded two independent clones of 820 and 980 nucleotides having an identical in-frame deletion (Fig. 9). These clones contain a wild-type upstream sequence from the 5′ untranslated region to the first 4 codons of exon 4. There follows a loss of 460 nucleotides, including most of exons 4 and 5. The last codon of exon 5 is preserved, as are downstream exon 6 sequences. Interestingly, the nucleotide sequences at the two borders of the deletion are identical, so that codon GAC could represent either nucleotides 1004–1006 (aa 258) of exon 4 or nucleotides 1463–1465 (aa 411) of exon 5 (33). This cDNA would encode a mutant ER of 442 aa instead of the normal 595 aa, having a 153 aa deletion from the end of the DNA-binding domain C, through the hinge region D, to the mid-hormone-binding domain E (34). The deletion originates in the sequence encoding the putative nuclear localization signal (aa 256–263; R-K-D-R-R-G-G-R) (42). However, the aa sequence encoded by the deletion mutant (R-K-D-R-N-Q-G-K) preserves 4 of the 5 basic aa residues of the wild-type sequence.

**DISCUSSION**

A great deal of work, with emphasis on the role of oncopgenes, has focused on the factors that initiate the neoplastic process (46–48). However, established tumors are not static entities but change during the course of the disease with grave consequences to the patient and formidable challenges to the physician. In breast cancer, a major sign of tumor progression is the development of resistance to hormonal treatments. The mechanisms underlying progression to hormone resistance are unknown, although the ER-negative, PR-negative phenotype in metastatic disease appears to be the conclusive stage (7).

**Genetic Instability and Hormone Resistance.** The FCM assay for simultaneous measurement of steroid receptors and DNA indices (31) allowed us to continuously evaluate the ploidy and
T47Dco Model for Hormone-Resistant Breast Cancer

PR status of several T47D sublines. In addition to the tetraploiddization described above, we have monitored the spontaneous appearance of HT populations in two other, independent, HD T47D stocks (not shown). This instability is not unique to T47D cells, since HD and HT variants of MCF-7 cells, the most widely studied breast cancer cell line, have also been reported (49, 50). We have now analyzed in detail subcloned HD and HT populations derived from the T47Dco variant, T47DV. Their extensive heterogeneity suggests that the intermediate states of hormone resistance, short of progression to the complete receptor-negative phenotype, may be quite complex. Rather than homogeneous models, T47Dco cells and other breast cancer cell lines in culture are models of heterogeneity and continued genetic change. For this reason, we plan to study the subclones of T47DV at early passages in an effort to minimize the effects of genetic drift. Some degree of heterogeneity is unavoidable, however, since ploidy changes can occur rapidly, as several of the T47DV subclones demonstrate.

Instability of T47D cells has been noted in other settings (26, 30). Reddel et al. (26) observed the formation of HT peaks in an HD estrogen-responsive T47D line and three of its subclones. In one of the stable HT clones, PR remained estrogen inducible, but growth regulation by estradiol or tamoxifen was lost, thus uncoupling the two responses. We have noted similar uncoupling in the T47DV subclones. Murphy et al. (30) described the emergence of a HD, ER- and PR-negative, estrogen-resistant line, following long-term estrogen deprivation of an HT, ER- and PR-positive, estrogen-responsive line. However, the changes appear to be reversible if the selective pressure is removed by return to an estrogenic medium.6 Their results suggest that ploidy and estrogen responsiveness may be subject to selective pressure on subpopulations of cells in culture and, perhaps, on tumors in vivo.

To encourage evolution to hormone resistance, other breast cancer cell lines have been grown in estrogen-depleted or antiestrogen-containing medium or in ovariectomized athymic mice. Results have been complex. These cells develop a phenotype that is not clearly hormone resistant. They often have rapid population doubling times and respond poorly, if at all, to the further growth stimulatory signals of estradiol. However, they contain functional ERs, are inhibited by antiestrogens, and estrogen regulation of PR or pS2 gene expression may be retained (49-54). Thus, they do not reproduce the hormone-resistant phenotype of breast cancer. Moreover, it is unlikely that estrogen withdrawal would serve as the natural selective force leading to irreversible resistance, since such tumors arise commonly in premenopausal women and there is no statistical evidence linking resistant tumors to older patients; if anything, the reverse is true (55). Clearly, tumors whose growth cannot

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Fig. 4. Effect of estrogens and antiestrogens on growth and PR levels of T47Dco HD subclone V36. Cells (1 x 10⁶) from subclone V36 were plated in duplicate T-175 flasks with CGM; 24 h later cells were refed with CGM or switched to medium containing DCC-stripped serum with no additions (OCC) or with 10 nM estradiol (E₂), 1 µM tamoxifen (TAM), or E₂ + TAM. Duplicate flasks were harvested at 6, 11, and 13 days following a 1-h treatment with 100 nM R5020. Aliquots were counted and PR quantitated in duplicate for each flask by the PR/DNA FCM assay as described before. A, mean number of cells/flask; B, mean PR-specific binding/cell in fluorescence units (PR SB/cell).

Fig. 5. Effect of estrogens and antiestrogens on growth and PR levels of T47Dco HT subclone V4. Cells from subclone V4 were plated and grown in the five media conditions as described in Fig. 4. Duplicate flasks were harvested at 7, 11, and 13 days, and growth (A) and PR (B) were quantitated as described in Fig. 4.

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6 V. C. Jordan, personal communication.
The link between genetic instability and aneuploidy is well established in other cancers. For example, the appearance of aneuploidy at a premalignant state is a cornerstone of the colorectal model of tumorigenesis, in which it is proposed that DNA hypomethylation leads to aneuploidy, while mutations in key genes lead to the malignant phenotype and, eventually, to metastasis (59). The molecular forces driving the continued chromosomal losses and rearrangements that end in rapidly proliferating aneuploid tumors are unknown; however, Schackney et al. (60) have proposed a link between growth-promoting genes and aneuploid populations. They recently described a computer model for the evolution of human solid tumors in which tetraploidy of diploid cells, followed by random chromosome loss, leading to activation of at least two growth-promoting genes (or loss of two tumor-suppressing genes) allowed distinct aneuploid populations to appear (60). This model was based on FCM and cytogenetic data from solid tumors and closely predicted the evolution of a human undifferentiated large cell carcinoma of the lung in culture (61). Thus, cell culture models may mimic closely the natural progression of solid tumors (60, 61), and the T47Dco model, and its clonal HD and HT derivatives, may serve this purpose for Stage IV breast cancers. We believe that, in breast cancer, the selective pressure leading to hormone resistance is triggered by the dual factors of genetic instability and mutations in genes that regulate growth. In breast cancers, the most important growth regulatory gene described to date is that for ER.

Genetic Instability and Aneuploidy. What is the role of aneuploidy in breast tumors? Is genetic instability in cell lines merely an artifact of culture or does it reflect tumor biology? Studies of ploidy in several stages of breast cancer suggest that aneuploidy in the primary tumor increases with malignant progression: 36% in mammographically detected lymph node-negative breast cancer, 57% in palpable lymph node-negative tumors, 71% in lymph node-positive patients, and 91% in patients with metastatic disease at the time of diagnosis (40, 56–58). Moreover, aneuploidy confers an increasingly poor prognosis. Compared to diploid tumors, HD primary tumors impart a 2- to 3-fold increased risk of death, and HT primary tumors impart a 3- to 5-fold increase (40).

Fig. 6. DNA sequence of wild-type ER cDNA and a frame-shift/translation termination mutant. Sequences (5' bottom to 3' top) of wild-type MCF-7 ER cDNA (33, 35) and of a clone isolated from T47Dco library I. *, inserted or mutated residues. Insertion of two T-residues (arrowheads) generates a TGA stop codon in the T47D clone. The exon 3 boundary [nucleotide 990/991: (33, 41)] is also marked. Sequences 3' of the boundary in the T47D clone are not found in exon 4 of wild-type ER cDNA (33, 35) or in intron 3 (41).

Fig. 7. Nucleotide and amino acid sequences at the translation termination sites of two mutant ER cDNAs compared to wild-type (W.T.) ER. Bold type, inserted or substituted nucleotides and mutant amino acids. Top, the DBD mutant shown in Fig. 6. Cys 245 in the DBD mutant is the last cysteine of the second DNA-binding finger. Bottom, the HBD mutant shown in Fig. 8. *, deleted G residue [nucleotide 1463; (33)] that occurs just before sequences encoding 7 unique aa, followed by a stop codon, as shown in bottom.

be stimulated by estradiol, but can be suppressed by tamoxifen, should not be classified as hormone resistant. Such tumors are modeled by clone V24 (Fig. 2B).

Fig. 8. DNA sequence of wild-type ER cDNA and a frame-shift/translation termination mutant. Sequences (5' bottom to 3' top) of cDNA from wild-type T47Dco ER cDNA and a mutant clone, both isolated from library II, are shown. Arrow and *, the deleted G residue [nucleotide 1463; (33)] that occurs just before the exon 5/exon 6 boundary. The deletion results in a frame-shift, leading to sequences encoding 7 unique aa, followed by a stop codon, as shown in bottom, Fig. 7.
Wild Type  

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<td>4</td>
<td>HORMONE</td>
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DBD  

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In-frame Deletion  

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but lack all or part of the hormone-binding domain, rendering them incapable of binding estradiol. Similar natural ER mutants have escaped detection in breast cancers. The explanation may lie in the protein itself: a hormone-binding domain mutant would be undetected by ligand binding and would be falsely classified as ER negative. Additionally, it would be undetected by H222, the anti-ER antibody currently in clinical use, whose epitope is in the hormone-binding domain (34, 45). Thus, other methods must be used to search for ER mutants. Recently, several groups have suggested the existence of variant ERs in breast cancers based on restriction fragment length polymorphism analysis of the ER gene (8) and on detection of structurally abnormal (16, 62) or truncated (17) ER mRNAs. However, given the lack of suitable antibodies, no group has yet detected the abnormal proteins.

We have now isolated and sequenced several natural mutant ER cDNAs from T47Dco cells, whose putative protein structures are shown in Fig. 10. We do not know whether the abnormal proteins are expressed. However, based on deletion mutagenesis analyses (22, 34), we can begin to predict the consequence to the cells of such mutant ERs. Especially in the HT subpopulations, which contain 4–5 alleles of the ER gene (29), cells having a mixture of wild-type and mutant receptors could exist. Heterodimers of the wild-type and mutant monomers, having dominant positive or dominant negative activity (63), could override the estrogen requirement of the wild-type receptor. This would result in ER-positive but estrogen-resistant cells, a phenotype that describes 50% of hormone-resistant breast cancers.

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