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Use of Chromatofocusing to Distinguish Estradiol Receptor from Ovarian-dependent and -independent Rat Mammary Tumors[1]

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ABSTRACT

Cytosol estradiol receptor from MTW9-D (ovarian dependent) and MTW9-MtT (ovarian independent) rat mammary tumors were fractionated by chromatofocusing, a procedure which separates proteins on an ion-exchange column as a function of isoelectric point. Receptor from MTW9-D usually fractionated as three peaks with mean pH at peak height of 7.5, 6.8, and 6.0. The intermediate peak at pH 6.8 was present in 90% of MTW9-D tumors examined but in only 20% of MTW9-MtT tumors. Treatment of cytosols with 20 mM sodium molybdate or 50 mM leupeptin did not change the chromatofocusing profiles.

The pI 6.8 fraction of receptor bound quantitatively to DNA-cellulose after ammonium sulfate precipitation, while receptor in the other two peaks bound much less. The quantitative binding of a fraction of estradiol receptor characteristic of MTW9-D to DNA is consistent with the greater binding of unfractionated cytosol receptor from MTW9-D to DNA than the binding of receptor from MTW9-MtT. Sucrose gradient analysis of the pI 6.8 receptor showed a sedimentation coefficient slightly less than ovalbumin with a Stokes radius of 26 Å as determined by agarose chromatography. The correlation of receptor binding to DNA with response to ovariectomy might make this form of receptor a potential marker of hormonal responsivity in mammary tumors.

INTRODUCTION

Rat mammary tumor MTW9 can be grown in an ovarian-dependent form, MTW9-D,[3] or an ovarian-independent form, MTW9-MtT (11). We have shown that both tumors have ER, that ER from the ovarian-dependent MTW9-D binds significantly more to DNA-cellulose than does the ER from the ovarian-independent MTW9-MtT, and that the difference is related to the capacity of the tumor to show ovariectomy-induced regression (16). The binding of ER to DNA is largely electrostatic, and study of the molecular charge of the receptors should aid in understanding the reaction of ER with DNA. Chromatofocusing is an ion-exchange column procedure which separates proteins largely on the basis of isoelectric point. Proteins are eluted from the resin at, or close to, their isoelectric point by means of a linear pH gradient elution procedure (28, 29). In this report, we demonstrate that the chromatofocusing patterns of ER from the ovariectomy-responsive and -resistant tumors are different and that this difference derives largely from a fraction of receptor from MTW9-D which binds quantitatively to DNA.

MATERIALS AND METHODS

Chemicals. [^{3}H]Estradiol (137 Ci/mmol) was obtained from New England Nuclear. DNA-cellulose (400 µg/ml) was prepared by the method of Alberts and Herrick (1) using calf thymus DNA (P-L Biochemicals, Inc.) and Cellex grade cellulose (Bio-Rad Laboratories). Norit A charcoal (Fisher Scientific Co.) and dextran T-70 (Pharmacia) were used to prepare DCC as described (13). Chromatofocusing reagents were obtained from Pharmacia. Testosterone, estradiol, progesterone, cortisol, and diethylstilbestrol were purchased from Sigma Chemical Co. Leupeptin was a gift from the USA-Japan Cooperative Cancer Research Program.

Standards used for sucrose gradients and agarose columns were: bovine thyroid thyroglobulin, horse spleen ferritin, and bovine liver catalase (Pharmacia); horse heart myoglobin (Sigma); ^{14}C-bovine serum albumin, ^{14}C-hen ovalbumin, and [^{14}C]alanine (New England Nuclear).

Animals. Female W/Fu rats were used as hosts for mammary tumor MTW9 and the mammosomatotropic tumor MtTW10. The transplantation and growth of hormonally sensitive (MTW9-D) and resistant (MTW9-MtT) tumors have been described (10, 11).

Preparation of ER. When mammary tumors were 1.5 to 2.0 cm in diameter, the rats were decapitated, the tumors rapidly removed and trimmed of fat and connective tissue, and the tumor tissue finely minced and washed with 4 volumes of Buffer A [20 mM Tris-30 mM KCl-1 mM EDTA-1 mM mercaptoethanol (pH 7.4) at 20°]. The tissue was pelleted, resuspended in 2 volumes Buffer A, and homogenized with 3 × 3-sec bursts of a Polytron 10ST homogenizer (Brinkmann Instruments). In some experiments, Na_2MoO_4 or leupeptin (final concentration, 20 and 50 mM respectively) were added to the homogenized tissue. The homogenates were then centrifuged at 190,000 × *g* for 30 min. The supernatants were made 10 nM in [^{3}H]estradiol with and without the addition of 100-fold excess nonradioactive estradiol and incubated for 90 min at 0°. The labeled cytosol was then incubated with a pellet of DCC (from 2 volumes of DCC suspension) for 10 min at 0°, and the suspension was recentrifuged. The labeled supernatants were used for chromatofocusing and measurement of DNA binding.

Binding of ER to DNA. Cytosolic ER was made 25% saturated in ammonium sulfate by addition of an appropriate volume of saturated ammonium sulfate solution, at pH 7.8. After 30 min, the suspension was centrifuged at 10,000 × *g* for 20 min, and the pellet was resuspended in Buffer B (Buffer A plus 0.2% bovine serum albumin and 10% glycerol). The preparation was recentrifuged to remove insoluble material, and the resulting solution was analyzed for DNA binding by the procedure of Yamamoto and Alberts (36) with some modification (13).

Chromatofocusing. The procedure was adapted from Sluyterman *et al.* (28, 29) with the guidelines in the Pharmacia brochure (23). All operations were done at 5°. The exchange resin PBE 94 was equilibrated with starting buffer (0.025 M Tris-acetate, pH 8.3). Elution buffer [70% (v/v) Polybuffer 74 and 30% Polybuffer 94 adjusted to pH 5.0 with acetic acid] was adsorbed into the column [0.7 x 32 cm], the sample of ER was applied in a volume of 1 to 3 ml, and the column was developed

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[3] The abbreviations used are: MTW9-D, a transplantable mammary carcinoma MTW9 with growth supported by administration of the dopamine antagonist drug No. R 6,366 (Janssen Pharmaceutica Inc.); MTW9-MtT, a transplantable mammary carcinoma MTW9 with growth supported by coimplantation of the mammosomatotropic tumor MtTW10; ER, estrogen receptor; DCC, dextran-coated charcoal; pI, isoelectric point.

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with elution buffer at a flow rate of 35 to 40 ml/hr. Fractions of 2 ml were collected, and aliquots were used for measurement of radioactivity, pH, and protein; proteins were estimated by absorbance (A_{260}/A_{280}) because ampholytes interfere with the Lowry colorimetric procedure. In most experiments, 3 ml cytosol containing 33 to 45 mg protein were applied, and about 70 to 80% was recovered in the separating region of the gradient (pH 8.0 to 5.5). Recovery of radioactivity applied to the column was about 65% in the pH 8.0 to 5.5 region.

Following chromatofocusing of ER, peaks of radioactivity were collected at 0°, made 0.02% in bovine serum albumin, and adjusted with NaOH to pH 7.8, and solid ammonium sulfate was added to 80% saturation. The precipitated proteins were kept at 0° overnight (at least 12 hr) and centrifuged at 10,000 × *g* for 20 min, and the supernatants were discarded. The pellets were resuspended in Buffer A, which was 30% saturated with ammonium sulfate, and the suspension was recentrifuged. The resulting pellets were dissolved in Buffer B, centrifuged to remove insoluble material, and analyzed for binding to DNA-cellulose as described above.

RESULTS

Chart 1 shows a typical chromatofocusing pattern of ER from the ovariectomy-responsive MTW9-D. The top line shows the excellent linearity of pH in the range pH 8.0 to 5.5 when effluent pH is plotted against fraction number. In this separating range, 3 peaks of radioactivity, with pH at peak height of 7.7, 7.0, and 6.0, are seen. The dotted line shows the radioactivity in fractions from the same ER labeled in the presence of excess nonradioactive hormone. The first peak is suppressed about 70%, and Peaks 2 and 3 are entirely suppressed. There is always some radioactivity in fractions collected before the linear gradient forms. In this experiment, the amount is small and represents mostly nonspecific binding. Material emerging outside the linear gradient has not been studied. Chart 2 shows a typical study of the ovarian-independent tumor MTW9-MtT. Peak 1 is similar to that seen in Chart 1, but Peak 2 is absent, and Peak 3 is much smaller; radioactivity in Peak 3 is specifically bound. When cytosols from either tumor are incubated with [^{3}H]estradiol in the presence of 100-fold excess of nonradioactive testosterone, progesterone, or cortisol, no suppression of binding is observed; however, diethylstilbestrol suppresses binding to the same extent as estradiol. Scatchard analysis of estrogen binding in cytosols shows only one class of high-affinity sites (K_d 2.0 to 4.0 × 10^{-10} M).

Table 1 shows the mean and standard deviation of pH for the 3 peaks. Peak 1 ER from both tumors emerges at pH 7.6 and was present in every experiment. Peak 2 was present in 18 of 20 MTW9-D and in 2 of 10 MTW9-MtT tumors. When Peak 2 was present in a MTW9-MtT tumor, it showed a mean pH at peak height of 6.8, identical to that from MTW9-D. Peak 3 shows a small but significant difference ($p < 0.001$) in mean pH between the 2 tumors; the difference is so small (5.98 *versus* 6.07) that it is difficult to assess. When individual peaks were isolated and DCC treated, only 9.5 ± 3.4% (S.D.) (Peak 1), 30.4 ± 1.8% (Peak 2), and 20.0 ± 2.0 (Peak 3) of total radioactivity was recovered; this large loss of steroid binding is indicative of the instability of partially purified steroid receptors (5, 8). When the

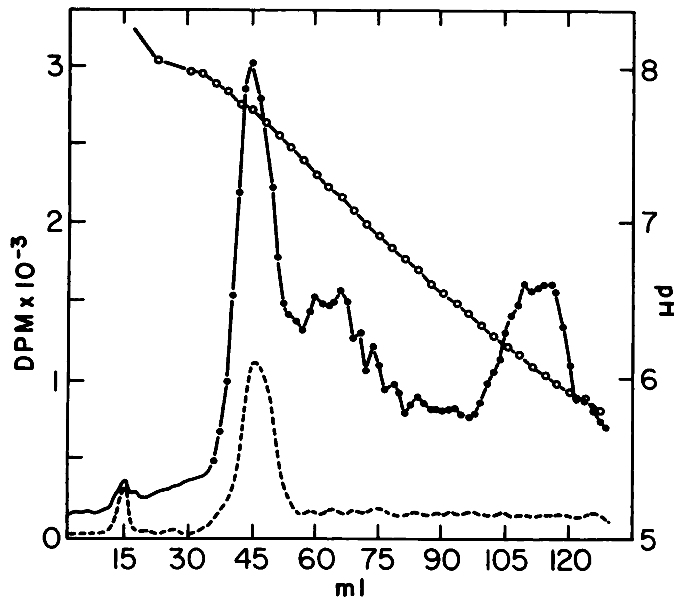

Chart 1. Chromatofocusing of [^{3}H]ER from MTW9-D cytosol. Cytosol (1 ml) from MTW9-D (13.7 mg protein per ml) was labeled with [^{3}H]estradiol in the presence (- - -) or absence (●) of 100-fold excess of nonradioactive estradiol. After DCC treatment to remove unbound steroid, the preparations were chromatofocused as described. Fractions (2 ml) were collected, and pH (O) and radioactivity were measured in each fraction. Total bound steroid equals 210,000 dpm/ml.

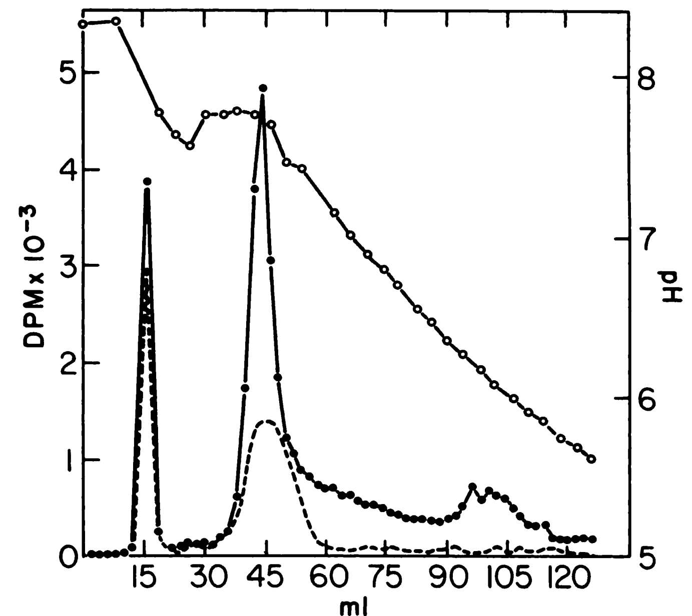

Chart 2. Chromatofocusing of [^{3}H]ER from MTW9-MtT cytosol. Cytosol (1 ml) from MTW9-MtT (12.1 mg protein per ml) was labeled with [^{3}H]estradiol in the presence (- - - -) or absence (●) of 100-fold excess of nonradioactive estradiol. After DCC treatment to remove unbound steroid, the preparations were chromatofocused as described. Fractions (2 ml) were collected, and pH (O) and radioactivity were measured in each fraction. Total bound steroid equals 149,000 dpm/ml.

Table 1

Mean pH at peak height of chromatofocused ER

pH values of fractions were measured at room temperature.

Peak	MTW9-D	MTW9-MtT
1	7.52 ± 0.13[a] (20/20)[b]	7.60 ± 0.08 (10/10)
2	6.84 ± 0.21 (18/20)	6.89 ± 0.35 (2/10)
3[c]	5.98 ± 0.05 (20/20)	6.07 ± 0.05 (10/10)

[a] Mean ± S.D. of pH at the height of each of 3 chromatofocusing peaks.

[b] Numbers in parentheses, number of times the peak was observed per total number of experiments.

[c] Difference between tumors for Peak 3 is $p < 0.001$.

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DCC-treated peaks are rechromatofocused, the radioactivity emerges at the expected pI. Treatment of MTW9-D and MTW9-MtT homogenates with 50 mM leupeptin or 20 mM sodium molybdate did not change the chromatofocusing profiles; MTW9-D retained Peak 2, which was absent in MTW9-MtT (data not shown).

The chromatofocused ER from MTW9-D was analyzed for DNA binding activity (Table 2). The binding of Peak 2 ER to DNA is quantitative; the binding of Peak 1 or 3 ER is less than 20%. Chart 3 shows the relationship between DNA concentration and binding of Peak 2 ER. Since MTW9-MtT lacks Peak 2 ER, one would predict that activated receptor preparations from this tumor would have a more limited ability to bind to DNA, and indeed this is the case. When crude cytosol ER was analyzed for DNA binding activity, 21.8% of ER from MTW9-D (S.D. 6.2; $n = 8$) could bind in contrast to only 11.8% of MTW9-MtT receptor (S.D. 2.4, $n = 5$) ($p < 0.001$).

Charts 4 and 5 show the sucrose gradient and agarose chromatography profiles of Peak 2 ER from MTW9-D tumor. The sedimentation coefficient for this material is slightly less than ovalbumin (3.5*S*), and the Stokes radius is 26.0 ± 0.3 Å.

DISCUSSION

Chromatofocusing is a useful tool for separation of proteins on the basis of molecular charge. The procedure is rapid, does not give extremes in pH or temperature, and can accommodate cg quantities of protein and cM concentrations of electrolyte. Simpson and DeLuca (27) used the procedure for characterization of partially purified steroid receptor, and Bjork *et al.* (4) used the procedure for separation of a human prostatic estramustine-binding protein. In the present study, chromatofocusing was used to separate ER from MTW9 cytosol into 3 components which migrated at pH 7.5, 6.8, and 6.0. These peaks most likely represent specific estrogen binding to receptor, and not to some other protein(s), on the basis of steroid specificity, and high affinity for estrogens.

Table 2

Binding of peak fractions of chromatofocused ER from MTW9-D to DNA

Binding to DNA-cellulose was measured as described under "Materials and Methods."

Peak	% of ER bound
1	18.2 ± 15.0[a]
2	100
3	16.9 ± 7.5

[a] Mean ± S.D. of 3 experiments.

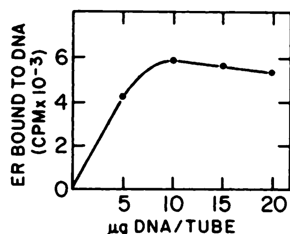

Chart 3. Binding of Peak 2 ER to DNA cellulose as a function of DNA concentration. Peak 2 ER from chromatofocusing columns was activated by precipitation with $(NH_4)_2SO_4$ as described. Aliquots (200 µl) were then incubated for 20 min at 20° with 150-µl DNA-cellulose suspension containing varying concentrations of DNA. After the incubation, the DNA-cellulose pellets were thoroughly washed with buffer, and bound radioactivity was extracted with ethanol (13). DNA-bound ER was estimated by subtracting the cpm bound to plain cellulose (background) from the cpm bound to DNA-cellulose (total binding). Input was 4,760 cpm. *Points* average of 2 determinations.

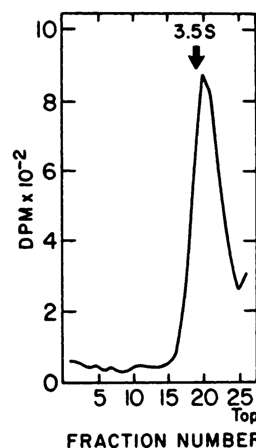

Chart 4. Sucrose gradient analysis of Peak 2 ER. Peak 2 ER was collected from chromatofocusing columns, and 10% glycerol, 1 mM mercaptoethanol, and 10 mM sodium molybdate were added to the preparation; the pH was then adjusted to 7.8 to 8.0, and 0.2-ml aliquots were applied to linear 5 to 20% sucrose gradients made up in 0.025 M Tris-acetate buffer, pH 8.3, containing 10% glycerol, 1 mM mercaptoethanol, and 10 mM sodium molybdate. Gradients were run at 44,000 rpm for 18 hr in an SW 50.1 rotor; following the run, tubes were punctured, and 3-drop fractions were collected and analyzed for radioactivity. ^{14}C-ovalbumin (3.5S) was added to samples as an internal marker.

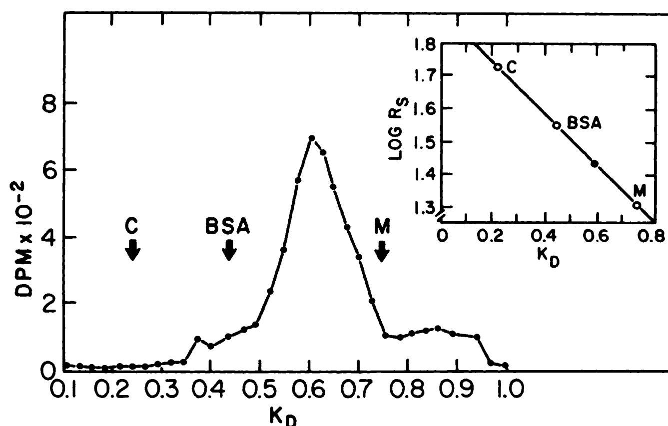

Chart 5. Gel filtration of Peak 2 ER. Peak 2 ER was collected from chromatofocusing columns, and 10% glycerol, 1 mM mercaptoethanol, and 10 mM sodium molybdate were added to the preparation; the pH was then adjusted to 7.8 to 8.0, and aliquots (usually 2 ml) were analyzed on agarose 1.5 m (100 to 200 mesh). The agarose column (1.7 x 80 cm; flow rate 13 ml/hr) was equilibrated with 0.025 M Tris-acetate buffer, pH 8.3, containing 10% glycerol, 1 mM mercaptoethanol, and 10 mM sodium molybdate. The void volume (V_0) was determined with blue dextran 2000; total volume (V_t) was taken as the elution volume of [^{14}C]alanine. The column was calibrated with catalase (*C*) (51.3 Å), bovine serum albumin (*BSA*) (35.9 Å), and myoglobin (*M*) (20.1 Å). K_d of standard proteins was defined as ($V_e - V_0$) divided by ($V_t - V_0$), where V_e is the elution volume of the standard (26). *Inset:* ●, position of ER.

The experiments described here also show that ER from ovariectomy-responsive MTW9-D and ovariectomy-resistant MTW9-MtT are different; while both tumors have receptors which focus at pH 7.5 and 6.0, ER from the former tumor also contains a component which focuses at pH 6.8 which is usually absent in ER derived from the latter tumor. The quantitative binding of this component to DNA is consistent with the greater binding of ER from MTW9-D cytosol to DNA (16).

To determine whether the difference in ER content might be

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due to a difference in proteolytic activity between the tumors, 20 mM molybdate or 50 mM leupeptin were added to tumor homogenates; no effect of these protease inhibitors were obtained on the chromatofocusing profiles. Moreover, sucrose gradient analysis of MTW9-D and MTW9-MtT cytosols showed that both tumors contained a major receptor component sedimenting at 9.7S as well as a 4S species (9, 12); the size distributions on agarose chromatography were also similar.[4] Since proteases tend to cleave ER to smaller fragments (25, 26), it is unlikely that differences in protease activity in the 2 tumors are responsible for the difference in ER content.

The separation of several specific estradiol binding proteins in MTW9 by chromatofocusing must be compared to data from isoelectric focusing separations. Two species, with pIs of 6.8 and 7.3, were described in oviduct (17), 2 (6.6, 6.8) in calf uterus (21, 22), 3 (5.6, 5.8, 6.5) in human myometrium (24), but only one (5.8) in rat uterus (20). dimethylbenz(*a*)anthracene rat mammary tumors were found to have ER with pI of 6.4 and 6.8 (2), and we have determined the pI of ER in MTW9-D to be 5.6 and 6.3 by isoelectric focusing in gel. Isoelectric focusing of human mammary tumor ER showed specific binding proteins with pI of 5.9 to 6.1 and 6.5 to 6.7; trypsinization eliminated the more acidic component (34). Wittliff (32) has also found multiple forms of ER in human breast cancer. It remains to be determined whether the different values obtained by these various laboratories represent tissue and/or species differences or differences in experimental conditions.

The steroid binding locus of receptors is distinct from the DNA-binding site (3, 13, 15, 18, 35). Therefore, one might predict that some steroid receptors found in nature might be physiologically inactive because of altered interaction with the genome. Indeed, Yamamoto *et al.* (37) described lymphoid tumor cells resistant to the cytolytic action of glucocorticoid, the corticoid receptor of which failed to translocate or translocated to a greater degree than receptor from steroid-sensitive cells. Stevens and Stevens (30) demonstrated that glucocorticoid receptor from steroid-sensitive murine lymphoma P1798 eluted more readily from tumor nuclei or DNA-cellulose than did receptor from steroid-resistant tumor. It has also been shown that androgen receptor from normal mouse kidney binds differently to DNA-cellulose and has a different pI than does the apparently inactive androgen receptor from mice with the feminizing testis syndrome (14).

We have found that resistance of MTW9 to ovariectomy-induced regression depends on the presence of a coimplanted MtTW10; responsivity can be reversibly controlled, and binding of ER to DNA varies *parri passu* with the capacity for ovariectomy response (16). Biological response and binding of receptor to DNA are related in this system. In this regard it is interesting to note the work of Boyd and Spelsberg (5–7), who find 2 components in chick oviduct progesterone receptor having pI values of 6 and 7. The level of the pI 7 component varied seasonally, being much lower in progesterone receptor preparations isolated during the winter; this fluctuation correlated nicely with the observed seasonal changes in receptor binding to chromatin both *in vivo* and *in vitro* and the effect of progesterone on RNA polymerase II activity. The pI 7 component appears identical to the DNA-binding A subunit of the progesterone receptor (8). The size of this protein (sedimentation coefficient slightly less than ovalbumin (5), as well as the pI, make it very similar physically to Peak 2 ER from MTW9. The small molecular size of Peak 2 ER (<3.5S, 26 Å) is consistent with results obtained on DNA-binding subunits of receptors in other systems as well (30, 31, 33).

In crude cytosols of MTW9-D, ER exists predominantly as a 9.7S entity. Chromatofocusing of this molecule shows the presence of all 3 peaks in varying amounts;[4] in contrast, 4S ER chromatofocuses as Peak 1 and Peak 3. It has been proposed that the 4S ER in crude mammary tumor cytosol is biologically inactive, and that only the large form (*i.e.*, 8S) is capable of promoting estrogen action (19, 32). Our results, showing an absence of the DNA-binding component in 4S material, tend to support this concept. Moreover, the finding that Peak 2 ER is consistently observed in MTW9-D but not MTW9-MtT suggests that this molecule may be a useful marker of hormonal responsivity.

[4] A. H. Coufalik, M. Feldman, M. Platica, L. Toth, D. Dreiling, and V. P. Hollander, unpublished observations.

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