Proteins Associated with Untransformed Estrogen Receptor in Vitro. Perturbation of Hydrophobic Interactions Induces Alterations in Quaternary Structure and Exposure of the DNA-Binding Site†

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ABSTRACT: Estrogen receptors from calf uteri have been analyzed by high-performance size-exclusion chromatography, chromatofocusing, and DNA affinity chromatography using conditions designed to evaluate the relative contribution of hydrophobic interactions between the steroid-binding subunit and other receptor-associated proteins. The single large (untransformed) species of soluble estrogen–receptor complex consistently ($n = 9$) found in calf uteri displayed a rapid change in Stokes radius from 8.0 to 3.5 nm upon exposure to elevated ionic strengths (0.4 M KCl). However, equilibration of the estrogen–receptor complex into urea (up to 6 M) did not dissociate the untransformed receptor into the 3.5-nm receptor form (subunit) observed in hypertonic (0.4 M KCl) buffers. Exposure to 6 M urea did result in conversion of the untransformed receptor (8.0 nm) to a 6.0–6.5-nm receptor form not previously observed in either hypotonic or hypertonic buffers. In the presence of both 6 M urea and 0.4 M KCl, the untransformed estrogen–receptor complex was converted to a smaller receptor form intermediate in apparent size (4.5–5.0 nm) to that observed in 6 M urea or 0.4 M KCl alone. The formation of this 4.5–5.0-nm receptor form was partially estrogen dependent as determined by parallel analyses of unliganded receptor in urea/KCl buffer. The urea-induced change in apparent size (8 nm to 6.0–6.5 nm) at low ionic strength was accompanied by little or no detectable change in net surface charge as determined by chromatofocusing but a complete exposure of the DNA-binding site as evidenced by nearly *quantitative* interaction with DNA–agarose. We conclude from these studies that hydrophobic-type interactions within or between the steroid-binding subunit and other receptor-associated proteins are significant for receptor structure and function as defined in vitro and may reflect the mechanism and potential for as yet undisclosed biospecific associations in vivo.

A significant gap in our current understanding of receptor-mediated steroid hormone action is related to the structure of the native receptor form. Indeed, the native receptor configuration remains unknown for each class of steroid hormone receptor (Sherman & Stevens, 1984; Grody et al., 1982; Sherman et al., 1983; Joab et al., 1984), including the estrogen receptor (Muller & Traish, 1986; Greene & Press, 1986; Dunaway et al., 1985; Hutchens et al., 1985; Sakai & Gorski, 1984). There is a disparity between estimates of steroid receptor size determined under nondenaturing, "stabilizing" conditions (300 000–320 000 daltons) and structure-dissociating or denaturing conditions (70 000–115 000 daltons). The potential biological significance of these differences is currently the focus of considerable attention. Many quaternary receptor structures have been proposed [e.g., see Sherman and Stevens (1984) and Grody et al. (1982)]. Several current models of receptor structure recognize the possibility of both homologous and heterologous components or subunit compositions. These ideas are experimentally based, to a large extent, on observed variations in size of the steroid–receptor complex associated only with altered ionic strengths. There has been particular interest in those ionic strength dependent changes in quaternary structure associated with the acquisition of high(er) affinity for DNA or other nuclear acceptor sites [e.g., see Dahmer et al. (1984) and Milgrom (1981)].

While the effects of increased ionic strength on receptor size continue to be evaluated, the relative contributions of hydrophobic interactions toward the maintenance of any receptor subunit organization have been virtually ignored. We have utilized high-performance size-exclusion chromatography and chromatofocusing to investigate rapid alterations in receptor size and surface charge associated with exposure to increasing concentrations of urea in both hypotonic and hypertonic buffer systems. Urea was chosen for the these studies because chaotropic ions of the Hofmeister series (Hofmeister, 1888) are known to influence protein structure and intermolecular associations by disrupting not only hydrophobic interactions but also electrostatic interactions (Sawyer & Puckridge, 1973). Also, we have found the estrogen–receptor interaction to be relatively stable in high concentrations (up to 6 M) of urea (Hutchens et al., 1987). We now report physicochemical evidence for two structurally and functionally distinct receptor forms, the generation of which is induced by varying the receptor's hydrophobic environment. This process appears to be partially steroid dependent. Since the urea-induced alterations in receptor size/shape described here were accompanied by a complete acquisition of high affinity for DNA, these data provide new insights into receptor structure and the mechanism of steroid-induced receptor transformation.

Experimental Procedures

Materials. Altex TSK-3000SW (30-nm pore size) and Bio-Sil TSK-400 (40-nm pore size) high-performance size-exclusion columns (600 mm × 7.6 mm i.d.) were purchased from Beckman Instruments and Bio-Rad Laboratories, re-

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spectively. Bio-Gel HT hydroxylapatite was from Bio-Rad Laboratories. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer) was from Research Organics Inc. 17β-[^{3}H]Estradiol (90–100 Ci/mmol) was purchased from New England Nuclear. Trizma (Tris) base, diethylstilbestrol (DES), dithiothreitol, double-stranded calf thymus DNA–cellulose (5–6 mg of DNA/g of DNA–cellulose), and glycerol were from Sigma Chemical Co. Disodium ethylenediaminetetraacetic acid (Na_2-EDTA) and Tween 80 detergent were from Fisher Scientific. Sephadex G-25 (PD-10) columns (prepacked), Polybuffer Exchanger 94 (PBE 94), Polybuffers 96 and 74, and Blue-dextran 2000 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ultrapure urea and single-stranded calf thymus DNA–agarose (0.66 mg of DNA/mL of gel) were obtained from Bethesda Research Laboratories. All buffer solutions were freshly prepared, filtered through HVLP filters (0.2-μm pore size; Millipore), and degassed just before use.

Source of Receptor. Uteri from small, immature calves were obtained from a local abattoir. Uteri were rinsed immediately after removal in ice-cold saline, cut into 1-g pieces, frozen in liquid nitrogen (or on solid CO_2), and stored frozen at −85 °C.

Preparation of Cytosol and Labeling of Estrogen-Binding Proteins. All procedures were performed in a cold room at 0–6 °C. Pieces of frozen uteri were minced before being homogenized in 2 volumes of 10 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) buffer (pH 7.4 at 0 °C) containing 1.5 mM Na_2-EDTA, 1 mM dithiothreitol (DTT) and 20% glycerol (by volume). Cytosol (18–22 mg of protein/mL) was obtained by high-speed centrifugation (>100000*g*; 60 min) of the homogenate and labeled at 0 °C with 5–10 nM 17β-[^{3}H]estradiol in the presence (nonspecific binding) and absence (total binding) of a 100-fold molar excess of the radioinert competitor DES. At various times before high-performance size-exclusion chromatography, radiolabeled steroid–protein complexes were quickly (<5 min) separated from excess free steroid and equilibrated into different buffers by rapid chromatography on small columns (9 mL) of G-25 Sephadex (Pharmacia PD-10 columns).

Buffers Utilized. The cytosol receptor preparations were equilibrated into various different buffers for ligand-binding evaluations (Scatchard, 1949; Braunsberg & Hammon, 1980), size-exclusion chromatography, and DNA affinity chromatography. The buffers utilized for these investigations are referred to as follows: control buffer, 50 mM potassium phosphate (pH 7.4 at 0 °C), 1 mM dithiothreitol, 1.5 mM Na_2-EDTA, and 10% (w/v) glycerol; KCl buffer, control buffer containing 0.4 M KCl; urea buffer, control buffer containing 6 M urea; urea/KCl buffer, control buffer containing both 6 M urea and 0.4 M KCl.

All buffers containing urea were freshly prepared (0–6 °C) for each experiment using precautions to prevent cyanate formation (Stark et al., 1960). Different buffer systems were employed for chromatofocusing.

High-Performance Size-Exclusion Chromatography. High-performance size-exclusion chromatography (HPSEC) was performed at 0–6 °C using a Beckman Model 110A high-performance liquid chromatography (HPLC) pump, an Altex TSK-3000SW column, and a Bio-Sil TSK-400 column each equipped with in-line frit filters (Supelco) and a 10-cm guard column. Where noted, cytosolic estrogen-binding proteins prelabelled with 17β-[^{3}H]estradiol were cleared of excess free 17β-[^{3}H]estradiol and introduced into the appropriate HPSEC buffer by rapid chromatography on small (9 mL) Sephadex G-25 columns. Unliganded estrogen receptor preparations were treated similarly only in the absence of 17β-[^{3}H]estradiol. Sample aliquots (500 μL) were injected with Rheodyne 7125-S injectors, and 1.0-min fractions were collected at 0.38 mL/min using a Pharmacia Frac-100 fraction collector. The elution profile of unliganded receptor was determined by postlabeling collected fractions with 10 nM 17β-[^{3}H]estradiol in the presence and absence of a 100-fold molar excess of DES. Protein-bound estradiol in each fraction was then quantified by hydroxylapatite adsorption (Erdos et al., 1970; Pavlick & Coulson, 1976) as modified and described by us previously (Dunaway et al., 1985).

The columns (TSK-3000SW and TSK-400) were equilibrated with HPSEC buffer [50 mM potassium phosphate buffer (pH 7.4 at 0 °C) containing 1.5 mM Na_2-EDTA, 1 mM DTT, and 10% glycerol] including either 0.4 M KCl or 6 M urea or both 0.4 M KCl and 6 M urea as indicated. Column void volumes (V_0) were determined by exclusion of Blue-dextran 2000. The columns were calibrated with the following purified proteins (Sigma Chemical) of known Stokes radii [e.g., see Sherman et al. (1980, 1984) and Edelhoch (1960)]: thyroglobulin (8.6 nm), ferritin (6.15 nm), catalase (5.13 nm), aldolase (4.5 nm), IgG (4.4 nm), bovine serum albumin (3.59 nm, ovalbumin (2.86 nm), myoglobin (2.0 nm), and cytochrome *c* (1.8 nm). Protein elution was monitored by using a Beckman Model 153 analytical UV detector (260 nm) with an 8-μL flow cell and an Omniscribe D5000 strip chart recorder (Industrial Scientific). The retention time of small molecules eluting in the inclusion volume was determined by using β-mercaptoethanol, cyanocobalamin (vitamin B_{12}), and 17β-[^{3}H]estradiol. Radioactivity was determined at 26–35% efficiency) by scintillation counting using a Beckman Model LS 250 scintillation counter. The columns were frequently cleaned with 1 M KCl and stored in methanol when not in use. Column calibration was monitored periodically for each separate equilibration buffer, and plots of log Stokes radii vs. retention time (or elution volume) were linear with correlation coefficients (*r* values) typically better than 0.97.

Chromatofocusing. The development and details of both high-performance and conventional (minicolumn) chromatofocusing procedures for the analysis of estrogen receptor surface charge heterogeneity have been described previously (Hutchens et al., 1983, 1984, 1985, 1986c). The present investigation utilized small (3–5 cm × 0.7 cm i.d.) open columns of Pharmacia PBE 94 as the stationary phase. The PBE 94 columns were equilibrated to pH 8.0 by using 25 mM Tris-HCl buffer containing 1 mM dithiothreitol, 10% (v/v) glycerol, and 2–6 M urea. Chromatography was performed in a cold room (4–6 °C), and buffer pH was adjusted and measured at 0–4 °C. Internal pH gradient formation was initiated by using either commercially available polyampholytes (Polybuffers 96 and 74) or our nonpolymeric focusing buffer exactly as described previously (Hutchens, 1986; Hutchens et al., 1986b,c) except for the presence of urea. Eluate fractions (1 min) were collected at a flow rate of 0.6–1.0 mL/min. Only previously unutilized PBE 94 was used for these experiments.

DNA Affinity Chromatography. All procedures were performed in a cold room at 0–6 °C. Both DNA–cellulose (Alberts & Herrick, 1971) and DNA–agarose were routinely used to monitor transformed (DNA-binding) receptor status. Columns (0.9–1.5 cm i.d.) were packed with affinity gel to a bed volume of 2–3 mL. Receptor samples (0.5–2 mL) were applied to DNA affinity columns equilibrated in either control buffer or urea buffer as indicated. After unbound sample was

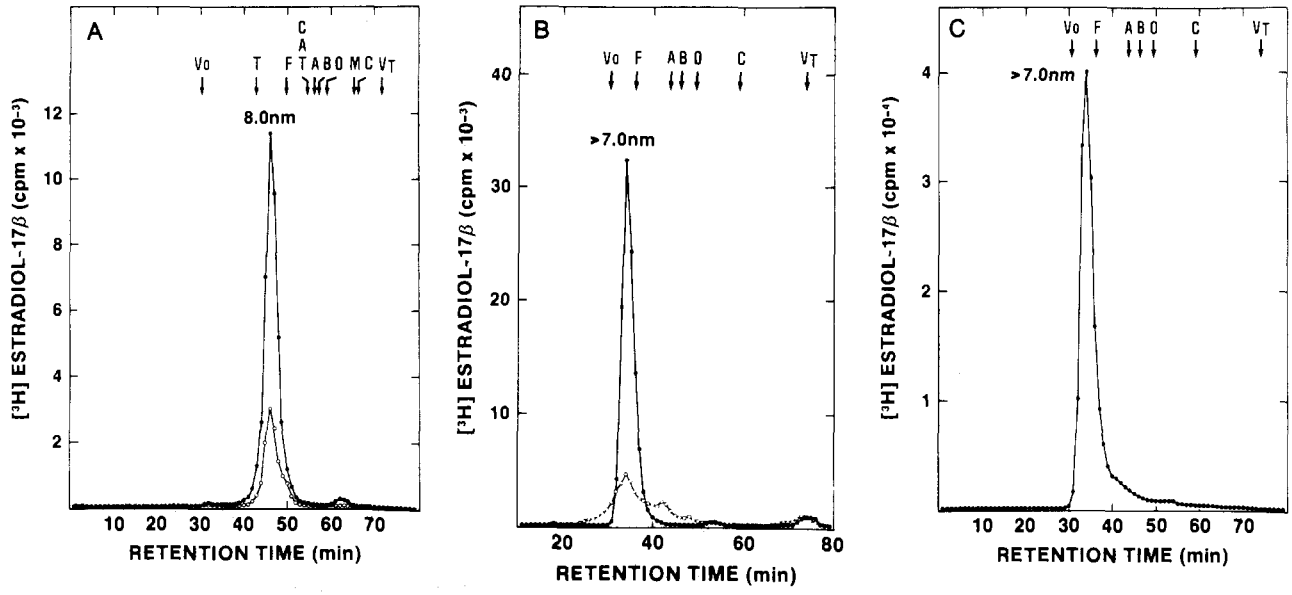

FIGURE 1: Single, large species of untransformed receptor revealed by high-performance size-exclusion chromatography. Estrogen–receptor complexes were analyzed on a TSK-400 column using control buffer (A). Parallel analyses of receptor preparations labeled with 17β-[^{3}H]estradiol in the absence (●) and presence (○) of excess DES reveal a lack of appreciable nonspecific binding. Estrogen–receptor profiles obtained with TSK-3000 columns are also shown for the same buffer system in both the absence (B) and presence (C) of 10 mM sodium molybdate. The column void volumes (V_0) and inclusion volumes (V_T) as well as the elution volumes of several marker proteins are as indicated: thyroglobulin (T), ferritin (F), catalase (CAT), aldolase (A), bovine serum albumin (B), ovalbumin (O), myoglobin (M), and cytochrome *c* (C). The Stokes radii of these proteins are provided along with procedural details under Experimental Procedures.

washed through with 4–6 column volumes of homologous buffer, bound receptor forms were eluted quantitatively with urea/KCl buffer.

RESULTS

In an effort to better determine the quaternary organization of untransformed receptor and the relative contribution of hydrophobic interactions toward the stabilization of this structure, increasing concentrations of urea (2–6 M) have been systematically included in our chromatofocusing and size-exclusion buffers. The uncertainties regarding receptor structure often associated with prolonged biochemical analyses [e.g., see Hutchens et al. (1984)] have been minimized by taking advantage of the much abbreviated analysis times afforded by high-performance chromatographic techniques. The calf uterine estrogen receptor was chosen as an appropriate model system for these studies because it is well characterized in other respects (Puca et al., 1972; Notides & Nielsen, 1974; Erdos & Fries, 1974; Notides et al., 1981; Redeuilh et al., 1981; Muller et al., 1983, 1985; Auricchio et al., 1986), and unlike the estrogen receptor from rat uteri (Giannopoulos & Gorski, 1971) or human uteri (Hutchens et al., 1984) and from breast tumors (Sherman et al., 1980; Miller et al., 1981), it is relatively stable in the untransformed state in vitro with little or no evidence for secondary receptor forms or proteolytic breakdown products under the control conditions outlined here.

The size-exclusion data to be presented here were collected in parallel by using two different HPSEC column types. Even though there is considerable overlap in their effective fractionation ranges, stationary phases with average pore diameters of 30 and 40 nm were chosen to provide maximum resolution of the entire spectrum of receptor forms observed in both hypotonic and hypertonic buffers.

Untransformed Receptor: A Single, Large Receptor Form of Unknown Subunit Structure. Certain associations between varied ionic strength and altered receptor size have come to be nearly definitive over years of investigation [e.g., see Muller and Traish (1986)]. Therefore, to provide the proper perspective and verify the physical characteristics of our particular receptor preparations, analyses by HPSEC were performed in both hypotonic (control) and hypertonic (KCl) buffers for each experiment. As shown in Figure 1 and Table I, in the absence of elevated ionic strengths, urea, or other structure-dissociating reagents, the calf uterine estrogen–receptor complex existed as a single, large species with a Stokes radius of 7.8–8.0 nm. Nonspecific estrogen-binding components were usually minimal and typically absent. The consistent elution of this receptor form as a sharp, symmetrical peak between the marker proteins thyroglobulin and ferritin is typified by the TSK-400 elution profile shown in Figure 1A. The lack of evidence for smaller receptor forms in either the absence (Figure 1A,B) or the presence of 10 mM sodium molybdate (Figure 1C) suggests the lack of proteolytic degradation of this receptor form (7.8–8.0 nm) under these conditions. This Stokes radius is in agreement with the apparent large size of the estrogen receptor as well as other steroid receptor proteins (approximately 8.0 nm) when characterized by conventional size-exclusion chromatoraphy using receptor-stabilizing conditions (Sherman & Stevens, 1984; Sherman et al., 1983;

Table I: Structure-Dissociating Effects of Urea and/or KCl on Untransformed Receptor

sample and column equilibration buffers[b]	Stokes radius of estrogen–receptor complex (nm)[a]	
	Altex[c] TSK-3000SW	Bio-Sil[d] TSK-400
control (±molybdate)	>7.0 (9)	7.8–8.0 (5)
urea	4.2–5.2 (4)	6.0–6.5 (4)
urea/KCl	4.1–4.6 (3)	5.4 (5)
KCl	3.6 (2)	3.4 (2)

[a] The Stokes radii of distinct receptor forms were determined individually from separate plots of retention time vs. log Stokes radii of standard proteins for each set of experimental buffer conditions. The number of experiments for each determination is indicated in parentheses. [b] HPSEC column equilibration buffers are described under Experimental Procedures. [c] Packing material Toya Soda G 3000SW; 30-nm pore size. [d] Packing material Toya Soda G 4000SW; 40-nm pore size.

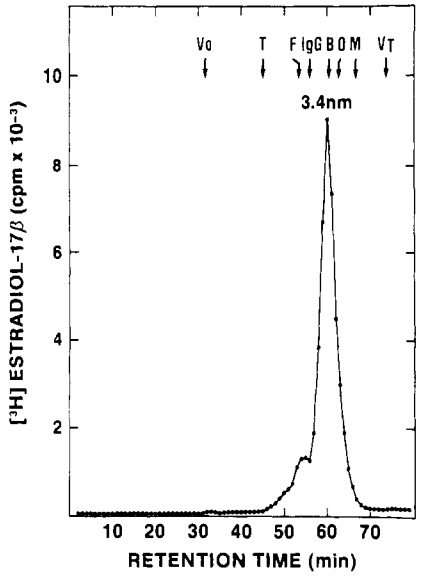

FIGURE 2: Salt-induced dissociation of untransformed receptor. High-performance size-exclusion chromatography of estrogen–receptor complexes on a TSK-400 column using KCl buffer. The column void volume (V_0) and inclusion volume (V_T) as well as the elution volume of several marker proteins are as indicated: thyroglobulin (T), ferritin (F), immunoglobulin (IgG), bovine serum albumin (B), ovalbumin (O), and myoglobin (M). The Stokes radii of these proteins are provided along with procedural details under Experimental Procedures.

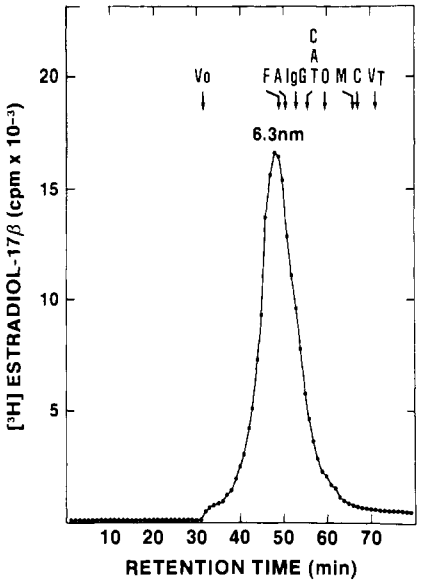

FIGURE 3: Effect of urea on untransformed receptor. High-performance size-exclusion chromatography of estrogen–receptor complexes on a TSK-400 column using 6 M urea buffer as described under Experimental Procedures. The column void volume (V_0) and inclusion volume (V_T) as well as the elution volume of several marker proteins are as indicated: ferritin (F), aldolase (A), immunoglobulin (IgG), catalase (CAT), ovalbumin (O), myoglobin (M), and cytochrome *c* (C). The Stokes radii of these proteins are provided along with procedural details under Experimental Procedures.

Redeuilh et al., 1981). We have also confirmed the 7.8–8.0-nm Stokes radius of the untransformed estrogen receptor from calf uterus by chromatography on Sephacryl S-300 in the presence of sodium molybdate. The untransformed status of the 8.0-nm receptor form was verified by its lack (<5–8%) of interaction with immobilized DNA. The addition of 10 mM sodium molybdate to both homogenizing and HPSEC buffers did not alter the elution profile and demonstrates the utility of rapid analysis times in preserving receptor structure even in the absence of such receptor-stabilizing agents.

Dissociation of Untransformed Receptor Induced by Elevated Ionic Strength. Results shown in Table I further confirm that elevated ionic strengths (0.4 M KCl) induce the rapid structural dissociation of the untransformed receptor complex to a second major form with a Stokes radius of 3.4–3.6 nm. Both HPSEC column types yield similar results. The profile in Figure 2 suggests that while 0.4 M KCl was very effective in dissociating the 8-nm receptor form observed in hypotonic buffers, the process was not entirely complete as evidenced by the presence of minor residual activities in two earlier eluting regions. These results were consistent regardless of whether receptor was introduced into 0.4 M KCl buffer prior to chromatography or only during chromatography. The estrogen–receptor complexes equilibrated into KCl buffer were also analyzed by DNA affinity chromatography after desalting. Only 20–30% of the KCl-treated receptors were ever retained on DNA–cellulose or DNA–agarose. These results are in agreement with several previous reports typically showing a submaximal 2–4-fold increase in DNA binding upon KCl- or temperature-induced transformation of cytosol estrogen receptors (Muller et al., 1983; Bailly et al., 1980; Alberga et al., 1976; Park & Wittliff, 1977).

Alterations in Structure of Untransformed Receptor Induced by Urea. Analyses of these same calf uterine estrogen receptor preparations by HPSEC in up to 6 M urea revealed a single form of receptor intermediate in apparent size to those observed in low and high ionic strengths (Table I). The retention time of receptor in 2 M urea was very close to that of receptor in control buffer. Figure 3 shows the alterations in receptor elution properties induced at the more elevated concentration of urea. While the shift in retention time was relatively small, it was reproducible (n = 8) and was most distinct in the higher concentrations of urea. The peak shape of eluting receptor was also affected. As in the case of receptor exposure to 0.4 M KCl, the size-exclusion results obtained in urea buffer were consistent regardless of preincubation time (up to 22 h) in urea. The generality of this observation is presently unknown as we are aware of no other reports characterizing the relative size of steroid–receptor complexes under these conditions. Interestingly, equilibration of calf uterine estrogen–receptor complexes into 6 M urea caused only slight loss (5–10%) of specific steroid-binding activity yet nearly all lower affinity, nonspecific binding was eliminated. Equilibrium steroid-binding analyses of calf uterine estrogen–receptor interactions indicated only a slight decrease (7–10-fold) in receptor affinity for steroid under these conditions. Data consistently obtained from 13-point Scatchard analyses showed the equilibrium dissociation constant (K_d) to increase from 0.36 ± 0.09 nM (n = 14) to 3.45 ± 0.86 nM (n = 6) in 6 M urea (±0.4 M KCl). In related studies (Hutchens et al., 1987), this effect was found to be completely reversible.

In the presence of urea, a disparity in apparent receptor size was obtained when the two different HPSEC column types as shown in Table I were used. The reproducibility of this phenomenon and our repeated inability to detect aberrations in the column calibration profiles (see Experimental Procedures) under these same conditions make the precise origin of this difference difficult to identify. Apparently, the presence of 6 M urea results in a change in receptor properties (e.g., altered surface hydrophobicity) that differentially affects its chromatographic behavior on the two column types (even though these columns are preared by the same manufacturer). Given their reportedly identical particle size, the difference in average pore size would mean a difference in surface area available for interaction with receptor. Potential interaction mechanisms between receptor and the coated silica surface

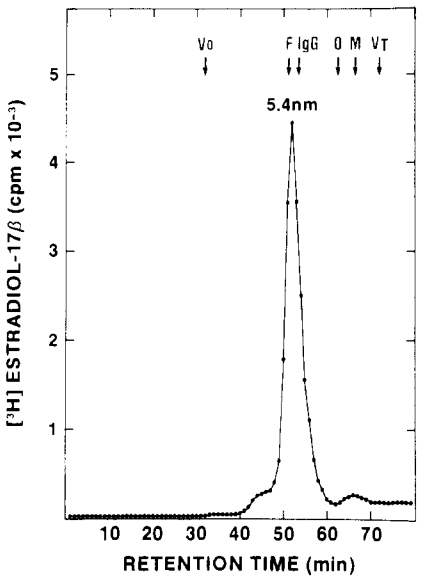

FIGURE 4: Dissociation of untransformed receptor with hypertonic urea buffer. High-performance size-exclusion chromatography of estrogen-receptor complexes on a TSK-400 column using the urea/KCl buffer described under Experimental Procedures. The column void volume (V_0) and inclusion volume (V_T) as well as the elution volumes of several marker proteins are as indicated: ferritin (F), immunoglobulin (IgG), ovalbumin (O), and myoglobin (M). The Stokes radii of these proteins are provided along with procedural details under Experimental Procedures.

Table II: Steroid-Dependent Effects of 6 M Urea/0.4 M KCl on Receptor Structure

	Stokes radius (nm)	
expt[a]	steroid–receptor complex	unliganded receptor
1	4.1–4.3	5.1
2	4.3–4.6	5.3
3	4.6 and 3.5	5.0

[a] An Altex TSK-3000SW column was used for these experiments as described under Experimental Procedures. Stokes radii were estimated based upon retention time at the median peak width.

include dipole–dipole interactions and sharing of π-electrons available from exposed aromatic residues as in the case of charge transfer (Porath & Larsson, 1978% Egly & Porath, 1979; Porath, 1979; Ochoa et al., 1980). Nevertheless, the *relative* elution order (apparent size) of receptor from each column type remained unchanged as a function of buffer constituent (i.e., Stokes radius decreases in the order control buffer > urea buffer > urea/KCl buffer > KCl buffer). By design, resolution of the Bio-Sil TSK-400 column in the 4.5–6.5-nm region is superior due to its increased average pore diameter, so the 6.0–6.5-nm value obtained for the 6 M urea-equilibrated receptor may be more accurate.

The receptor form observed by HPSEC in 6 M urea at elevated ionic strengths (0.4 M KCl) appears further reduced in size (Figure 4) relative to that seen in urea alone but not nearly as much as is observed in 0.4 M KCl alone. The presence of 6 M urea prevents the full dissociating effects of 0.4 M KCl on receptor structure. Using the TSK-3000SW HPSEC column, we found that the dissociating effect of urea/KCl on receptor structure was partially steroid dependent. Figure 5 illustrates the differential effects of urea/KCl on receptor retention time in the presence and absence of bound hormone. Table II summarizes the results of three such experiments. These results suggest that receptor interaction with estradiol modifies the contribution of hydrophobic interactions in the maintenance of any quaternary receptor structure.

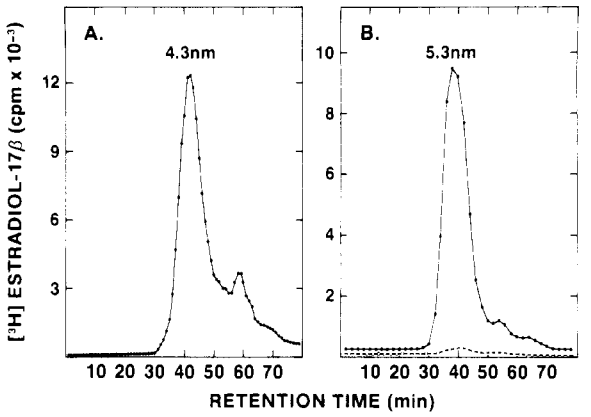

FIGURE 5: Steroid dependence of urea/KCl-induced dissociation of untransformed receptor components. High-performance size-exclusion chromatography (TSK-3000 column) of estrogen receptor in the presence and absence of bound estradiol. Cytosol was prepared and either prelabeled with 10 nm 17β-[^{3}H]estradiol (A) or analyzed in the unliganded form (B) using postcolumn receptor labeling techniques as described under Experimental Procedures. Labeled and unlabeled receptor preparations were equilibrated by rapid G-25 chromatography into urea/KCl buffer. The lack of nonspecific estrogen-binding components (O) was also verified in postlabeled fractions. Procedural details are provided under Experimental Procedures.

Conservation of Receptor Surface Charge in Urea. The surface charge properties of the estrogen–receptor complexes were evaluated by rapid chromatofocusing in both the absence and presence of up to 6 M urea. Chromatofocusing was chosen as a highly resolving technique able to distinguish proteins and receptor forms not separated by ion-exchange chromatography or isoelectric focusing (Sluyterman & Elgersma, 1978; Sluyterman & Wijdenes, 1978; Hutchens et al., 1984). Figure 6 shows the lack of appreciable change in elution pH for the major estrogen–receptor form when analyzed in 6 M urea compared to control buffers. The chromatofocusing elution profiles for receptor analyzed in 2 M urea were indistinguishable from those obtained using control buffers. Furthermore, in each case there is a lack of evidence for significant surface charge heterogeneity. The results shown are representative of eight similar chromatofocusing comparison experiments using polyampholyte buffer systems. Chromatofocusing experiments performed using our nonpolymeric focusing buffer (Hutchens et al., 1986a–c) containing 6 M urea revealed similar results, suggesting the lack of urea-promoted interactions between the estrogen–receptor complex and polymeric ampholyte species present in Polybuffers 96 and 74.

Urea-Induced Exposure of the DNA-Binding Site. The urea-induced alterations in receptor size as described here were also accompanied by an acquisition of high affinity for DNA, *without* previous exposure to elevated temperatures or increased ionic strengths. The affinity of estrogen–receptor complexes for immobilized DNA was evaluated in the continued presence of 6 M urea buffer to avoid imposing nonequilibrium conditions on receptor structure. Undiluted, untreated (i.e., no urea) controls and estrogen–receptor complexes chromatographed on Sephadex G-25 or diluted in control buffer alone had little or no affinity for DNA–cellulose or DNA–agarose. Estrogen–receptor complexes were sequentially chromatographed on G-25 Sephadex (up to 4 times) using control buffer before repeated exposure to the DNA affinity columns (four to six passages) in attempts to reveal maximal binding. In the absence of urea, only 7 ± 1% (n = 8) and 9 ± 3% (n = 3) of the total estrogen–receptor complexes were bound to DNA–agarose and DNA–cellulose, respectively. In contrast, the estrogen–receptor complexes equilibrated into 6 M urea as for size-exclusion analyses were quantitatively

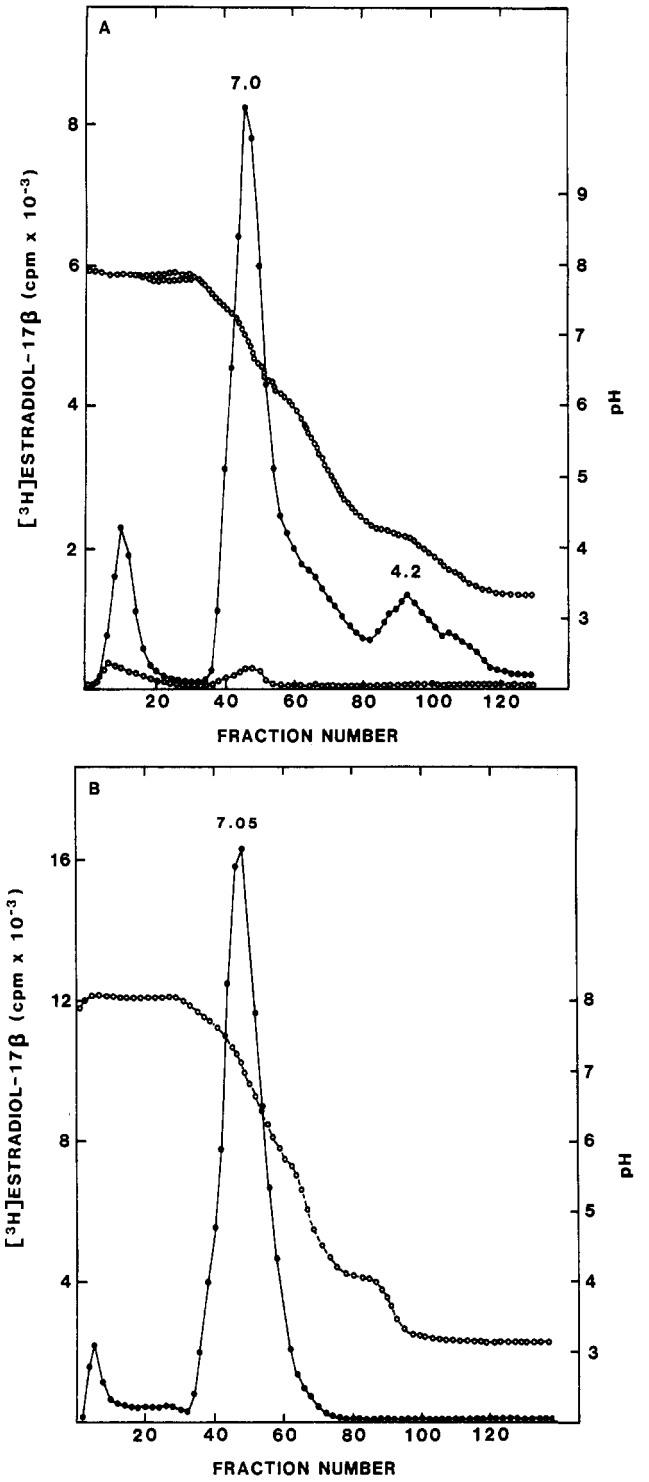

FIGURE 6: Urea effects on receptor surface charge properties. Estrogen–receptor complexes were analyzed by chromatofocusing on PBE 94 in both the absence (A) and presence (B) of 6 M urea. Parallel analyses were performed by using estrogen receptor preparations labeled with 17β-[^{3}H]estradiol in the presence (○) and absence (●) of a 100-fold molar excess of DES to detect nonspecific binding components. The descending elution pH values were nearly identical for the two experiments shown in (A). Unbound steroid was removed by Sephadex G-25 chromatography immediately prior to chromatofocusing. Nonspecific binding was absent in the presence of 6 M urea. The specific elution pH of estrogen–receptor forms is indicated. Procedural details are provided under Experimental Procedures.

(92 ± 8%, n = 12) adsorbed to DNA–agarose and significantly (84 ± 10%, n = 6) adsorbed to DNA–cellulose. Concentrations of urea from 2 to 6 M were equally effective in promoting the quantitative interaction of estrogen–receptor complexes with DNA–agarose. Exposure of the estrogen–receptor complex to urea immediately prior (approximate exposure time 15–20 min) to DNA–agarose chromatography is sufficient to promote maximal interaction. By comparison, exposure of the estrogen–receptor complex to 0.4 M KCl resulted in only 23 ± 8% (n = 4) binding to either DNA affinity matrix. Nonreceptor estrogen-binding proteins present in cytosol labeled in the presence of excess DES did not bind (<1%) to either DNA–cellulose or DNA–agarose under any of the conditions evaluated. Similarly, free 17β-[^{3}H]estradiol did not (<0.5%) interact with the DNA affinity resins.

Urea not only induces but also sustains the interaction of estrogen–receptor complexes with DNA. Removal of urea from the affinity column buffer precluded extraction of DNA-bound estrogen receptors with increased ionic strength (up to 2 M NaCl). Quantitative elution was only achieved by using the urea/KCl buffer as described under Experimental Procedures. Thus, 6 M urea causes relatively small changes in receptor size (8.0 nm to 6.0–6.5 nm), affinity for estradiol, and apparent net surface charge compared to its dramatic effect on exposure of the DNA-binding site. High-performance size-exclusion chromatography of the receptor form eluted from DNA demonstrated no further change in Stokes radius. Receptor elution profiles in urea buffer were identical before and after DNA affinity purification. The resistance of urea-transformed receptor elution from immobilized DNA with high salt (in the absence of urea) suggests that nonelectrostatic interactions may be involved.

Discussion

The only consensus concerning the structure and quaternary organization of native (untransformed) estrogen receptor is that it is not known (Sherman & Stevens, 1984; Grody et al., 1982; Muller & Traish, 1986). The last 20 years of investigation indicate that perturbation of electrostatic interactions alone may not provide all the necessary clues.

The exact subunit composition of the untransformed receptor remains unclear; however, our attempts to dissect its component parts with urea have revealed the presence of two intermediate-sized, high-affinity DNA-binding receptor forms distinctly different from those identified previously by manipulation of ionic strength alone. Furthermore, we have presented evidence that the configuration of the single, large (8.0 nm) species of untransformed receptor is differentially affected by the structure-dissociating effects of 6 M urea in hypotonic vs. hypertonic buffers. This suggests a contribution of hydrophobic interactions toward the stabilization of untransformed receptor and masking of the DNA-binding site. There was no evidence to suggest that any smaller receptor forms acted as structural intermediates during the formation of the 6.0–6.5-nm DNA-binding receptor form observed in 6 M urea. It is significant that 2–6 M urea induced exposure of the DNA-binding site without a detectable increase in net positive surface charge as determined by chromatofocusing. These results may indicate that the presence of urea promotes only the redistribution of surface charge and/or the exposure of noncharged receptor–DNA interaction sites.

The preserved high affinity of receptor for estradiol in 6 M urea buffer reported recently (Hutchens et al., 1987) and confirmed in these studies suggests that the essential steroid-binding structure of receptor is not necessarily compromised under these conditions. Bound steroid, however, influences urea effects on other aspects of receptor structure, for example, DNA binding and size (see Table I; Hutchens et al., 1986d). Preliminary investigations (Hutchens et al., 1985; Dunaway et al., 1985) to establish rapid methods of isolating various unliganded receptor forms provide additional evidence for

steroid-dependent as well as steroid-independent alterations in receptor structure (size and surface charge) under diverse experimental conditions. Since then, Hansen and Gorski (1985, 1986) have reported the use of aqueous two-phase partitioning techniques to suggest that estrogen interaction with the unliganded rat uterine receptor caused a change in hydrophobic but not surface charge properties. Although the physical properties (e.g., size and surface charge) of the unliganded, cytosolic receptor forms partitioned were not established under the conditions utilized, or, alternatively, before and after exposure to the actual experimental conditions employed, the authors do present arguments suggesting that only the monomeric receptor form was present. Regardless of interpretations regarding the particular receptor structure(s) involved, ligand binding noticeably influences receptor structure in vitro, thus demonstrating the need to more thoroughly evaluate unliganded receptor properties.

A significant problem has been encountered during numerous previous attempts to address those physicochemical properties of native receptor which are altered during the steroid-induced acquisition of high affinity for DNA. This problem has been the ambiguity associated with the use of nonhomologous buffer systems to first identify specific receptor forms and then monitor their interactions with DNA. For example, while exposure of steroid–receptor complexes to increased ionic strengths is universally recognized as a means of affecting receptor transformation [e.g., see Muller and Traish (1986), Dahmer et al. (1984), and Milgrom (1981)], the ionic strength is typically lowered significantly prior to measuring subsequent receptor–DNA interactions. Since ionic strength dependent changes in receptor size can be reversible, the receptor form observed by density gradient centrifugation or size-exclusion chromatography in high-salt buffers most probably does not represent that form of receptor introduced at low ionic strength to the DNA. We believe that an important quality of the results shown here is the simultaneous assessment of receptor size, surface charge, and DNA-binding properties in homologous buffers. The same conditions that induced and maintained increased affinity of receptor for DNA were in fact maintained during size-exclusion chromatography, chromatofocusing, and ligand-binding (Scatchard) analyses.

The mechanism by which 2–6 M urea promotes an interaction of hormone–receptor complex with DNA which is subsequently "salt-resistant" remains unclear at this time. A series of comparative experiments performed recently demonstrate that nuclear estrogen–receptor complexes resistant to extraction with 0.4 M KCl are completely extracted with the urea/KCl buffer as described here for elution of DNA-bound receptor (unpublished results). Hemminki (1976) used DNA intercalating reagents as well as detergents to suggest the involvement of hydrophobic receptor domains during binding to nuclei and chromatin. There would seem to be more possibilities for sequence-specific base recognition based upon hydrogen-bonding, charge-transfer, and/or hydrophobic-type interactions than pure electrostatic interaction with the polyanionic phosphate extremities alone. The linear sequence of amino acids in the putative DNA-binding region C [see Krust et al. (1986)] of the chicken and human estrogen receptor contains several highly conserved nonpolar and aromatic residues as well as basic residues. It is well-known that hydrophobic interactions can affect not only local DNA structure but also DNA–protein recognition events [e.g., see Jernigan et al. (1986)]. Porath has investigated aqueous protein and nucleic acid charge-transfer interactions (Porath & Larsson, 1978; Egly & Porath, 1979; Porath, 1979; Ochoa et al., 1980) and, along with his colleagues, has eloquently described the electron donor–acceptor properties of urea and its role in mediating these reactions (Ochoa et al., 1980). To this end, Ochoa noted that the quantity and selectivity of polynucleotide adsorption on acriflavin–Sepharose 4B were dramatically altered using 6 M urea buffers with and without 0.4 M NaCl. In this context, it is interesting that, in contrast to urea, previous receptor-transforming manipulations not involving purification (i.e., increased temperature or ionic strength) are known to increase hydrophobic interactions (Hjerten, 1981). Interestingly, Buchou et al. (1983) reported that exposure of untransformed progesterone receptor to 2–3 M urea induced the conversion of receptor to a smaller form, but receptor affinity for DNA or other polyanions was not evaluated. We are now investigating the mechanism of urea-induced interaction of estrogen receptor with several different polynucleotide models. Recent proposals suggesting that DNA-binding proteins may locate specific sequences by facilitated diffusion along "nonspecific" sequences (Hannon et al., 1986) necessitate the evaluation of receptor–DNA interaction mechanisms using both nonspecific and putative "specific" nucleotide sequences.

Beyond the utility of urea (e.g., 2 M) as an in vitro probe of biophysical relationships, speculation concerning biological structures or altered microenvironments able to induce similar changes in receptor properties must be somewhat limited. Given the complex intracellular infrastructure and subcellular compartmentalization which clearly exist and the propensity of steroid receptors toward association with anionic and cationic as well as uncharged surfaces, it seems entirely inappropriate to imply any particular value of ionic strength and/or relative hydrophobicity as "physiological" in this context. The value of these parameters in the microenvironment of a solution/solid phase interface is simply unknown.

Previous conclusions regarding the structure and subunit organization of the estrogen receptor have relied quite heavily upon velocity sedimentation profiles obtained at various ionic strengths. Considerable effort has been directed toward an understanding of the generic 4S to 5S shift in sedimentation coefficient on high-salt gradients after exposure to elevated temperature. Less effort has been directed toward the larger, discrete receptor species consistently observed under structure-stabilizing conditions (Sherman & Stevens, 1984; Sherman et al., 1983). Similar large, untransformed receptor forms have been identified for each class of steroid hormone receptor (Sherman & Stevens, 1984). In recent years, it has been established that those experimental conditions which dissociate the larger (8.0 nm; 8–10 S), untransformed estrogen receptor into the 4S estrogen-binding subunit(s) do indeed cause the altered estradiol dissociation kinetics and increased affinity for DNA which define estrogen receptor transformation (activation). Thus, aside from that form of receptor which exists prior to hormonal perturbation (i.e., unliganded), the large (8.0 nm), untransformed estrogen–receptor complex described here and by others [e.g., see Sherman and Stevens (1984)] seems worthy of characterization as the potential native form of receptor in vitro. Little is known about steroid receptor structure in vivo. In fact, the subcellular location of unliganded steroid receptor proteins still remains unsettled. However, assuming these receptor proteins are synthesized in the cytoplasm and given the effects of steroid ligand binding on receptor affinity for DNA and/or other nuclear constituents both in vitro and in vivo, a central question that remains is one of ligand influence on receptor structure/function relationships relative to cellular infrastructure. Steroid binding and DNA binding represent receptor functions, but the rela-

tionship of these functions to unliganded/liganded receptor structure is largely unknown due to the lack of a structural definition for receptor beyond that of the steroid-binding monomer or subunit as defined under structure-dissociating conditions. Certainly, the biological significance of quaternary receptor structures observed in vitro will be difficult to establish since the possibility for "artifacts" seems high [e.g., association of heat shock proteins with receptor; for a review, see Lindquist (1986)]. Regardless of stoichiometry, a noncovalent quaternary organization of the steroid-binding subunit with other non-steroid-binding proteins or biomolecules should not be ignored as a potential means of structural and/or regulatory control.

In summary, rapid monitoring of hydrophobic perturbations in the quaternary organization of untransformed receptor may reveal insights into receptor structure/function (e.g., DNA binding) relationships not readily apparent using altered ionic strengths alone. Gentle manipulation of hydrophobic domains within the untransformed receptor completely exposes the DNA-binding site without the more extensive structure-dissociating effects observed during exposure to 0.4 M KCl. The urea effect on receptor size (and/or shape) is relatively slight in low ionic strength buffers but more extensive at elevated ionic strengths. Urea effects on receptor surface charge were undetectable by chromatofocusing. The urea-induced and maintained increase in the affinity of estrogen–receptor complexes for single-stranded DNA additionally provides a powerful receptor immobilization and purification tool. Since DNA-unwinding proteins are defined by their affinity for single-stranded DNA (Chase & Williams, 1986), it would appear that our data may support speculation that steroid receptor proteins act in this capacity. Overall, our results suggest that further understanding the relative contribution of hydrophobic-type interaction forces may facilitate the identification of components necessary for the structural definition of the native receptor form as well as our understanding of the mechanism(s) by which steroid binding induces increased affinity of receptor for DNA.

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Homology-Dependent Changes in Adenosine 5′-Triphosphate Hydrolysis during *recA* Protein Promoted DNA Strand Exchange: Evidence for Long Paranemic Complexes†

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ABSTRACT: As a first step in DNA strand exchange, *recA* protein forms a filamentous complex on single-stranded DNA (ssDNA), which contains stoichiometric (one *recA* monomer per four nucleotides) amounts of *recA* protein. *recA* protein monomers within this complex hydrolyze ATP with a turnover number of 25 min^{-1}. Upon introduction of linear homologous duplex DNA to initiate strand exchange, this rate of ATP hydrolysis drops by 33%. The decrease in rate is complete in less than 2 min, and the rate of ATP hydrolysis then remains constant during and subsequent to the strand exchange reaction. This drop is completely dependent upon homology in the duplex DNA. In addition, the magnitude of the drop is linearly dependent upon the *length* of the homologous region in the linear duplex DNA. Linear DNA substrates in which pairing is topologically restricted to a paranemic joint also follow this relationship. Taken together, these properties imply that all of the available homology in the incoming duplex DNA is detected very early in the DNA strand exchange reaction, with the linear duplex DNA paired *paranemically* with the homologous ssDNA in the complex throughout its length. The results indicate that paranemic joints can extend over thousands of base pairs. We note elsewhere [Pugh, B. F., & Cox, M. M. (1987b) *J. Biol. Chem. 262*, 1337–1343] that this duplex acquires resistance to digestion by DNase with a much slower time course (30 min), which parallels the progress of strand exchange. Together these results imply that the duplex DNA is paired with the ssDNA but remains outside the nucleoprotein filament. Finally, the results also support the notion that ATP hydrolysis occurs throughout the *recA* nucleoprotein filament.

The *recA* protein of *Escherichia coli* promotes a series of in vitro DNA strand exchange reactions that mimic its in vivo function in homologous genetic recombination (DasGupta et al., 1980; Cox & Lehman, 1981a). The best characterized of these reactions is a three-strand exchange in which the *recA* protein promotes the exchange of complementary strands between linear duplex and circular single-stranded DNA molecules derived from bacteriophages. The products of this reaction are a nicked circular heteroduplex DNA and the displaced linear single-stranded DNA (ssDNA).[1] The reaction can be divided into three experimentally distinguishable phases. The first is presynapsis, the cooperative binding of stoichiometric amounts of *recA* protein to ssDNA in the presence of ATP (Cox & Lehman, 1982; Radding et al., 1983; Flory et al., 1984; Morrical et al., 1986). This complex exhibits a stoichiometry of one *recA* monomer per four nucleotides (Cox & Lehman, 1987) and exhibits a filamentous structure in the electron microscope (Flory & Radding, 1982; Dunn et al., 1982). The second phase is synapsis, the search for homology and eventual alignment of the duplex DNA with homologous sequences on the ssDNA (Shibata et al., 1979;

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[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; M13mp8(+), circular single-stranded genome of bacteriophage M13mp8; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; bp, base pair(s); SSB, *Escherichia coli* single-stranded DNA binding protein; SDS, sodium dodecyl sulfate.

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