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Stimulation of oligonucleotide binding of estradiol receptor complexes by accessory proteins

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ABSTRACT

During purification of E_2R using oligo(dT)-cellulose chromatography, a receptor accessory factor (RAF) was identified in the cytosol of mouse kidney. This factor stimulates the binding of purified E_2R to oligo(dT)-, oligo(dC)-, and oligo(dA)-cellulose as well as to DNA cellulose. It is a heat-stable, trypsin-resistant protein with an apparent molecular weight of between 10 and 30,000 daltons. Although structurally unrelated, similar stimulation of oligonucleotide binding was seen with calf thymus histones and, to a lesser extent, egg white lysozyme. Individual histones, especially H2a, H2b, and H3, also facilitate rebinding of purified E_2R to oligo(dT)-cellulose, while H1 is less effective. Furthermore, histones stabilize the holoreceptor during sedimentation at 4° and 12°C. The N- and C-terminal half molecules of H2b were generated by cyanogen bromide-mediated cleavage and the N-terminal half was found to duplicate the effects of the parent molecule, both in binding and holoreceptor stabilization. These data suggest that the *in vivo* binding of E_2R to DNA can be modulated by accessory proteins of cytosol and nuclear origin.

INTRODUCTION

Despite the absence of information on the detailed structures of pure estradiol receptor proteins, studies using cruder preparations indicate at least two distinct domains on these molecules, the binding sites for steroid ligands and those for the polydeoxyribonucleotides. In regard to the latter, studies have demonstrated that the binding was nonsaturable and nonspecific as to the source of the DNA (1-3). Yamamoto and Alberts (4) postulated that estradiol receptors resemble the *lac* repressor by binding to both sequence specific and nonspecific sites in DNA. They calculated that as many as 10^3 specific sites could be masked by nonspecific binding using current methods of analysis. Yet nonspecific binding itself is a potential determinant in the action of a putative modulator of transcription such as the estradiol receptor complexes (E_2R).

Furthermore, the term nonspecific is an inaccurate description of the nonsaturable binding of E_2R to DNA. A preference in binding was reported for

native over denatured DNA (5, 6), poly(dA-T) over other synthetic polydeoxyribonucleotides (6), bromuridine-substituted DNA over unsubstituted DNA (7), and oligo(dT)- and oligo(dC)-cellulose over oligo(dA)-cellulose (8). The nonrandom affinities for poly- and oligonucleotides suggest a complimentary configuration on the receptor protein which functions as a recognition site. The separateness of the polynucleotide recognition site from that of steroid binding in E_2R, as well as other steroid receptor complexes, was demonstrated by the effects of limited proteolysis (9-11). Under these conditions, DNA binding was destroyed without affecting retention of the steroid ligand. Similarly agents such as pyridoxal-5-phosphate (12), aurintricarboxylic acid (13) and Cibacron blue F3GA (14) inhibit the DNA or nuclear binding of glucocorticoid and estradiol receptor complexes without altering the association of the bound steroid.

The polynucleotide recognition site presents a potential locus for the modulation of E_2R activity. However, little information is available on the role of cellular components which may influence the DNA binding of these complexes. Thrower and co-workers (15) showed that the binding of rat uterine E_2R to oligo(dT)-cellulose was parallel to its binding to DNA cellulose in the amount of E_2R uptake and the salt concentration required for elution. In addition, they found that oligo(dT)-cellulose binding was stimulated by a cytosol factor under different conditions. We have also used oligo(dT)-cellulose chromatography for the purification of E_2R previously identified in mouse kidney and uterine cytosols (16, 17). Initial experiments indicated that a component of mouse kidney cytosol, which bound tightly to oligo(dT)-cellulose, stimulated the oligonucleotide binding of partially purified E_2R. This receptor accessory factor (RAF) was partially purified and found to be a heat-stable, trypsin-resistant protein. In assessing the specificity of stimulation of the rebinding of E_2R to oligo(dT)-cellulose, the calf thymus histones were effective at low concentrations, while lysozyme and gamma globulin were not. These results indicate that polynucleotide binding of E_2R can be modulated by endogenous components of the cytoplasm and of the nucleus of target cells.

MATERIALS AND METHODS

Nya:NYLAR mice, males weighing 20 g and 21-day-old females of 12 g, were obtained from the Griffin Laboratories of this Division. [2,4,5,7,16,17-^{3}H]-Estradiol-17β, specific activity 145 Ci/mmole, was purchased from Amersham and tested for purity by thin layer chromatography. Nonradioactive steroids were obtained from Steraloids, Inc., Wilton, NH. Oligo(dT)-, oligo(dA)-, and oligo(dC)-celluloses were the products of P-L Biochemicals, while DNA cellulose,

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prepared according to the method of Alberts and Herrick (18), was from Enzo Laboratories, New York, NY. Worthington Biochemical Corporation was the source of trypsin, DNase, RNase, lysozyme, the calf thymus histone mixture and the individual histones (H1, H2a, H2b, and H3). Gamma globulin and pepsin were from Sigma. Pronase was from Calbiochem, proteinase K from Merck and Staphylococcus aureus proteinase V from Miles. SP-Sephadex and Sephadex G-25 were from Pharmacia while Bio-Rad was the source of Biogels P-10, P-30, P-60 and P-100.

The methods of preparation of mouse kidney and uterine cytosol, receptor binding of $[^3H]$estradiol and oligodeoxyribonucleotide binding have been published (8). A concentration of 0.15 M KCl was routinely used in the nucleotide binding assays. The dissociation of $[^3H]$estradiol from $[^3H]E_2R$ was measured by a modification of the method of Penefsky (19, 20). The buffers which were used are TED (Tris-HCl, pH 7.6, 0.01 M; EDTA, 0.001 M; DTT, 0.001 M) and $T_{20}EDG_{20}$ (Tris-HCl, pH 7.6, 0.02 M; EDTA, 0.001 M; DTT, 0.001 M; glycerol, 20% w/v).

Enrichment of E_2R using oligo(dT)-cellulose binding

$[^3H]E_2R$ of either kidney or uterine cytosol, 0.2 ml, was added to 1.5 ml polyethylene tubes (Beckman) which contained 250 μg of oligonucleotide covalently bound to 5 mg of cellulose in 0.4 ml of 0.225 M KCl-TED. The tubes were agitated using a vortex and then rotated at low speed on a multipurpose rotator (Scientific Industries) for 1 hr at 4°C, following which the mixtures were centrifuged at 10,000 x g for 3 min and the supernatants were discarded. The pellets were resuspended in 0.6 ml of 0.15 M KCl-TED and centrifuged again. The supernatants were discarded and the pellets suspended in 0.3 ml of 0.5 M KCl-$T_{20}EDG_{20}$. The mixtures were rotated at low speed for 2 hr at 4°C, centrifuged and the supernatant retained. The partially purified E_2R present in the supernatant contained 1600 fmoles/mg protein compared to 20 fmoles/mg in the initial kidney cytosol, an enrichment of 80-fold. The values for uterine E_2R were 1088 fmoles/mg protein in the 0.5 M KCl eluent compared to 150 fmoles/mg in the cytosol, an enrichment of 7-fold. In some experiments, the partially purified kidney E_2R was concentrated by slowly adding solid $(NH_4)_2SO_4$ to 60% (w/v) of saturation. The pH was maintained at 7.6 by addition of concentrated NH_4OH. The mixture was equilibrated for 1 hr at 4°C and then centrifuged at 100,000 x g for 30 min using an SW56 Ti rotor (Beckman). The pellet was suspended in a minimal volume of $T_{20}EDG_{20}$ buffer.

Enrichment of RAF

The kidney cytosol was prepared according to the method of Thanki et al.

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(8) and designated as fraction I. The cytosol was transferred to glass tubes, 2.5 x 9.5 cm, and immersed in a boiling water bath for 10 min; after cooling, the 30 ml mixture was clarified by centrifugation at 10,000 x g for 10 min. The clear supernatant was retained as fraction II. Two alternate methods were used at this point for further purification: (a) Fraction II, 1 ml, was added to 10 mg SP-Sephadex and rotated slowly for 1 hr at 4°C. The gel was pelleted by low speed centrifugation and washed once with TED. Elution of the RAF activity was done by addition of 0.5 M KCl-$T_{20}EDG_{20}$ to the SP-Sephadex pellets and subsequent rotation of the mixtures for 60 min at 4°C. The eluate obtained by centrifugation was designated fraction SP. (b) Fraction II was dialyzed against two changes of distilled water for 16-24 hr and the dialyzed fraction was lyophilized. The slightly yellowish white powder was designated fraction III and stored at -20°C.

The cyanogen bromide-mediated cleavage of calf thymus histone 2b and recovery of its half molecules was the procedure described by Adler et al. (21). Gel electrophoresis of histone 2b, as well as the other histones used, and of the half molecules of histone 2b was by the method of Bonner et al. (22). The sedimentation studies of the $(NH_4)_2SO_4$ precipitated enriched E_2R utilized 5-20% (w/v) sucrose linear gradients in 0.15 M KCl-TED buffer. The gradients were centrifuged in an SW56 swinging bucket rotor (Beckman) at varying temperatures for a pre-set 155,280 $rad^2/sec \times 10^7$. The gradients were fractionated from the top using an Auto-Densi-Flow IIC pump (Searle-Buchler Instruments) into about 25 samples. Ten ml of Aquasol (New England Nuclear Co.) were added to radioactive samples and these were counted in an LS-250 Beckman scintillation counter with an efficiency of 30% for ^3H. Approximately 100 cpm equaled 1 fmole of macromolecular-bound [^{3}H]estradiol. The protein concentrations were determined by the method of Schaffner and Weissman (23). Cellulose-bound oligodeoxynucleotide phosphates were determined by the method of Chen et al. (24). [^{14}C]Bovine serum albumin was prepared by the method of Rice and Means (25).

RESULTS

Identification of an oligonucleotide binding stimulatory factor

During purification of mouse kidney cytosol E_2R, using oligo(dT)-cellulose batch chromatography, an unexpected result was encountered in the initial experiments. Of the input E_2R about 30% was bound to the oligonucleotide and of that bound, 60-70% was eluted with 0.5 M KCl. This resulted in an 80-fold increase in E_2R specific activity if cytosol was used and 200-fold if an

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intervening 0-30% $(NH_4)_2SO_4$ fractionation step was included. However, when the purified E_2R was assayed by readsorption to oligo(dT)-cellulose, the amount retained was the same as that observed with the initial crude cytosol and not the increase in binding anticipated for a molecule purified through affinity interaction. This was not due to dissociation of the steroid from the receptor protein as determined by gel filtration. If the oligo(dT)-cellulose, recovered from one cycle of E_2R adsorption and elution, was substituted for fresh affinity matrix, there was a 2- to 3-fold enhancement of purified E_2R rebinding (Table 1). The improved binding was not seen if cytosol E_2R were used instead of the partially purified complex. These results suggested that a factor (or factors) was retained on the oligonucleotide cellulose which stimulated E_2R binding to this ligand.

Addition of mouse kidney cytosol indicated the presence of an oligonucleotide binding stimulatory activity when tested with purified kidney E_2R (Table 1,

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Table 1. Evidence that a cytosol factor stimulates the binding of partially purified E_2R to oligo(dT)-cellulose

Experimental condition	Input (fmoles)	Specific oligo(dT) binding (fmoles)	E_2R bound % of input
1. Fresh oligo(dT)-cellulose	9	3	33
Used oligo(dT)-cellulose	9	7	78
2. No addition	7	1.8	26
+ 0.1 ml fraction I	7	3.8	54
+ 0.1 ml fraction II	7	3.6	51
+ 0.1 ml fraction III	7	3.2	46

Partially purified E_2R was bound to oligo(dT)-cellulose, plus or minus the designated additions, under conditions described in the section on Methods in ref. (8). In Experiment 1, the used oligo(dT)-cellulose was the pellet following one cycle of E_2R adsorption and elution. Residual radioactivity of 310 cpm was subtracted from the observed radioactivity of the second binding to yield the percent of input E_2R which was bound. RAF fractions I, II, and III preparation is described in the section on Methods. The amount of protein added per experiment was 100 μg for fraction I, 6 μg for fraction II, and 4 μg for fraction III. In this and other binding experiments, the nonspecific binding to unmodified cellulose was subtracted from the oligo(dT)-cellulose-bound radioactivity to yield the selective binding and each point is the mean of duplicate assays.

part 2). A 2-fold increase in binding was seen with addition of the cytosol. Neither exposure to 100°C for 10 min nor boiling followed by dialysis against distilled H_2O significantly reduced the cytosol stimulatory activity. The RAF effect was not restricted to kidney receptor complexes but was also apparent when the oligonucleotide binding of purified uterine cytosol E_2R was tested (Kumar, S. A., Beach, T. A. and Dickerman, H. W., unpublished results). In this experiment, uterine cytosol E_2R was adsorbed onto DNA cellulose and eluted with 0.4 M KCl using a batch procedure identical to the oligo(dT)-cellulose chromatography described in the section on Methods. The percentage of input E_2R bound to oligo(dT)-cellulose prior to DNA cellulose chromatography was 35% and following elution, 12%. However, the addition of a boiled kidney supernatant increased the binding of the eluted E_2R to 32% of input. Addition of the RAF fraction to nonpurified uterine E_2R had no effect on its oligonucleotide binding.

RAF activity was not restricted to the kidney but was also detected in the cytosols of the mouse uterus and, to a lesser extent, lung. No significant stimulation was obtained by addition of serum or the cytosols of skeletal muscle or brain (Table 2).

The stimulation of kidney E_2R binding by kidney RAF was not restricted to oligo(dT)-cellulose but was also apparent with oligo(dC)-, oligo(dA)-, and DNA

Table 2. Effect of extracts from various tissues on partially purified kidney E_2R binding to oligo(dT)-cellulose

Tissue extract added to E_2R	fmoles E_2R bound
None	2.1
Kidney	4.3
Uterus	3.2
Lung	2.8
Brain	2.0
Muscles	2.1
Serum	2.0

The extracts from tissue cytosols and serum were prepared the same as was kidney RAF fraction II (section on Methods). Aliquots containing 100 μg protein of these extracts were added to the binding mixtures containing 7 fmoles of partially purified E_2R.

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celluloses (Table 3). The lowered binding to oligo(dA)-cellulose as opposed to the oligopyrimidine ligands has already been reported with crude cytosol receptor complexes (8). The RAF effect could be duplicated by preincubation of the oligo(dT)-cellulose with the factor, subsequent pelleting of the cellulose, washing with 0.15 M KCl-TED and then addition of an aliquot of purified E_2R to determine the extent of binding. The RAF effect on subsequent E_2R binding was essentially the same if the preincubation was done at KCl concentrations ranging from 0.05 to 0.5 M. These data indicate that part of the RAF effect was the direct interaction of the factor with the oligonucleotide and that this interaction was relatively insensitive to salt concentration. Under comparable salt concentrations, kidney cytosol E_2R underwent a 2- to 3-fold increase in binding to oligo(dT)-cellulose at 0.1–0.2 M KCl with an abrupt decrease above 0.2 M (8).

Partial purification and characterization of kidney RAF

The RAF of kidney cytosol was enriched using Procedure a described in the section on Methods. Addition of the SP-Sephadex eluate, over a 6-fold concentration range, to purified kidney E_2R led to an increase in oligo(dT) binding to almost 80% of the input complex (Fig. 1). The approximate molecular weight of RAF, fraction III (method b), was obtained by a modification of the method of Penefsky (19, 20) using sedimentation through a graded series of gels, P-10, P-30, P-60, and P-100. The retention of activity in the void volumes as percent of the input was as follows: P-10, 100%; P-30, 50%; P-60, 6%; and

Table 3. The effect of RAF on partially purified E_2R binding to oligodeoxynucleotides and DNA celluloses

Cellulose type	fmoles E_2R bound	
	RAF	No RAF
Oligo(dT)	4.0	1.9
Oligo(dC)	3.7	2.1
Oligo(dA)	2.5	0.9
DNA	3.0	2.0

In this experiment, the cellulose matrices contained either 250 μg of covalently linked oligodeoxynucleotides or 50 μg of native calf thymus DNA which was not covalently bound. The input of partially purified kidney E_2R was 7 fmoles and, where designated, 100 μg of protein (RAF fraction II) was added.

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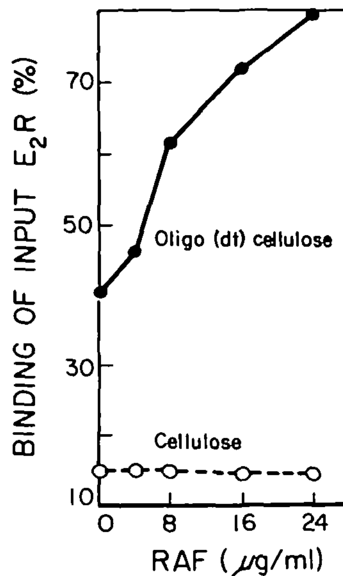

Figure 1. The binding of E_2R to oligo(dT)-cellulose as a function of RAF concentration. $[^3H]E_2R$ was purified using oligo(dT)-cellulose and RAF using SP-Sephadex as described in the section on Methods. The input of $[^3H]E_2R$ was 7 fmoles.

P-100, 0%. This suggests that RAF is a molecule of between 10 and 30,000 daltons molecular weight.

The chemical identity of RAF was determined using the effects of degradative enzymes on factor activity. Bullock and Bardin (16) reported that estradiol retention by mouse kidney cytosol receptors was sensitive to pronase digestion; in our laboratory, binding of crude or partially purified E_2R to oligo(dT)-cellulose was eliminated by the actions of pronase, proteinase K or trypsin. However, the heat stability of RAF allowed the testing of the factor's sensitivity to proteolysis without compromising E_2R. In the experiment described in Table 4, the proteases were added to partially purified RAF (Procedure a, section on Methods) and the mixture was incubated for 30 min at 20°C. The reactants were then boiled for 10 min, cooled, centrifuged to remove any precipitate and an aliquot transferred to the binding reaction mixtures. The action of pronase and proteinase K resulted in a loss of stimulatory activity while exposure to trypsin, even at a marked excess, had no effect. Repetition of the preincubation of RAF with trypsin at 37°C did not

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Table 4. Effect of proteolytic enzymes on [^{3}H]E_2R and RAF

Protease treatment of RAF fraction II	Protease treatment of [^{3}H]E_2R	Percent binding of [^{3}H]E_2R
No RAF	None	28
None	None	56
None	Pronase	0
None	Proteinase K	0
None	Trypsin	0
Pronase	None	22
Proteinase K	None	26
Trypsin	None	49

RAF fraction II, 100 µg protein, was incubated with 5 µg of proteolytic enzyme or TED buffer at 4°C for 1 hr. The mixtures were then boiled for 10 min and added to the incubation tubes containing [^{3}H]E_2R and oligo(dT)-cellulose and in some of the proteolytic enzymes. The input was 8 fmoles of partially purified E_2R.

alter this experimental result. Chymotrypsin was also without an effect on RAF activity. In contrast, exposure of RAF to pepsin or Staphylococcal aureus protease V led to a loss of activity suggesting an involvement of glutamyl and aspartyl residues. RNase A and DNase I exposure did not lead to a loss of activity. These results indicated that RAF was a protein or a small molecule intimately bound to a protein.

Effect of other proteins on binding of E_2R to oligo(dT)-cellulose

The finding that a relatively low molecular weight, heat-stable protein-stimulated E_2R binding to oligo(dT) led to an examination of other proteins as modulators of the binding reaction. Figure 2 represents the change in extent of binding as a function of varying concentrations of calf thymus histones, egg white lysozyme and human gamma globulin. The total histones, at a concentration range of 30 to 70 µg/ml, led to a 2-fold increase in E_2R binding with saturation occurring at about 100 µg/ml. The basic protein, lysozyme, also led to an increased binding but only at concentrations 2.5 greater than that of the histones while gamma globulin was essentially without effect.

The stimulation by basic proteins suggested that the effect was due to their content of lysine and/or arginine residues and this was tested by com-

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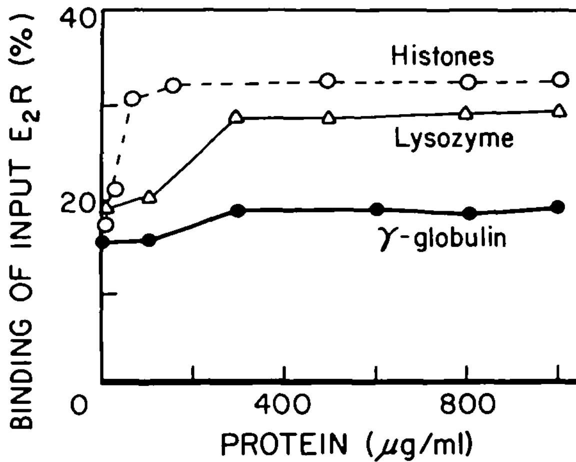

Figure 2. The effect of different proteins on the binding of E_2R to oligo(dT)-cellulose. The input of $[^3H]E_2R$ was 6 fmoles.

paring the effects of the individual histones, H1 (lysine-rich), H2a and H2b (slightly lysine-rich) and H3 (arginine-rich). Of the input 7 fmoles, the amount of E_2R bound to oligo(dT)-cellulose was 2.2 in the absence of histones, 3.0 for H1, 5.6 for H2a, 5.6 for H2b, and 4.8 for H3. The amount of histones added was 50 μg/binding reaction mixture. When the amount of added histones was 5 μg/mixture, the bound E_2R was 2.7 for H1, 4.3 for H2a, 4.4 for H2b, and 3.7 for H3. A more complete comparison of the effects of H1 and H2a is shown in Fig. 3. Although H1 increased the oligonucleotide binding of purified E_2R, it was not as effective as H2a. The latter led to a 2-fold increase in binding at 40 μg or more of added protein. An apparent saturation in the stimulatory effect was seen with both H1 and H2a. These data indicated that the histone effect was not due to the lysine content of the proteins alone but that the nucleosomal components (H2a, H2b and H3) were more effective than extranucleosomal lysine-rich H1. In this regard, poly-L-lysine, 140,000 molecular weight, decreased the oligo(dT)-cellulose binding of E_2R from a control level of 19% of input to 10% when 50 μg of the polycation was added and 5% when 100 μg was added. Exposure of the histones to trypsin however decreased the stimulatory activity as follows: H1, 38%; H2a, 59%; H2b, 46% and H3, 46%. Precautions, including adding a 5-fold excess of soybean trypsin inhibitor and boiling for 10 min, were taken to eliminate any effect the protease might have on the receptor complex. Under the same conditions, there was no decrease in the

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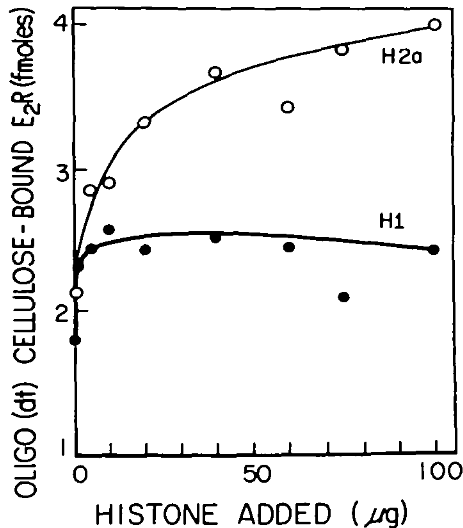

Figure 3. The effect of calf thymus histones, H1 and H2a, on binding of E_2R to oligo(dT)-cellulose. The commercially obtained histones were analyzed by acid-urea gel electrophoresis (22) and found to be essentially uncontaminated.

stimulatory activity of kidney RAF, fraction III (Method b).

The above data indicate that the stimulation of oligonucleotide binding by the histones was not a function of their basic amino acid content alone, although the sensitivity to trypsin did show that the lysine/arginine content was a factor. A possibility was that the histones were active through neutralization of the negative charges of the ionized phosphates of the oligo(dT) residues in a manner similar to histone:DNA interaction. Furthermore, the oligonucleotide residues exist in an ordered structure at the conditions of temperature and ionic strength of the binding reaction, not as a random coil (26). The histones might stabilize such a structure in the same manner that they do nuclear DNA (27). However, an additional factor in the stimulation of binding might be the interaction of basic proteins, such as histones, with the receptor complex as well as with the oligonucleotide. This was tested by adding the individual histones to $(NH_4)_2SO_4$ precipitated purified E_2R and observing their effect on the stability of the hormone-receptor complex during sedimentation in a 5-20% sucrose linear gradient containing 0.15 M KCl at various temperatures. The results are shown in Fig. 4. If no histone was added, the E_2R complex observed following centrifugation at 4^oC was present as a 4S peak

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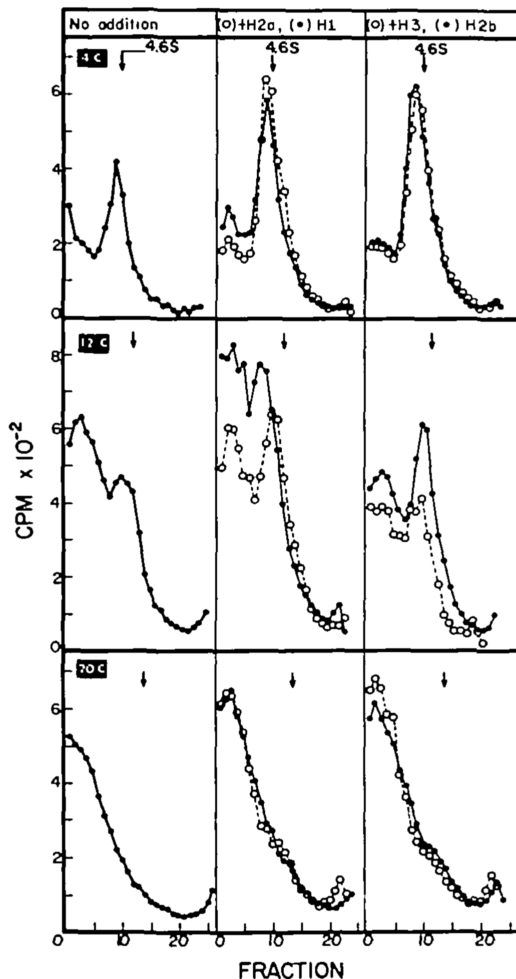

Figure 4. The stabilization of holoreceptor by individual histones during sedimentation. [^{3}H]E_2R was enriched and concentrated using $(NH_4)_2SO_4$ as described in the section on Methods. The 5-20% linear sucrose gradients were equilibrated at the temperature used in the subsequent centrifugation for 4 hr. The amount of [^{3}H]E_2R applied to the gradients was 8150 cpm, 4°C; 9500 cpm, 12°C; and 8000 cpm, 20°C. Fifty μg of the histones were incubated with E_2R for 30 min at 0°C prior to transfer to the gradients. The 4.6S position was identified by the peak of [^{14}C]bovine serum albumin.

with a significant zone of dissociated [^{3}H]E_2 ranging back towards the meniscus. However when any of the histones were added to E_2R prior to centrifugation, there was an increase in the area of the 4S peak relative to the amount

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of dissociated steroid. At a centrifugation temperature of 12°C, a diminished 4S peak was apparent in the absence of histones due to dissociation of the complex. When H2a and H2b were present, the 4S peak was better preserved although H3 and H1 also demonstrated some stabilizing effects. At a temperature of 20°C, there was essentially no recovery of a 4S peak with the exception of a small shoulder in the sample to which H2b had been added. These data indicate that exposure of purified E_2R to all the histones tested led to an increased amplitude of the 4S hormone-receptor complex. Addition of RAF, procedure (a) fraction SP, did not lead to an enhanced recovery of E_2R at any of the operational temperatures, nor did equivalent amounts of lysozyme or gamma globulin.

The effects of the N- and C-terminal half molecules of H2b on E_2R binding and stability

There is an asymmetry in the distribution of basic charges in the five types of histones and the possibility existed that the histone effects on E_2R binding and/or stability were a function of those positions of the molecules with either a high or low basic charge density. Histone 2b has a very uneven distribution of lysine and arginine residues and is subject to a single cleavage with cyanogen bromide (28). The resultant products are an N-terminal half molecule, N, which contains residues 1-58 and 32% of the residues are basic and a C-terminal half molecule, C, with 20% of charged residues. Data from several studies indicate that the N fragments of the histones bind to DNA while the C fragments of the nucleosomal histones interact with each other and, possibly, regulatory protein molecules (21, 29, 30). The fragments were recovered after cleavage and column chromatography. On gel electrophoresis, the C fragment was essentially pure as was the intact molecule, while the N fragment was contaminated with less than 10% C. These products were tested for their ability to stimulate the oligo(dT)-cellulose binding of purified E_2R and the results are shown in Fig. 5. Although both the N and C fragments stimulated the binding, the former was more active in the extent of E_2R which was bound. These data indicate that, at least for H2b, the N-terminal portion of the molecule was more active in stimulating the interaction of E_2R and the oligonucleotide.

The N- and C-terminal half molecules were tested for their effect on the stabilization of purified steroid receptor complexes during sedimentation in the same manner as were the individual histones. The results are shown in Fig. 6. At a sedimentation temperature of 4°C, the increased recovery of radioactivity at a 4S position and the corresponding decrease in dissociated

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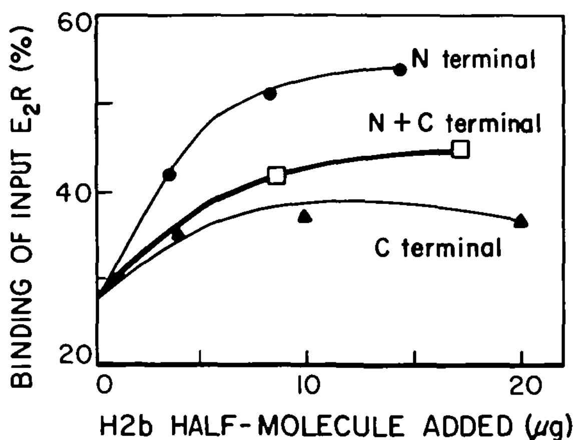

Figure 5. The effect of N- and C-terminal half molecules of H2b on oligo(dT)-cellulose binding of E_2R. The fragments were generated and recovered as described by Adler et al. (21). The input of [^{3}H] E_2R was 6 fmoles. The symbols represent the N-terminal half molecule, ●—●—●; the C-terminal half molecule, △—△—△ and equal mixtures of the fragments, □—□—□ .

steroid was seen with H2b. The N-terminal half molecule approximated the effect seen with untreated H2b while the C-terminal half molecule led to an aggregation of E_2R as well as an increase in pelleted radioactivity (data not shown). At 12°C, there was a marked decrease in the amplitude of the 4S peak in the absence of any histone while H2b addition again led to retention of a well-defined peak. At the higher temperature, addition of the N-terminal half molecule yielded results comparable to that of the parent molecule while the more helical C-terminal fragment again led to aggregation of purified E_2R. As with the intact histone molecules, the amplitude of the 4S E_2R complex was increased but there was no shift in the sedimentation coefficient. These results indicate that the estradiol receptor protein contains a cation reactive site which affects the retention of the steroid.

DISCUSSION

The identification of a DNA binding site on E_2R from various tissues (9-14), in addition to the steroid binding domain, suggests yet another locus for modulation of steroid-dependent processes. Using the *in vitro* binding to oligo(dT)-cellulose as a simplified model for DNA interaction, a group of proteins were found to stimulate the uptake of mouse kidney E_2R. Their characteristics are listed in Table 5. The cytosol factor of mouse kidney (RAF) is a heat-stable,

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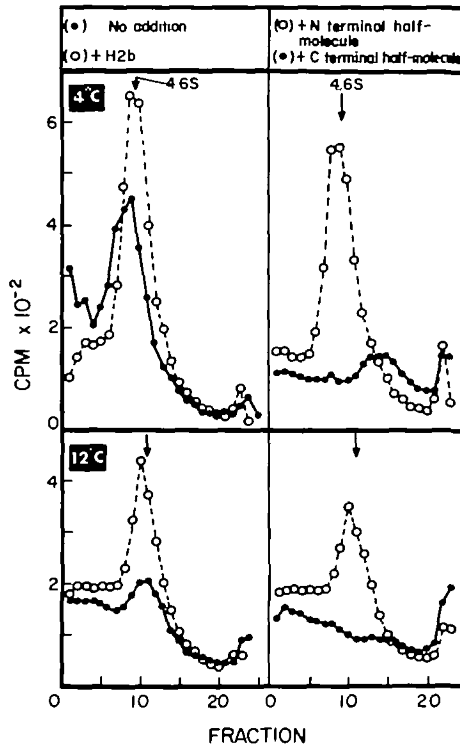

Figure 6. The stabilization of holoreceptor by the N- and C-terminal half molecules of H2b during sedimentation. The procedure was as outlined in the legend of Fig. 4. The amount of [^{3}H]E_2R applied to the gradients was 8000 cpm, 4°C and 5100 cpm, 12°C.

trypsin-resistant protein whose activity is dependent on the integrity of aspartyl and/or glutamyl peptide bonds. Similar heat-stable activities were found in the cytosol of mouse uterus and lung. Although impure, RAF is apparently a macromolecule of 10 to 30,000 daltons on the basis of exclusion from a series of cross-linked gels. Its action is at the level of the oligonucleotide template, possibly through stabilization of the ordered nucleotide structure which exists at conditions of temperature and ionic strength of the binding reaction (26). There is no indication of a direct effect of RAF on the receptor complex. Others have suggested that a rate-limiting factor, present in the cytosol of target cells, facilitates the nuclear binding of E_2R and that this factor can be assessed by measuring the stimulation of factor-

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Table 5. Properties of receptor accessory proteins

Protein	Oligo(dT)* binding	Trypsin resistance	Cell site	Molecular weight
RAF	++	yes	cytoplasm	10 to 30,000
H1	+	no	nucleus	21,500
H2a	++	no	nucleus	14,000
H2b	++	no	nucleus	13,700
H3	++	no	nucleus	15,300

*The binding at maximal response is designated: +, <50% above control; ++, >50% above control.

deficient receptor complex binding to oligo(dT)-cellulose by cytosol (15). The fluctuations in the cytosol activator fraction were used to explain the nuclear-cytoplasmic distribution of E_2R in rat uterus and female neonate hypothalamus at different stages of the estrous cycle (31, 32). Although there are similarities between the cytosol activity described in these studies and mouse kidney RAF, i.e., cytosol localization and requirement for factor-depleted E_2R, the studies are too preliminary to conclude that they are identical. It remains to be proven that RAF has a physiologic role in the nuclear uptake of E_2R but the potential exists for positive regulation of DNA-E_2R binding by specific cytosol proteins.

Another group of proteins, the histones of calf thymus, were found to be effective in the stimulation of E_2R binding to oligonucleotides. There are previous reports of interaction between E_2R and histones, as well as other basic proteins. Precipitation of rat uterine nuclear E_2R occurred with total calf thymus histones, protamine sulfate and poly-L-lysine (33). A small but significant increase in precipitant activity was observed with H3 compared to H1. Puca and his co-workers (34, 35) reported nonspecific, salt-insensitive interactions between histone 2a and uterine E_2R complexes during DNA cellulose, DEAE cellulose and histone 2a agarose chromatography but the results are difficult to interpret because of the loss of DNA binding consequent to the preparation of the 4.5S complex (9).

When the extent of binding of partially purified E_2R to an oligonucleotide ligand was measured, calf thymus histones were found to be stimulatory at concentrations far below those at which lysozyme, an extracellular basic protein,

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was effective. Human gamma globulin was without effect indicating that the stimulation was not a general protein effect. Comparison of histones H1, H2a, H2b, and H3 indicated that those which are part of the nucleosome were more effective than the lysine-rich extranucleosomal H1. This is of interest because if target cell nuclei are partially digested with DNase I, E_2R was located at the released mononucleosomal peak or at slightly heavier position (36-38). Further elucidation of the histone effect using the well-defined half molecules of histone H2b indicated that the N-terminal half was more effective in promoting binding to oligo(dT) than was the C-terminal half molecule. This basic amino acid-rich sequence has been shown to be the part of the molecule which interacts with DNA by thermal denaturation (29), NMR (30) and CD (21) studies while the C-terminal half has been assigned a function in histone:histone interactions. In addition to promoting oligonucleotide binding, histones, especially H2a and H2b, impair the dissociation of estradiol from partially purified kidney E_2R complexes. Again, the extended chain N-terminal half of H2b duplicates the effect of the intact molecule while the more helical C half apparently leads to aggregation of the receptor complex. Although histone:E_2R interactions were detected by E_2R stabilization, there was no change in the apparent sedimentation coefficient of the receptor protein. This is possibly due to the weakness of the interaction between histones and E_2R which preclude recovery after sedimentation in a dilute medium or to the imperceptible difference in sedimentation which a 1:1 complex of the molecules would yield, i.e., with histone 2b, an increase of the coefficient of 4 to 4.6 (39). While the N-terminal half molecule was more active in oligonucleotide binding and stabilization of E_2R, there are structural determinants other than the basic charge densities of the polypeptides. The lysine-rich H1 was only slightly active in stimulation of binding over a wide concentration range while the synthetic poly-L-lysine was inhibitory.

There are two possible consequences of the E_2R:histone interaction. Clark and his co-workers have reported that the uterotropic effects of estradiol administration are related to retention of receptor in the nucleus 6 hr after injection and not to the initially high nuclear content of E_2R (40). If nuclear retention is dependent upon chromatin-bound holoreceptor, and not on dissociated aporeceptor, then juxtaposition of nuclear E_2R to the N-terminal portions of the nucleosomal histones could prolong the time of chromatin occupancy by stabilization of the holoreceptor. Conversely, the interaction of chromatin-bound E_2R may influence the bonding of nucleosomal histones to

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DNA. Beard (41) has demonstrated that nucleosomes migrate in a slow *in vitro* reaction from SV40 chromosomes onto naked DNA covalently joined to the chromatin. He favors a rolling mechanism of nucleosomal movement as opposed to nucleosome release and rebinding or a displacement transfer to an adjacent DNA strand. Central to a mechanism of nucleosomal migration is the necessity for disruption of the strong binding between the histones and DNA. While this binding is a composite of several ionic bonds, the individual bonds are weak and in constant equilibrium between formation and disruption. Intervention of a nonhistone protein, such as the estradiol receptor protein, capable of interacting with both DNA and the nucleosomal histones may facilitate nucleosomal migration and consequently effect the quantity and/or quality of transcription.

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FOOTNOTES

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