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Selective complexing of the "nuclear" 5S estradiol receptor by a serum component, 5S-CA

(steroid hormones/receptor transformation/tissue and species comparison/controls for antisera)

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ABSTRACT **A component present in several sera interacts selectively with the 5S "nuclear" form of estradiol receptor but not with the 4S "cytosol" form. This component is therefore referred to as "5S-complexing activity (5S-CA)." This selective interaction occurs with 5S receptor from mouse, calf, or rat. In addition, 5S-CA recognizes the nuclear form of the receptor from hypothalamic as well as uterine tissue. Thus, 5S-CA indicates a general similarity in the chemical nature of 5S nuclear receptors prepared from several sources. Using 5S-CA as a chemical probe for the nuclear form of the estradiol receptor, I have shown the chemical as well as physical similarity of 5S receptors prepared *in vivo* (by injection of [^{3}H]estradiol and subsequent isolation from nuclear/myofibrillar extracts) and *in vitro* (by labeling of cytoplasmic extracts and subsequent chromatography on columns of DNA-cellulose). These results indicate a distinctive chemical property of nuclear 5S forms of estradiol receptor. The data are interpreted with regard to models for 5S receptor formation. 5S-CA was found in sera from animals immunized against various antigens. The importance of testing for 5S-CA in antisera directed against steroid derivatives, steroid receptors, and other steroid-binding proteins is discussed.**

Binding proteins with high affinities for selective steroid hormones—termed steroid "receptors"—have been described for many tissues (1). One of these, the estradiol receptor, exhibits a transformation from a "cytosol" form to a "nuclear" form (2). Understanding the details of this process may tell us about receptor chemistry, nuclear retention of hormone receptor complexes, and the action of these complexes in the cell's nucleus. When examined under conditions of moderate to high ionic strength (as in Tris buffers containing 0.15 to 0.4 M NaCl), these cytosol and nuclear forms sediment with rates of "4S" and "5S," respectively (1, 3). However, cytosol receptors in crude extracts can sediment with a range of constants (4–9 S) depending on total extract concentration and ionic strength (4, 5). For these reasons, studies of the formation of nuclear estradiol receptor would be greatly aided by physical criteria that distinguish the nuclear 5S receptors from those forms of cytosol receptors that happen also to sediment at 5 S.

I have described a serum component that fulfills these criteria (6, 7). This component selectively complexes nuclear 5S estradiol receptor and is termed "5S-complexing activity (5S-CA)." In the present study I used the 5S-CA as a probe to demonstrate a structural similarity among 5S receptors from several species, from different tissues, and for both *in vivo*- and *in vitro*-derived nuclear estradiol receptors.

METHODS

Preparation of 5S-CA. The 5S-CA was prepared from a goat anti-rabbit gamma globulin serum, a gift from T. Carnow and M. Schachner. Other similar sera were obtained from Antibodies, Inc. (Davis, CA). The 5S-CA was prepared by the methods used previously to fractionate rabbit sera containing 5S-CA (6). After precipitation with 1.75 M ammonium sulfate and subsequent dialysis against 0.01 M sodium phosphate buffer (pH 7.5), the dialysate was fractionated by DEAE-cellulose chromatography. The fraction used in these experiments eluted at 0.05 M NaCl and contained 24 mg of protein per ml. Some experiments were performed with unfractionated sheep antisera against 5α-dihydrotestosterone hemisuccinate attached to albumin (gift of W. Moyle) or against 17β-estradiol hemisuccinate on albumin (gift of C. D. Toran-Allerand).

Preparation of Extracts. Cytosol extracts of uteri from (C57BL/6J × C3H/HeJ)F_1 mice, calves (J. Trelegan, Cambridge, MA), and rats (Charles River CD outbred albino, Sprague–Dawley derived) and of mouse hypothalamus were prepared as described (8). The extraction buffer contained 0.01 M Tris·HCl (pH 8.1; 25°), 1 mM EDTA, 1 mM mercaptoethanol, 10% (vol/vol) glycerol, and 0.15 M NaCl. Buffer for elution of DNA-cellulose columns and for sucrose density gradients was prepared with the same components, except that it contained 0.4 M NaCl.

In vivo labeled nuclear receptors from rat uteri were obtained after injecting five 25-day-old female rats with 50 μCi of 17β-[2,4,6,7,16,17-^{3}H]estradiol (152 Ci/mmol, New England Nuclear, Boston, MA) in 0.9% NaCl and 2% ethanol. One hour after injection, the rats were etherized and perfused with 0.9% NaCl. Uteri were removed, minced, and homogenized in the extraction buffer. Subsequent steps were performed at 2°. The homogenate was centrifuged for 15 min at 25,000 rpm (56,700 × g max, Beckman rotor 40) and the pellet was rehomogenized in extraction buffer and again centrifuged. The washed pellet was homogenized in extraction buffer adjusted to contain 0.4 M NaCl. After incubation with stirring for 1 hr, the homogenate was centrifuged for 60 min at 40,000 rpm (145,000 × g max) to obtain the particle-free supernatant, which includes nuclear estradiol receptor.

DNA-Cellulose Chromatography. The cytosol extracts from all tissues were chromatographed on DNA-cellulose essentially as described (8). Extracts were labeled with either 17β-[2,4,6,7-^{3}H]estradiol (91.3–98.5 Ci/mmol, New England Nuclear) or the hexasubstituted isotope described above. The labeled extracts were chromatographed on DNA-cellulose that had been equilibrated with extraction buffer containing 0.2 mg of crystallized bovine albumin (Pentex, Miles Laboratories) per ml and were eluted in extraction buffer containing 0.4 M NaCl.

Analysis by Sucrose Gradient Sedimentation. Sucrose gradients (5 ml, 5–20% wt/vol) were prepared in extraction buffer containing 0.4 M NaCl. Aliquots of receptor fractions (50–200 μl) were mixed in polystyrene tubes with equal vol-

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Abbreviation: 5S-CA, 5S-complexing activity.

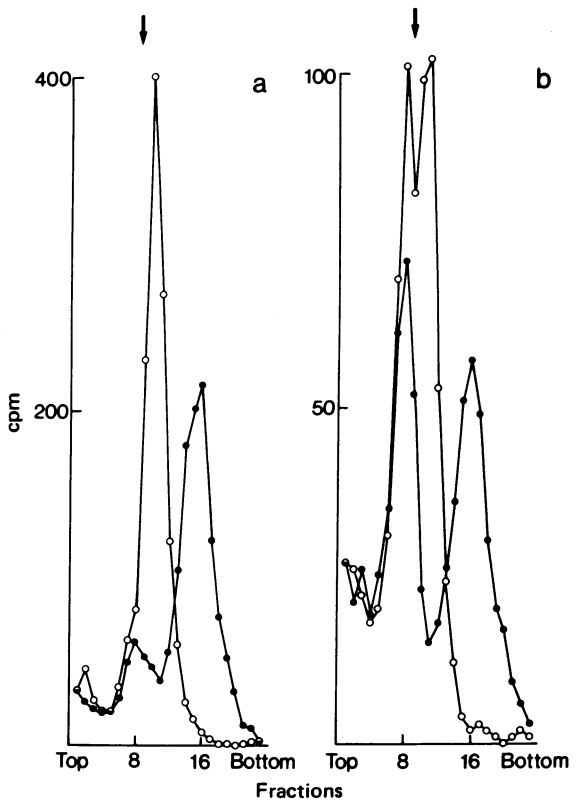

FIG. 1. Selective interaction during sedimentation of 5S-CA with uterine 5S estradiol receptors from mice (*a*) and calves (*b*). Parallel samples of receptor fractions eluted from DNA-cellulose were centrifuged without (open circles) or with (solid circles) 5S-CA. The arrows indicate the location of the internal marker, dansylated bovine serum albumin (approximate sedimentation rate, 4.7 S).

umes of partially fractionated goat serum 5S-CA and with 2 μl of 5-dimethylamino-1-naphthalenesulfonyl chloride-conjugated bovine serum albumin as internal fluorescent marker (approximate sedimentation rate, 4.7 S). These samples were then layered on the sucrose density gradients and centrifuged for 18–21 hr at 50,000 rpm (300,000 × *g* max, Beckman rotor SW 50.1). Radioactivity was determined in Omnifluor (New England Nuclear)/toluene scintillation counting fluid. The dansylated albumin did not interfere with these measurements. All graphs were plotted after subtracting the background (8 cpm) detected in blank samples.

RESULTS

The selective complexing of 5S estradiol receptor by several sera from immunized rabbits, goats, and sheep was demonstrated previously (6). For the rabbit serum, the 5S-CA was demonstrated in a fraction that was precipitated by ammonium sulfate (1.75 M), retained during dialysis, not retained on DEAE-cellulose, and retained by an Amicon ultrafiltration PM-10 filter. As expected for this protocol, that fraction also contained rabbit gamma globulins as evidenced by a strong precipitin band with goat anti-rabbit gamma globulin antisera and the detection with Coomassie brilliant blue of only two protein bands (at approximately 25,000 and 50,000 daltons) on a polyacrylamide gel containing sodium dodecyl sulfate. This is the pattern typically obtained for light and heavy immunoglobulin chains and indicates that the factor either is an immunoglobulin or can be isolated with them (6).

Because many samples of goat anti-rabbit gamma globulin antisera that are available contain 5S-CA and because fractionated 5S-CA lacks other complicating activities of whole sera, such as steroid-binding components, this goat serum was fractionated for this study. The 5S-CA was found in the flow-through of the DEAE-cellulose column and, to a greater extent, in a fraction eluted with 0.05 M NaCl. On polyacrylamide gels with sodium dodecyl sulfate, Coomassie brilliant blue staining revealed primarily two bands at approximately 25,000 and 50,000 daltons [as for fractionated rabbit serum (6)] and several minor bands. This fraction was prepared in December 1975, has been stored at 2°–6° since, and is still active.

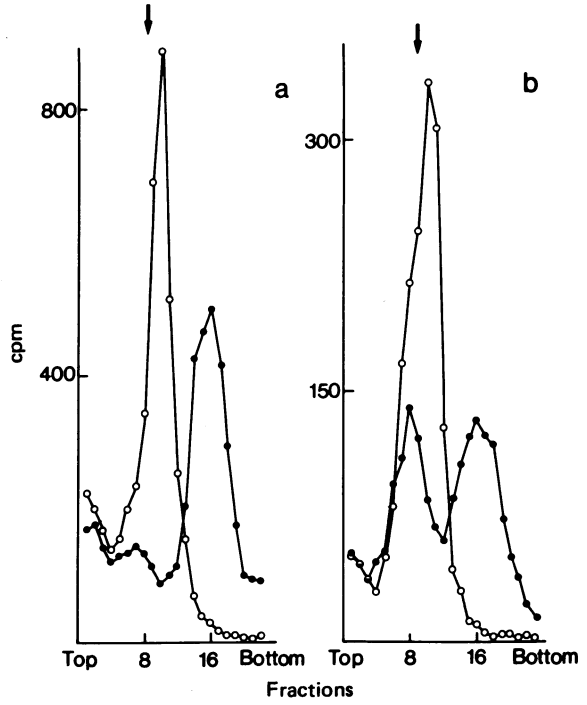

FIG. 2. Interaction during sedimentation of 5S-CA with rat uterine 5S estradiol receptor labeled *in vitro* (*a*) or *in vivo* and extracted from nuclear/myofibrillar pellets (*b*). Parallel samples eluted either from DNA-cellulose (*a*) or uterine pellets (*b*) were centrifuged without (open circles) or with (solid circles) 5S-CA. Arrows indicate dansylated bovine serum albumin (4.7 S)

Under the conditions of the assay, receptor–5S-CA complex is stable, as evidenced by the relatively sharp peak of complexed receptor after 18–21 hr of sedimentation. This complex sediments at more heterogeneous and intermediate rates when recovered from a sucrose gradient and run a second time on an identical gradient (6). Heating for 2 min to 80°, but not to 56°, treatment with trypsin, and dilution of 5S-CA all result in decreased complexing activity with decreased sedimentation rates (ref. 6; data not shown). Activity was observed with 1:10 to 1:20 dilutions of this 5S-CA fraction.

Similarity of 5S Estradiol Receptors from Several Species. When mouse uterine cytosol extract labeled with [^{3}H]estradiol is chromatographed on DNA-cellulose and eluted, a rapid transformation from the 4S to the 5S receptor form occurs. Usually this conversion goes nearly to completion (Fig. 1*a*) (6, 8). Calf uterine extracts yielded less complete formation of 5S receptor (Fig. 1*b*). In these gradients, however, the 4S and 5S peaks for the calf uterine extract were only partially resolved (Fig. 1*b*). The remaining 4S receptor in the mouse eluate appeared as a highly reproducible, although subtle, shoulder on the major 5S peak (Fig. 1*a*). A similar pattern was obtained for the eluate from DNA-cellulose of a rat uterine extract (open circles, Fig. 2a).

To resolve these estradiol receptor forms more fully and to

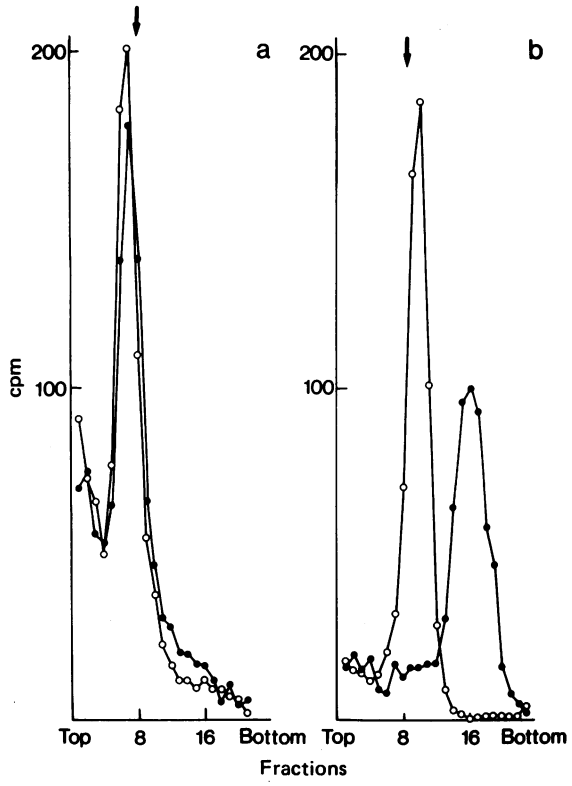

FIG. 3. Sedimentation with 5S-CA for cytosol and nuclear forms of estradiol receptor from mouse hypothalamus. Samples of hypothalamic extract were labeled with [^{3}H]estradiol and either chromatographed through Sephadex G-25 columns (6, 8) to remove excess unbound radioactivity (*a*) or were chromatographed on DNA-cellulose and exposed for 1 hr at 30° (6, 7) to produce 5S receptor (*b*). These samples were centrifuged without (open circles) or with (solid circles) 5S-CA. Arrows indicate dansylated bovine serumin albumin (4.7 S).

demonstrate a chemical as well as a physical difference between them, the same eluates were mixed with 5S-CA and sedimented in parallel sucrose density gradients. 5S-CA selectively interacted with the 5S receptor forms, causing them to sediment further (Figs. 1 and Fig. 2*a*). 5S-CA apparently does not interact with the 4S estradiol receptor form, which resolved in a distinct 4S peak. For all three mammalian species, 5S-CA caused the 4S and 5S forms of the estradiol receptor to sediment as distinct peaks.

In collaborative experiments conducted in our laboratory, Adele Salhanick and Ian Callard have demonstrated an estradiol receptor from turtle (*Chrysemys picta*) oviduct and shown that it partially converts to a 5S form on DNA-cellulose. This 5S species complexes with 5S-CA; the sedimentation profiles with and without 5S-CA are virtually the same as shown for calf uterine receptor (Fig. 1*b*). Thus, the effect of 5S-CA is the same for turtle oviduct as for mouse, calf, and rat uterine 5S estradiol receptors.

Interaction with 5S-CA of the 5S Estradiol Receptor Formed from Extracts of Mouse Hypothalamus. The cytosol estradiol receptor from mouse hypothalamus sediments at 4S (Fig. 3*a*) as also occurs for uterine cytosol (8). Under the standard conditions for DNA-cellulose chromatography (with all steps at 2°), the hypothalamic receptor also elutes as a 4S species (6, 7) whereas the uterine receptor elutes mostly as a 5S species (ref. 8; Fig. 1 and Fig. 2*a*). However, when [^{3}H]estradiol-labeled hypothalamic extract on DNA-cellulose was heated at 30°, washed, and then eluted, a 5S peak could be observed (Fig. 3*b*). In this experiment, the eluted receptor was completely in a 5S form.

Because the hypothalamic 5S receptor required heat (30°) for its formation on DNA-cellulose (7), it might be argued that this 5S peak is an artifact caused by the heat treatment. Furthermore, different *in vivo* (9) and chromatographic (6–8) behaviors of the hypothalamic and uterine receptors might suggest that the properties of the 5S receptors from the two tissues differ significantly. These issues were addressed by comparing the cytosol and nuclear receptors from hypothalamic extracts for their interactions with 5S-CA. The cytosol (4S) estradiol receptor sedimented at 4 S even in the presence of 5S-CA and therefore did not form a significant amount of complex with the serum complement (Fig. 3*a*). On the other hand, 5S hypothalamic receptor produced by the temperature-dependent DNA-cellulose process did react essentially completely with the 5S-CA (Fig. 3*b*). Consequently, 5S-CA reveals that the 5S forms of estradiol receptor obtained *in vitro* for both uterus and hypothalamus are similar to one another and both differ in the same manner from the 4S form.

Evidence for the Similarity of 5S Estradiol Receptors Formed *In Vivo* and *In Vitro*. The hypothesis that nuclear estradiol receptor formed *in vitro* from extracts is the same as the nuclear receptor that can be isolated from nuclei has rested on their similarity as judged by sedimentation criteria. To test this similarity further, the nuclear receptor formed by chromatographing [^{3}H]estradiol-labeled rat uterine extract on DNA-cellulose (Fig. 2*a*) was compared to nuclear receptor that was obtained by eluting nuclear/myofibrillar pellets from uteri of rats injected with [^{3}H]estradiol (Fig. 2*b*). Both extracts of nuclear receptor contained 4S and 5S species. 5S-CA added to each extract resolved the complexes into uncomplexed 4S peaks and complexed "5S" species that then sedimented to about 9 S. This provides a new criterion for comparing 5S nuclear receptors obtained *in vivo* with those that are formed by *in vitro* manipulations.

Inspection of the receptor obtained after *in vivo* injection of [^{3}H]estradiol (Fig. 2*b*) could yield the conclusion that nuclear estradiol receptor sediments as a 5S peak. Only after complexing with 5S-CA is it unmistakably obvious that this peak contains both 4S and 5S receptor species. I have obtained this same result for a nuclear/myofibrillar extract of uteri from mice injected with [^{3}H]estradiol (data not shown). Thus, 5S-CA allows a more precise description of the receptor forms in high-salt extracts from nuclei-containing cell fractions.

DISCUSSION

5S-CA, a partially purified serum factor, has been used to distinguish cytosol 4S and nuclear 5S receptors for estradiol. The interactions of 5S-CA with 5S receptors from different sources yield the following conclusions: (*i*) 5S forms of estradiol receptor from one nonmammalian and three mammalian species have a common interaction with 5S-CA serum factor; (*ii*) the 5S receptor formed *in vitro* from hypothalamic extract exhibits the same 5S-CA interaction as uterine 5S receptor, even though it derives from a separate tissue and forms with different parameters both *in vitro* and *in vivo* (7); and (*iii*) the rat 5S receptor formed *in vitro* on DNA-cellulose is selectively complexed by 5S-CA, as is the 5S receptor formed *in vivo* and recovered from nuclear/myofibrillar pellets, suggesting their chemical as well as physical similarity.

The results presented in this report demonstrate the generality of the 5S-CA interaction with nuclear (5S) but not cytosol (4S) receptor. The interaction is indistinguishable among several

species and for different tissues. The data also provide a new chemical criterion for accepting *in vitro*-produced 5S receptor as a model of *in vivo* nuclear estradiol receptor. This latter demonstration can be used for future analyses of *in vitro* 5S formation. This will be especially crucial for situations in which the required conditions differ from those for rat or mouse uterine receptor—for example, mouse hypothalamus *versus* uterus (6, 7, 9), and the nontypical conditions reportedly required (10) and sedimentation coefficient obtained (11) for human estradiol receptor. The selective interaction of 5S-CA for different species and tissues and for both *in vivo*- and *in vitro*-derived 5S receptor increases the likelihood that this receptor form is not simply an artifact of *in vitro* manipulations of the cytosol (4S) receptor. It is not yet known whether 5S-CA is a single entity or is a class of molecules that interact similarly with closely related, although possibly nonidentical, 5S receptors.

The 5S-CA effect supports the hypothesis that the 5S receptor form consists of 4S receptor coupled to a second factor, subunit X (12). This hypothesis results from experiments demonstrating that the transformation of 4S to 5S receptor precedes via a bimolecular reaction (13, 14). If subunit X is different from 4S receptor, and if 5S-CA physically recognizes subunit X, then the presence of subunit X in 5S, but not in 4S, estradiol receptor may explain the selective action of 5S-CA. Alternate possibilities include: an incipient binding site for 5S-CA, not present on 4S receptor or subunit X, may accompany 5S formation; or, if subunit X is identical to 4S receptor, making 5S a dimer, then bivalent binding to 5S-CA may be detected, whereas monomeric 4S may bind 5S-CA only weakly and nondetectably. To demonstrate that 5S-CA interacts with the receptor macromolecule, rather than with the bound estradiol hormone, gradients were run in the presence of a large excess (1–100 μM) of unbound, nonradioactive estradiol (ref. 6; data not shown). The 5S-CA effect was not altered. Furthermore, the serum fraction did not bind [^{3}H]estradiol that was added directly. Thus, 5S-CA may provide a readily obtainable reagent for directly tagging a portion of the estradiol receptor complex. With a direct probe for the 5S receptor it may be possible eventually to determine if this form actually exists within cells prior to fractionation and whether its existence is physiologically significant.

The widely varied sources of 5S-CA suggest caution in the use of antisera in steroid hormone and receptor research. Four years ago I undertook preliminary experiments to elicit antisera by using preparations of partially purified rat uterine estradiol receptor as antigen (6). An activity was observed in immune rabbit serum that selectively complexed the 5S nuclear receptor form but not the 4S cytosol form of mouse estradiol receptor. Subsequent experiments and controls with other sera indicated that 5S-CA is present in many sera from many animals immunized against an array of seemingly unrelated antigens. This activity has now been observed in sera from rabbits, goats, and sheep. The antigens that were used to immunize 12 sources from which 5S-CA was obtained include rabbit gamma globulins (in more than half a dozen different goats), basic myelin protein, and bacterial cell wall fractions (rabbits), and dihydrotestosterone and estradiol attached as hemisuccinates to albumin (in three sheep). It is an added technical advantage of 5S-CA that the interaction with 5S nuclear receptor occurs under the stringent conditions of high ionic strength. The interaction also occurs in 1.0 M NaCl. So far, only one serum from a rabbit that was not intentionally immunized has exhibited 5S-CA, and this complexing activity occurred only in 0.15 M NaCl and not in 0.4 M NaCl.

The presence of 5S-CA in several sera from animals immunized with many different antigens certainly suggests that some sera immunized with steroid analogues, receptors themselves, and other antigens might well contain this activity. Antisera against steroids have been used to compare bound and unbound hormones in labeled receptor extracts (15) and to reduce circulating levels of specific steroids in animals (16) and in tissue cultures (17). The demonstration of 5S-CA in some sera prepared by immunization against derivatives of dihydrotestosterone and estradiol (ref. 6; data not shown) requires consideration of possible interactions of serum components with receptors or their components.

It is especially interesting to consider the recent report of antibodies directed against uterine estradiol receptors (18). Using a sedimentation assay comparable to mine, these investigators demonstrated interactions of estradiol receptor-directed antisera with both cytosol and nuclear receptor forms. Curiously, their results may also indicate an additional and independent confirmation of the existence of 5S-CA. Besides the interaction of their antisera with both cytosol and nuclear receptor, they also showed a second complex of nuclear receptor, just as we see for several immune sera. Thus the antisera of Greene *et al.* (18) may contain not only antibodies that bind both cytosol and nuclear receptors but also a second component, perhaps 5S-CA.

A major discrepancy in the literature on estradiol receptors may have as one source the presence of factors like 5S-CA. Using antisera to rat α-fetoprotein, Michel *et al.* (19) showed no interaction of α-fetoprotein-directed antisera with estradiol receptor, whereas a later report (20) claimed an interaction of anti-α-fetoprotein with a form of rat uterine estradiol receptor. Although other studies have demonstrated nonidentity of estradiol receptor and α-fetoprotein for brain tissues (21, 22) and uterus (23), the serum reported (20) to interact with estradiol receptor may have contained a 5S-CA-like component. This is the type of result that one might predict, given the 5S-CA effects shown here. It may be especially necessary to check for these complications when potential antigenic entities are in very low (1 pM–1 nM) concentrations, as are the receptors in these experiments.

In addition to its use as an analytical tool, 5S-CA could also serve a physiological function. Wira and Sandoe (24) have suggested that uterine secretions accumulate immunoglobulins A and G in an estradiol-dependent fashion. They suggest that this may represent a functional interdependence of the endocrine and immune systems. It may be profitable to ask if 5S-CA is a component of such a proposed system or whether 5S-CA might be present in their sera and thus might be interacting with components of the uterine secretions. For these reasons it may be significant that 5S-CA is detected in several immune sera.

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