Proc. Natl. Acad. Sci. USA
Vol. 77, No. 10, pp. 5678–5682, October 1980
Biochemistry

Immunocytochemical localization of the sex steroid-binding protein of plasma in tissues of the adult monkey *Macaca nemestrina*

(prostate/epididymis/testes/androgen receptor/MCF-7 cells)

SANDRA BORDIN AND PHILIP H. PETRA*

Departments of Obstetrics and Gynecology and Biochemistry, University of Washington, Seattle, Washington 98195

Communicated by Hans Neurath, June 13, 1980

ABSTRACT **The sex steroid-binding protein (SBP) present in the serum of the monkey *Macaca nemestrina* is shown to exist in cells of tissue involved in reproduction. The localization was demonstrated by immunofluorescence with monospecific antibodies raised against homogeneous human SBP. These antibodies were previously shown to crossreact with monkey SBP. The protein appears to be located in the cytoplasm of epithelial cells lining the prostate alveoli, the ducti of the epididymis, and the seminiferous tubula of the testes of the monkey. The protein is also present in the cytoplasm of parenchymal cells of the liver, where SBP is believed to be synthesized, and in cells of the adrenal cortex, where steroids are known to be synthesized. Controls appear dark and illustrate specificity of the immunofluorescence, ruling out both tissue autofluorescence and other nonspecific interactions. In all cases, the relative intensity of fluorescence appears minimal in the nuclei of cells. Experiments performed with cultured MCF-7 cells indicate that SBP can across the plasma membrane and enter into the cytoplasm but not into the nucleus. Additional studies indicate that the monospecific antibodies do not crossreact with the monkey prostate androgen receptor, as shown by ultracentrifugation in sucrose density gradients. The physiological significance of these observations is not known; however, the existence of this protein in cells of target tissues for sex steroids introduces a new dimension in our thinking about the role of this protein in androgen action.**

The plasma of humans and that of most species tested contain a protein, SBP, which specifically binds 5α-dihydrotestosterone (DHT) and testosterone with high affinity and estradiol-17β with lower affinity depending upon the species. This protein has been purified to homogeneity from the plasma of humans (1–4), rabbits (5), cows (6), and dogs (7). The physicochemical characteristics of the protein isolated from each of these species have been described (3–9).

Various reports have appeared suggesting that, in humans, SBP may enter interstitial fluid and tissues and, therefore, may not be exclusively localized in the plasma (10–13). Others, however, have suggested that this observed presence of SBP in cell extracts is due to plasma contamination when the tissues are homogenized. Although the nature of the experimental evidence gathered from those studies does not allow us to distinguish between these two possibilities, it becomes imperative to establish whether or not SBP exists inside cells of target tissues. The consequence of tissue localization of this protein will undoubtedly affect our thoughts and future experimentation with regards to the physiological role of SBP in relation to sex steroid hormone action.

We have been able to prepare monospecific antibodies against homogeneous human SBP (14). These antibodies also crossreact with nonhuman primate SBP, such as that present in the plasma of the monkey (*Macaca nemestrina*) and the baboon (*Papio cynocephalus*) (14). The antibodies, however, do not crossreact with the plasma of any other animals tested, including rabbits, dogs, cats, sheep, goats, cows, and calves (14). Using the monkey model, we have presented preliminary evidence for the existence of SBP within tissue cells.† In this report, we formally describe our results in detail and discuss the possible implication of these findings in relation to already existing information.

METHODS

SBP was purified from human serum essentially as described (1) except for a modified affinity chromatographic procedure (2). In some cases, the purified protein was passed through an antitransferrin–agarose column to remove traces of transferrin. This immunoadsorbent was synthesized as follows: 12 ml of Affigel-10 (Bio-Rad) was washed rapidly with cold distilled water on a small fritted-disc funnel, dried gently, and added to a solution containing 2 ml (58 mg) of rabbit anti-human transferrin immunoglobulins (Bio-Rad) in 2 ml of 0.2 M $NaHCO_3$ (pH 8.0). The mixture was shaken gently at 4°C overnight. The next day, 0.2 ml of 1 M ethanolamine was added and the mixture was incubated for 1 hr at 25°C to inactivate all remaining reactive ester groups. The gel was equilibrated with 19.3 μM DHT in buffer A [0.1 M NaCl/10% (vol/vol) glycerol/10 mM Tris·HCl, pH 7.4] and poured into a column at 4°C. Purified human SBP was applied to the column in a volume equal to the void volume of the column (6 ml), stoppered, and eluted the next day. This step removes all traces of transferrin.

Human SBP antibodies were prepared and partially purified as described (14). The antibodies were further purified by immunoadsorption on a column of agarose containing covalently attached human SBP. This antigen column was prepared as follows: homogeneous human SBP (8 mg) was coupled to 12 ml of Affigel-10 as described above for the antitransferrin immunoadsorbent. Rabbit immunoglobulins, purified by $(NH_4)_2SO_4$ precipitation and DEAE-Sephadex A-50 chromatography, were applied to the SBP–agarose column and incubated overnight at 4°C. Unadsorbed immunoglobulins (lacking the specific SBP antibodies) were eluted the next day by washing the column with 50 ml of DHT/buffer A. This immunoglobulin fraction was used for the control immunofluorescence experiments. The specific antibodies were then eluted from the column by washing with 30 ml of DHT/buffer A

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: SBP, sex steroid-binding protein of serum; DHT, 5α-dihydrotestosterone; buffer A, 0.1 M NaCl/10% (vol/vol) glycerol/10 mM Tris·HCl, pH 7.4; buffer B, 10 mM Tris·HCl/1.5 mM EDTA/0.5 mM dithiothreitol/10% glycerol, pH 7.4; R-1881, methyltrienolone; ABP, androgen-binding protein.

* To whom reprint requests should be addressed.

† Bordin, S. & Petra, P. H. (1979) 61st Annual Meeting of the Endocrine Society, abstr. 526.

containing 0.2 M glycine (pH 2.2). The antibodies were immediately brought to pH 7.4, dialyzed for 24 hr against several changes of phosphate-buffered saline (pH 7.4), and concentrated to 0.5 mg of protein per ml by ultrafiltration. The isolated specific antibodies represent 1% of the total immunoglobulin fraction. The SBP–agarose column was re-equilibrated with DHT/buffer A and kept at 4°C.

Fresh tissue specimens of normal male monkeys (*M. nemestrina*, 5–6 years old) and normal baboons (*P. cynocephalus*, 5–6 years old) were obtained from the Regional Primate Center at the University of Washington. After surgical removal, tissues were extensively washed with cold phosphate-buffered saline, blotted dry, and frozen in liquid nitrogen. Frozen sections (4 μm thick) were cut in a cryostat at 20°C and mounted on glass slides wetted with a solution of 0.3% protamine sulfate (grade II, Sigma).

Immunofluorescence studies were performed by the indirect method (15). Frozen cut sections were fixed and dehydrated with 80% (vol/vol) acetone at 20°C for 5 min, then rinsed in phosphate-buffered saline. Sections were covered with 200 μl of antibodies (3 mg/ml) or affinity column-purified antibodies (50 μg/ml) diluted 1:10 in phosphate-buffered saline. Both test and control tissue sections were covered with 200 μl of fluorescein-conjugated goat antiserum to rabbit gamma globulins (Miles-Yeda, Rehovot, Israel) diluted 1:20 in phosphate-buffered saline, incubated at 25°C for 30 min, and rinsed with phosphate-buffered saline. The slides were mounted with 50% glycerol/phosphate-buffered saline and studied with a Zeiss microscope equipped with fluorescent optics. Tissue autofluorescence under the conditions of the experiment was determined by hydrating the tissues and mounting them for observation. Some preparations were counterstained with 0.1% toluidine blue to eliminate nonspecific background and enhance cellular details.

The androgen receptor in monkey prostate cytosol was determined as follows. Prostate tissue (≈3.5 g) was rinsed in ice-cold buffer B (10 mM Tris·HCl/1.5 mM EDTA/0.5 mM dithiothreitol/10% glycerol, pH 7.4), mixed, and homogenized in 3 vol of buffer B in a Polytron homogenizer (type PT-10) by two 10-sec pulses with a 50-sec pause between the pulses. The homogenate was centrifuged at 105,000 × *g* for 90 min at 0°C in a Beckman 50Ti rotor. Cytosol aliquots (300 μl) were incubated for 2 hr at 0°C with 5 nM [^{3}H]R-1881 (methyltrienolone; specific activity, 87 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) or 5 nM [^{3}H]DHT (specific activity, 123 Ci/mmol) in the presence or absence of 200-fold molar excess of unlabeled R-1881 or DHT. Unbound steroid was removed by adding each aliquot to a dextran–charcoal pellet (prepared from 0.5 ml of 0.005% dextran/0.5% charcoal in 10 mM Tris·HCl, pH 7.4), stirring for 10 min at 0°C, and centrifuging at 2000 × *g* for 10 min. Aliquots (200 μl) were layered on 5–20% sucrose density gradients prepared in buffer B and centrifuged at 322,000 × *g* for 16 hr in an SW65-Ti rotor. Fractions were collected in an ISCO model 640 fractionator; radioactivity was measured in 4 ml of scintillation fluid (4 g of Omnifluor per liter of toluene) after the two phases were mixed thoroughly (16). In order to demonstrate the presence of human SBP in these cytosols, a double-antibody incubation followed by sucrose density gradient centrifugation was done as follows. Cytosol aliquots (300 μl), already equilibrated with [^{3}H]R-1881 or [^{3}H]DHT and treated with charcoal, were further incubated with 10 μl (3 mg/ml) of human SBP antibodies for 12 hr at 4°C. An aliquot (10 μl) of goat antiserum to rabbit gamma globulins (162 μg/ml) was added and the solution was incubated an additonal 8 hr at 4°C. Aliquots (200 μl) were layered on 5–20% sucrose gradients in buffer B and centrifuged in a SW-50Ti rotor at 322,000 × *g*. A control of monkey plasma was carried through the same procedure as follows. Serum taken from the same monkey from which the prostate was removed was diluted 1:50 with 10 mM Tris·HCl (pH 7.4) and incubated for 1 hr at 25°C with 10 nM [^{3}H]DHT in the presence and absence of 200-fold molar excess of unlabeled DHT. Unbound steroid was removed with dextran–charcoal prior to all centrifugation. Half of the samples were carried through the double-antibody incubation, and aliquots (200 μl) were layered on sucrose density gradients and centrifuged.

MCF-7 cells, a gift of Charles M. McGrath (Michigan Cancer Foundation), were received at passage 239 and were serially cultured in our laboratory for 1 year. Cells were subcultured (1:5) every 3 weeks by using trypsin (0.05%) in Ca^{2+}- and Mg^{2+}-free balanced salt solution. Cells (1 × 10^5) were plated into Leighton tubes containing rectangular (5 cm^2 surface) coverslips and grown at 37°C in Eagle's minimal essential medium supplemented with 10% (vol/vol) calf serum (GIBCO) and 10 μg of insulin per ml (Sigma). Cultures were 50% confluent 3 days after plating. After the cultures were replenished with serum-free growth medium, homogeneous human SBP saturated with DHT was added at a concentration of 500 nM and incubated for 1 hr at 37°C. Cells were fixed in 80% cold acetone after they were washed with phosphate-buffered saline; they were then incubated with antibodies for immunofluorescence. For the peroxidase method, fixed cells were incubated with human SBP antibodies, washed, incubated for 30 min with peroxidase-conjugated anti-rabbit IgG (Miles) diluted 1:20, and washed three times with phosphate-buffered saline. Peroxidase activity was developed with 3,3-diaminobenzidine (0.5 μg/ml)

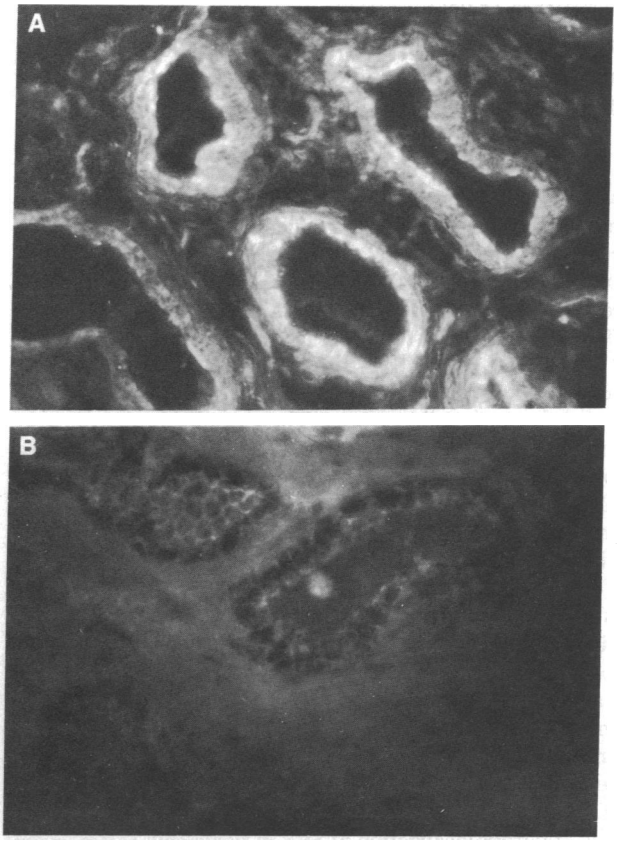

FIG. 1. (*A*) Immunofluorescent localization of SBP in cells of monkey prostate. (*B*) Same conditions except that rabbit Ig from immunized rabbits were passed through the antigen–agarose column. Controls for the other tissues shown in Fig. 2 and 4 also appear dark as in the case of prostate tissue. (×100.)

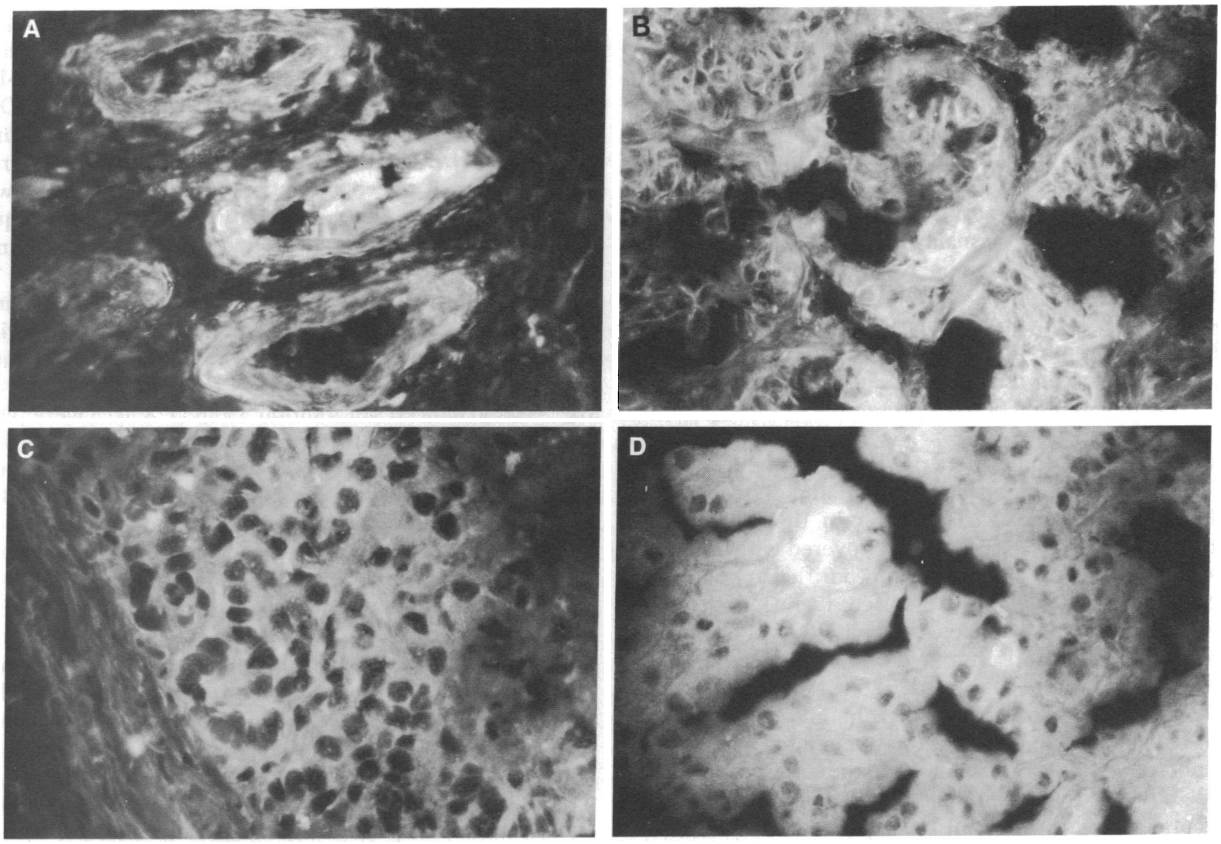

FIG. 2. Immunofluorescent localization of SBP in cells of monkey epididymis (*A*), testis (*B*), adrenal (*C*), and liver (*D*). Controls showed no fluorescence. (×100.)

in 50 mM Tris·HCl (pH 7.6) containing 0.005% hydrogen peroxide. Cells were examined under a light microscope (Zeiss).

RESULTS AND DISCUSSION

Figs. 1 and 2 illustrate the presence of SBP of *M. nemestrina* in cells of tissues involved in reproduction and known to contain receptors for DHT and estradiol-17β. The protein appears to be located in the cytoplasm of epithelial cells lining the prostate alveoli (Fig. 1*A*), the ducti of the epididymis (Fig. 2*A*), and the seminiferous tubula of the testes (Fig. 2*B*). The localization of SBP in the epithelial cells of the prostate does not agree with the data of Cowan *et al.* (13), who have suggested that the protein is located in the stroma. Their results, however, were obtained from disaggregated tissue from which cytosols were prepared and incubated with tritiated DHT to estimate steroid binding. Because the tissue was not intact during the procedure, interpretation of the results becomes questionable. In contrast, we find very little immunofluorescence in the stroma (Fig. 1*A*). The protein is also present in the cytoplasm of parenchymal cells of the liver (Fig. 2*D*), where SBP is believed to be synthesized, and in cells of the adrenal cortex (Fig. 2*C*), where steroids are known to be synthesized. Controls appear dark (Fig. 1*B*) and illustrate the specificity of the immunofluorescence, ruling out both tissue autofluorescence and other nonspecific interactions. In all cases, the relative intensity of fluorescence appears minimal in the nuclei of cells, particularly in the liver (Fig. 2*D*) and the adrenal cortex (Fig. 2*C*).

The apparent localization of SBP in tissue cells raises several questions because most hypotheses regarding its function have been formulated by assuming a physiological role solely in plasma. The first question deals with the specificity of the observed immunofluorescence. Although we know that the immunological reaction is very specific for human SBP (14), we cannot rule out the possibility that the antibodies may also recognize other proteins that share common antigenic determinants. Steroid-binding proteins can be divided into three major classes: extracellular proteins such as SBP and CBG (transcortin), the intracellular proteins such as steroid receptors, and enzymes that catalyze the metabolism of steroids. All three classes have specific steroid-binding sites and would be expected, in theory, to be more or less structurally related to one another. For instance, SBP, Δ^4-steroid 5α-reductase, and the prostate androgen receptor all recognize DHT and, consequently, must have common structural elements in the steroid-binding site. Conservation in protein structure would have arisen from divergent evolutionary processes allowed to take place through time on some ancestral steroid-binding protein. Although the steroid-binding site represents a relatively small portion of the protein molecule, it is possible for some antibodies to recognize it. In order to test this idea, an extract of monkey prostate cytosol containing the androgen receptor was incubated with human SBP antibodies and centrifuged in sucrose density gradients along with a monkey plasma control. Fig. 3*A* illustrates the control, in which monkey SBP, shown to sediment in the 4S area in the absence of SBP antibodies, migrated to the bottom of the tube as an SBP–antibody complex when the antibodies were added prior to centrifugation. Fig. 3*B* shows the analogous experiment in monkey prostate cytosol, in which the specific radioactivity originally in the 4S area, indicating the presence of SBP consistent with the immunofluorescence data, also migrated to the bottom of the tube when SBP antibodies were added. However, the specific radioactivity in the 8S area corresponding to the androgen receptor was not displaced, indicating that the antibodies did not crossreact with the androgen receptor. The relative concentration of the specific antibodies

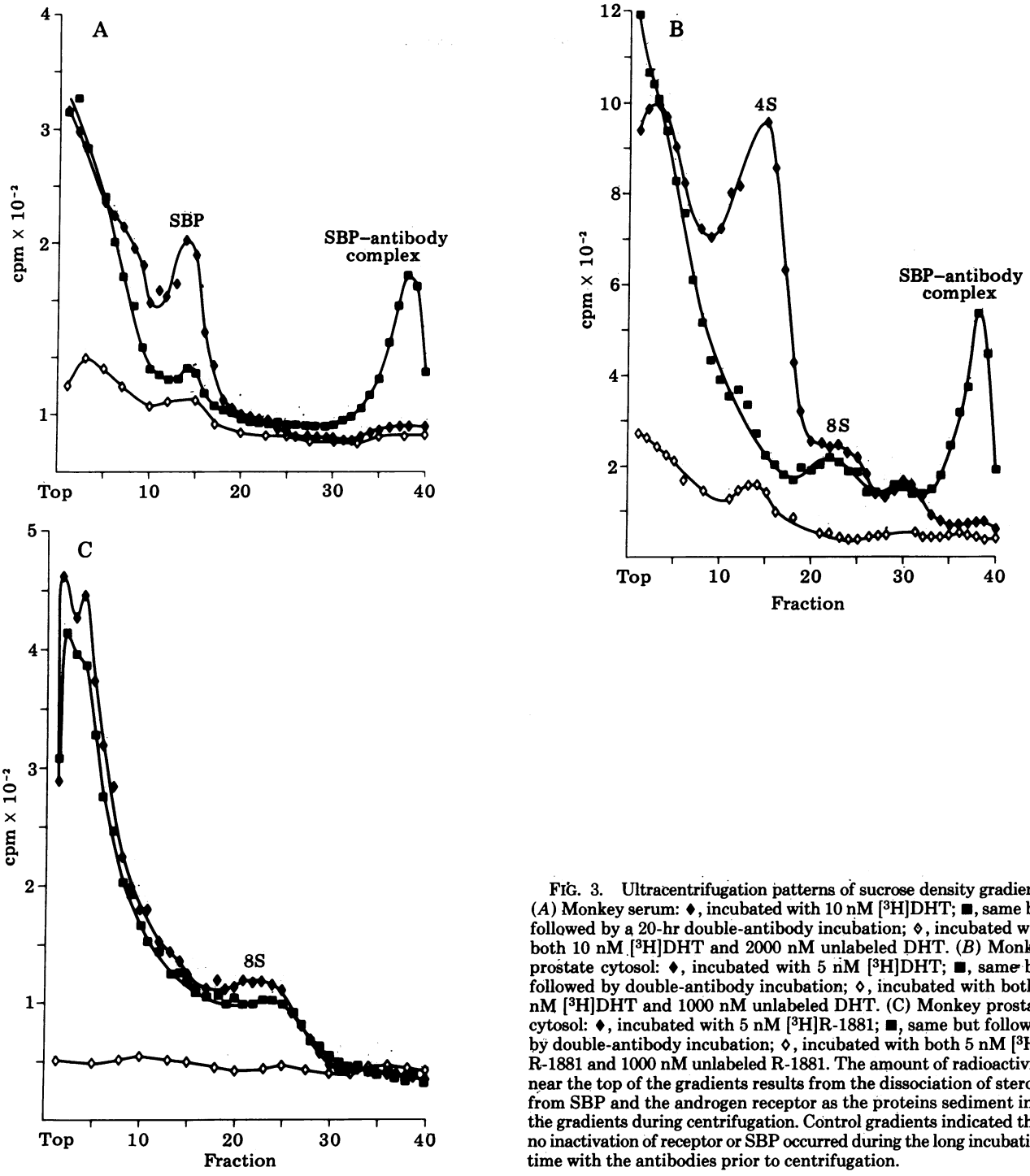

FIG. 3. Ultracentrifugation patterns of sucrose density gradients. (*A*) Monkey serum: ♦, incubated with 10 nM [^{3}H]DHT; ■, same but followed by a 20-hr double-antibody incubation; ◊, incubated with both 10 nM [^{3}H]DHT and 2000 nM unlabeled DHT. (*B*) Monkey prostate cytosol: ♦, incubated with 5 nM [^{3}H]DHT; ■, same but followed by double-antibody incubation; ◊, incubated with both 5 nM [^{3}H]DHT and 1000 nM unlabeled DHT. (C) Monkey prostate cytosol: ♦, incubated with 5 nM [^{3}H]R-1881; ■, same but followed by double-antibody incubation; ◊, incubated with both 5 nM [^{3}H]-R-1881 and 1000 nM unlabeled R-1881. The amount of radioactivity near the top of the gradients results from the dissociation of steroid from SBP and the androgen receptor as the proteins sediment into the gradients during centrifugation. Control gradients indicated that no inactivation of receptor or SBP occurred during the long incubation time with the antibodies prior to centrifugation.

directed at the binding site was apparently too small to interact with the receptor. The results in Fig. 3*C* with a synthetic androgen, R-1881, that binds to the androgen receptor but not to SBP (17) not only confirm those of Fig. 3*B* but also indicate that the displaced radioactivity in the 4S area of Fig. 3*B* originated from SBP and not from a 4S androgen receptor. The antibody–SBP complexes are radioactive and therefore consist of trinary complexes of DHT–SBP–gamma globulins, which suggests that the major interaction with antibodies does not affect steroid binding. In conclusion, these data, in conjunction with those of immunofluorescence, strongly indicate that SBP exists in tissue cells of the monkey prostate.

The next question to consider is whether or not SBP can be shown to enter into tissue cells. The immunofluorescence data presented in Figs. 1 and 2 only reveal the presence of SBP; they do not allow us to conclude anything about its origin. To this end we incubated serially cultured MCF-7 cells in the presence or absence of homogenous human SBP in the culture medium to find out if SBP can enter into the cells. This human breast cancer cell line was chosen because it originates from a tissue involved in reproduction and contains steroid receptors (18). Also, because the cells had been cultured for years through a total of 239 passages before we acquired them, they were no longer exposed to human SBP. Fig. 4 indicates the presence of SBP in cells exposed to the protein and supports the contention that SBP can indeed cross the plasma membrane and enter cells.

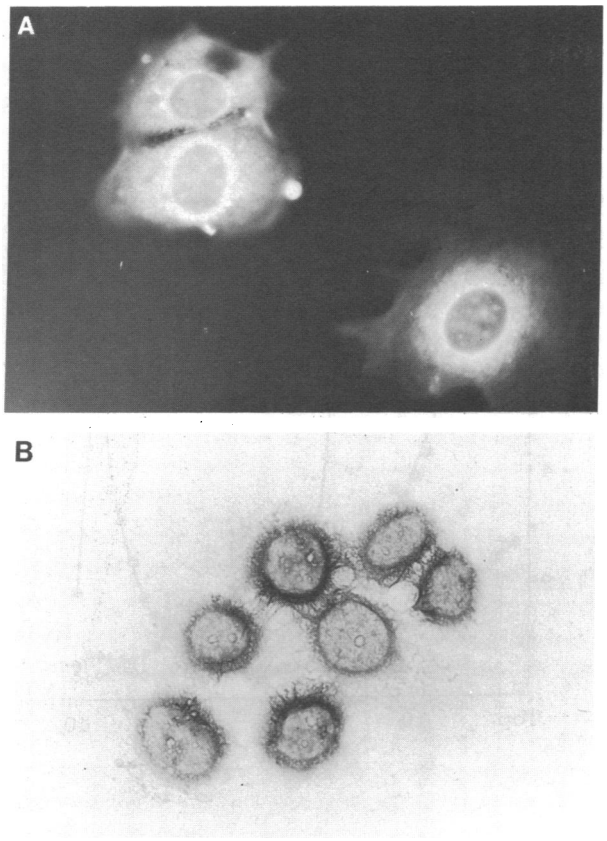

FIG. 4. (*A*) Immunofluorescent localization of SBP in MCF-7 cells (1×10^5 cells). The cells not exposed to SBP did not fluoresce. (*B*) Same except that the immunoperoxidase method of staining was used. Controls carried out with preimmune serum did not fluoresce and showed no staining with the immunoperoxidase method. (×240.)

Furthermore, the fluorescence and staining appear to be localized around the nucleus, which suggests a possible interaction with the nuclear membrane. HeLa cells incubated with SBP under the same conditions did not fluoresce.

There are several questions that remain to be resolved. One deals with the physiological and biochemical relationship between SBP and another androgen-binding protein, ABP, which appears to have identical biochemical properties. ABP has been shown to exist in the epididymis and testes of several species (19). In humans and nonhuman primates, however, there is controversy with regards to its existence. It has been suggested that the steroid-binding protein detected in primate testicular and epididymal cytosol is actually SBP arising from blood contamination when the tissues are homogenized (20). However, others have indicated that ABP and SBP can be distinguished electrophoretically due to differences in sialic acid content of their carbohydrate moiety (21). The data presented here cannot resolve the controversy. Certainly, the immunofluorescence data of Fig. 2 rule out blood contamination because the protein is found in cells. However, our method could not distinguish between the two proteins if both were to share a large number of common antigenic determinants. Such would be the case if both proteins were products of the same gene expressed in different tissues; variation only in carbohydrate content and the ubiquitous nature of the proteins support this view. Purification of the epididymal and testicular protein and comparison with SBP will be required before the issue can be resolved. Also, information on the localization of the biosynthesis of SBP will be needed to evaluate the proposal of multiple gene expression. We therefore conclude from the data presented here that SBP or a similar gene product, possibly ABP, exists intracellularly. For ABP, the protein would probably be synthesized within the tissue instead of originating from plasma. Although the physiological significance of these observations is not known, the presence of SBP in target tissues for sex steroids introduces a new dimension in our thinking about the role of this protein in androgen action. Intracellular localization means that the protein will necessarily have a more extensive role than that of simply controlling the unbound concentration of androgens in plasma. We believe that wherever androgens (and possibly estrogens) are located in the organism, SBP will be present by serving as a specific carrier of steroid from plasma into cells that possess steroid receptors and as a specific carrier of sex steroids into plasma from cells that synthesize them. More experimentation will be needed, particularly in primates, before we can formulate a rational hypothesis for SBP function.

This research was supported by National Institutes of Health Grant HD 07190 (to P.H.P.), the Regional Primate Research Center at the University of Washington, National Institutes of Health Grant 00166, and Institutional Cancer Grant IN-26 "S" from the American Cancer Society (to S.B.).

1. Mickelson, K. E., Teller, D. C. & Petra, P. H. (1978) *Biochemistry* **17**, 1409–1415.
2. Petra, P. H. & Lewis, J. (1980) *Anal. Biochem.* **105**, 165–169.
3. Mercier-Bodard, C., Renoir, J. M. & Baulieu, E.-E. (1979) *J. Steroid Biochem.* **11**, 253–259.
4. Mische, W., Weise, H. C., Graesslin, D., Rusch, R. & Tamm, Y. (1979) *Acta Endocrinol.* **90**, 737–742.
5. Mickelson, K. E. & Petra, P. H. (1978) *J. Biol. Chem.* **253**, 5293–5298.
6. Suzuki, Y., Itagaki, E., Mozi, H. & Hosoya, T. (1977) *J. Biochem.* **81**, 1721–1731.
7. Suzuki, Y., Okumara, Y. & Sinohara, H. (1979) *J. Biochem.* **85**, 1195–1203.
8. Petra, P. H. (1979) *J. Steroid Biochem.* **11**, 245–252.
9. Hansson, V., Ritzen, E. N., Weddington, S. C., McLean, W. S., Tindall, D. J., Nayfeh, S. N. & French, F. S. (1974) *Endocrinology* **95**, 690–700.
10. Rosen, V., Jung, I., Baulieu, E.-E. & Robel, P. (1975) *J. Clin. Endocrinol. Metab.* **41**, 761–770.
11. Steins, P., Krieg, H., Hollmann, H. Y. & Voigt, K. D. (1974) *Acta Endocrinol. Copenhagen* **75**, 773–784.
12. Jung-Testas, I., Mercier-Bodard, C. & Robel, P. (1976) *Ann. Endocrinol.* **37**, 97–98.
13. Cowan, R. A., Cowan, S. K., Giles, C. A. & Grant, J. K. (1976) *J. Endocrinol.* **71**, 121–131.
14. Bordin, S., Lewis, J. & Petra, P. H. (1978) *Biochem. Biophys. Res. Commun.* **85**, 391–401.
15. Coons, A. H. & Kaplan, M. H. (1950) *J. Exp. Med.* **91**, 1–13.
16. Namkung, P. C., Moe, R. E. & Petra, P. H. (1979) *Cancer Res.* **39**, 1124–1125.
17. Bonne, C. & Raynaud, J.-P. (1975) *Steroids* **26**, 227–232.
18. Brooks, S. M., Locke, E. R. & Soule, H. D. (1973) *J. Biol. Chem.* **248**, 6251–6253.
19. Hansson, V., Ritzen, E. M., French, F. S. & Nayfeh, S. N. (1975) *Handb. Physiol.* **5**, 173–201.
20. Vigersky, R. A., Loriaux, D. L., Howards, S. S., Hodgen, G. B., Lipsett, M. & Chrambach, A. (1976) *J. Clin. Invest.* **38**, 1061–1068.
21. Hsu, A.-F. & Troen, P. (1978) *J. Clin. Invest.* **61**, 1611–1619.