Epithelial-Mesenchymal Interactions in Prostatic Development. II. Biochemical Observations of Prostatic Induction by Urogenital Sinus Mesenchyme in Epithelium of the Adult Rodent Urinary Bladder

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ABSTRACT Adult bladder epithelium (BLE) is induced to differentiate into glandular epithelium after association with urogenital sinus mesenchyme (UGM) and subsequent in vivo growth in syngeneic male hosts. Alteration of epithelial cytodifferentiation is associated with the expression of prostate-specific antigens, histochemical and steroid metabolic activities. These observations suggest that the inductive influence of the UGM has reprogrammed both the morphological and functional characteristics of the urothelium. In this report, differences regarding the mechanisms and effects of androgenic stimulation of prostate and bladder are exploited to determine the extent to which UGM plus BLE recombinants express a prostatelike, androgen-dependent phenotype.

Results from cytosolic and autoradiographic binding studies suggest that androgen binding is induced in UGM plus BLE recombinants and that this activity is accounted for by the induced urothelial cells. In UGM plus BLE recombinants, androgen-induced [^{3}H]thymidine or [^{35}S]-methionine uptake analyzed by two-dimensional gel electrophoresis was qualitatively and quantitatively similar to that of prostate as opposed to bladder.

These studies indicate that expression within BLE of prostatic phenotype is associated with a loss of urothelial characteristics and that androgen sensitivity is presumably a function of the inductive activities of the stroma.

Adult bladder epithelium (BLE) is induced to differentiate into a glandular epithelium after association with urogenital sinus mesenchyme (UGM) (1, 2). This change in epithelial cytodifferentiation is associated with the expression of prostate-specific antigens as well as prostatelike histochemical and steroid metabolic activities (2; Neubauer, B. L., N. G. Anderson, G. R. Cunha, J. F. Towell, and L. W. K. Chung. Submitted for publication.). These later observations suggest the possibility that the inductive influence of UGM has reprogrammed both the morphological and the functional (biochemical) characteristics of the urothelium. In this report, differences regarding the mechanisms and effects of androgenic stimulation on prostate and bladder are exploited to determine the extent to which UGM plus BLE recombinants express a prostatelike, androgen-dependent phenotype.

MATERIALS AND METHODS

Animals: Inbred C57/6J BL mice (The Jackson Laboratory, Bar Harbor, ME) and CDF rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were used. Urogenital sinuses of male and female 15–16-d embryonic mice and 16–17-d embryonic rats (vaginal plug equals day 0), and urinary bladders obtained from adult mice (20–30 g) and rats (200–250 g) were separated into their epithelial and stromal (mesenchymal) components by methods previously described (2). Recombination of the UGM and BLE, in vivo grafting techniques, and the cleanness of separation of the tissue elements were all monitored by criteria outlined in the accompanying paper (2). Tissue recombinants were grown

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as subcapsular renal grafts in adult animals for 25–30 d before hormonal manipulation.

Cytosolic Androgen Receptor Assays: For the cytosolic androgen receptor assays, bladder and prostatic tissues from 28-d-old rats as well as UGM plus BLE recombinants were excised from hosts castrated 24 h before death. The tissues were homogenized at a ratio of 1:10 or 1:20 in TEDG buffer (10 mM Tris-HCl, 1.5 mM EDTA, and 1.0 mM dithiothreitol, 10% vol/vol glycerol, pH 7.4). Cytosols isolated by ultracentrifugation at 105,000 × *g* for 1 h were used immediately for receptor analysis. 175 μl of cytosol (0.25–0.84 mg protein) was incubated with TEDG buffer containing 17α-methyl-[^{3}H]trienolone ([^{3}H]R-1881, 87 Ci/mmol, 0.03125–2.0 nM, New England Nuclear, Boston, MA) in the presence or absence of unlabeled competing R-1881 (1 μM). The final volume of the incubate was 0.3 ml and equilibrium was achieved after 20 h at 0°C. Free ligand was removed by adding 0.6 ml of TEDG buffer containing dextran-coated charcoal (0.003% T-70 dextran [Sigma Chemical Co., St. Louis, MO] and 0.25% acid-washed activated charcoal [Sigma Chemical Co.]). Following a brief centrifugation (8,000 × *g*), aliquots were added to a 10.0-ml Maxifluor scintillation cocktail (J. T. Baker Chemical Co., Phillipsburg, NJ) and counted on a Beckman LS-3133P scintillation counter (Beckman Instruments, Inc., Irvine, CA). The counting efficiency for tritium was 60%. Data were analyzed by the method of Scatchard (3).

[^{3}H]Dihydrotestosterone (DHT) Autoradiographic Analysis of Androgen Receptors: Hosts bearing tissue recombinants for 25–30 d were killed, and UGM plus BLE, prostatic, and bladder specimens were removed and minced into 1–2-mm^2 pieces. The specimens were incubated in a Brunswick shaker (Brunswick Instruments, New Brunswick, NJ) for 1.5 h at 37°C in serum-free Ham's F-12 medium containing 17 nM [^{3}H]DHT (170 Ci/mmol, New England Nuclear) with or without a 600-fold molar excess of unlabeled DHT (10.2 μm). The samples were inserted into sealed Nitex (TETCO Inc., Elmsford, NY) 50-μl mesh (2.5 × 2.5 cm) bags and then washed in a 1-liter erlenmyer flask containing 500 ml of isotonic phosphate-buffered saline (PBS) (0.01 M NaH_2PO_4, 0.15 M NaCl, pH 7.4). During the 3-h wash with constant magnetic stirring, 20 liters of PBS was pumped through the flask with a peristaltic pump as described by Shannon et al. (4). Specimens of prostate, urinary bladder, and the UGM plus BLE recombinants were removed and flash frozen in liquid propane (−180°C) chilled with liquid nitrogen. Thaw-mount autoradiography was then performed on 4-μm frozen sections according to the procedures of Stumpf and Sar (5).

Protein Synthesis Analyzed by Two-Dimensional Gel Electrophoresis: Specimens of host prostate and bladder, as well as UGM plus BLE tissue recombinants, were obtained from mice 32–35 d after graft implantation. 7-d castrated hosts (25–27 d postimplantation) were injected subcutaneously with testosterone propionate (TP; 10.0 mg/kg; Sigma Chemical Co.) 24 h before sacrifice, which is the time point of maximal *de novo* protein synthesis (6). The optimal conditions for labeling total tissue proteins with [^{35}S]methionine are modifications of those established earlier in this laboratory (7). Upon sacrifice, the specimens were excised, finely minced with scissors, and placed in gassed (95% O_2:5% CO_2) Krebs-Ringer phosphate buffer (pH 7.4) at 37°C for 1 h to deplete endogenous methionine. Incubations were carried out in glass culture tubes sealed with polypropylene caps on a Labquake shaker (LABINDUSTRIES, Berkeley, CA). Specimens for each incubation contained approximately the same amount of DNA (15–25 μg) and weighed 4–6 mg for the prostate and UGM plus BLE recombinants and 8–10 mg for the bladder tissues. The samples were then incubated in one ml of Krebs-Ringer phosphate buffer containing [^{35}S]methionine (10.0 Ci/mmol, 1.0×10^{-5} M, New England Nuclear) for 3 h at 37°C. After incubation with the [^{35}S]methionine, the tissues were washed twice in 5 ml of ice-cold (4°C) Krebs-Ringer phosphate buffer, centrifuged at 1,000 × *g*, and frozen at −20°C until use.

100 μl of lysis buffer (9.5 M urea [Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY], 2% [wt/vol] Nonidet P-40 [LKB Instruments, Inc., Rockville, MD], and 2% [wt/vol] ampholines consisting of 1.6% [wt/vol] pH range 5–7 and 0.4% [wt/vol], pH range 3–10 [Bio-Rad Laboratories, Richmond, CA]) containing 10% [vol/vol] mercaptoethanol (Bio-Rad Laboratories) was added to each sample, and tissues were homogenized with a ground glass homogenizer (20 strokes) and allowed to sit at room temperature for 10 min. At this time, saturating amounts of urea crystals were added to the sample, the mixture was homogenized as before, and the sample loaded onto the isoelectric focusing gel (2 × 110 mm).

Electrophoresis was carried out using modifications of the method of O'Farrell (8). The isoelectric focusing gels (4.5% acrylamide) contained 2% ampholytes, pH 3–10 (Bio-Rad Laboratories). The gels were pre-electrophoresed at 200 V for 15 min, 300 V for 30 min, and 400 V for 30 min. Samples of 50 μl were applied to the gels, which were focused at 450 V for 15 h and 500 V for the final hour. After the isoelectric focusing, the gels were equilibrated by agitation in SDS sample buffer (10% [vol/vol] glycerol, 5% [vol/vol] mercaptoethanol, 2.3% [wt/vol] SDS [Bio-Rad Laboratories] and 0.0625 M Tris-HCl, pH 6.8) for 1 h. The proteins were electrophoresed the same day on SDS slab gels in the second dimension.

The second-dimensional slab gels (13 × 15 cm) were composed of a 5-% [wt/vol] acrylamide stacking gel and a 10-% acrylamide [wt/vol] separating gel. Proteins focused isoelectrically were transferred onto the SDS gels as described by O'Farrell (8) and run in the second dimension at 17 mA per gel in Tris-glycine buffer (0.025 M Tris-HCl, 0.192 M glycine, 0.1 M SDS, pH 8.3) for 4 h. Electrophoresis was terminated when the bromophenol blue tracking dye approached 1 cm from the bottom of the gel.

After electrophoresis in the second dimension, the SDS slab gels were fixed by agitation for 1 h in a solution of isopropyl alcohol (10% [vol/vol]) and acetic acid (7% [vol/vol]). The gels were then transferred to a solution of ENHANCE (New England Nuclear) and agitated for 1 h. After this step, the gels were washed by agitation for 1 h in distilled H_2O and dried on filter paper using an electrophoresis gel dryer (Hoefer Scientific Instruments, San Francisco, CA). A sheet of x-ray film (Cronex-4) was placed in contact with the gels in an air-tight exposure box at −80°C for 4–7 d. The apparent molecular weights of the proteins were estimated, using known molecular weight reference standards (bovine serum albumin, 68,000; glutamate dehydrogenase, 53,000; ovalbumin, 45,000; pepsin, 34,000; trypsinogen, 24,000; and ribonuclease A, 14,000). The apparent isoelectric points of the proteins were estimated from the pH of the isoelectric focussing gel.

Androgen-dependent DNA Synthesis As Measured by [^{3}H]Thymidine Incorporation into Tissue DNA: Host animals carrying the UGM plus BLE tissue recombinants for 21–23 d were castrated for 1 wk and then implanted with a pellet containing 50 mg of TP or lactose in the treated and control animals, respectively. At days 1, 2, 3, or 4 (day 0 is equal to the date of implantation), animals were killed and the specimens were minced with fine scissors at 4°C. Aliquots of minced host prostate, bladder, and UGM plus BLE tissue recombinants (5–10 mg) were incubated in gassed (95% O_2: 5% CO_2) Krebs-Ringer phosphate buffer (pH 7.4) containing [^{3}H]thymidine (400 mCi/mmole, 1×10^{-5} M, New England Nuclear) for 1 h at 37°C. After incubation with the [^{3}H]thymidine, the tissues were washed twice with 1.0×10^{-4} M unlabeled thymidine in ice-cold Krebs-Ringer phosphate buffer, centrifuged, and stored at −20°C until analysis. DNA was analyzed by the fluorometric assay of Hinegardner (9) with calf thymus DNA (Sigma Chemical Co.) as the reference standard. Sample aliquots were added to a 10-ml Maxifluor scintillation cocktail (J. T. Baker Chemical Co.) and counted as described earlier. The counting efficiency for tritium was 60%. The rate of DNA synthesis was expressed as specific activity (counts per minute per microgram of DNA per 60 min). Under these experimental conditions, it was established that the rate of DNA synthesis is linear with regard to the weight of the tissues and the duration of incubation. Values presented are expressed as the mean ± SEM of at least six observations. Data were analyzed by a one-way analysis of variance for groups of unequal size. Significant differences between TP- and lactose-treated groups were determined by Dunnet's multiple comparison method (10).

RESULTS

The results presented were based on the analysis of 98 UGM plus BLE recombinants grown in 18 hosts. 90 grafts of isolated UGM grown in 9 hosts served as controls for assessment of the cleanness of separation. Contamination of the UGM by residual urogenital sinus epithelium was observed in six specimens (6%).

Cytosolic Androgen Receptor Assays

To provide a firm basis from which to assess the expression of androgen receptor activity, both ventral prostatic and bladder tissues from 28-d-old male CDF rats, as well as 28-d-old tissue recombinants, were subjected to receptor analysis. Scatchard plots of the data (Fig. 1) reveal that young rat ventral prostatic cytosol contained substantial (37.4 fmoles/mg protein) high-affinity (the dissociation constant (K_d) is equal to 0.8 nM) binding sites, whereas cytosol from bladder was devoid of [^{3}H]R-1881 binding. The UGM plus BLE recombinant contained high affinity (0.3 nM) binding sites in reduced, yet measurable amounts (3.9 fmoles/mg protein). Total protein concentrations per sample in ventral prostate, bladder, and UGM plus BLE assays were 2.9, 1.2, and 0.8 mg/ml, respectively. In a separate experiment (Fig. 1), intact male Copenhagen rat cytosol was diluted to protein concentrations of 0.3–4.0 mg/ml. At sample protein concentrations <1 mg/ml, there

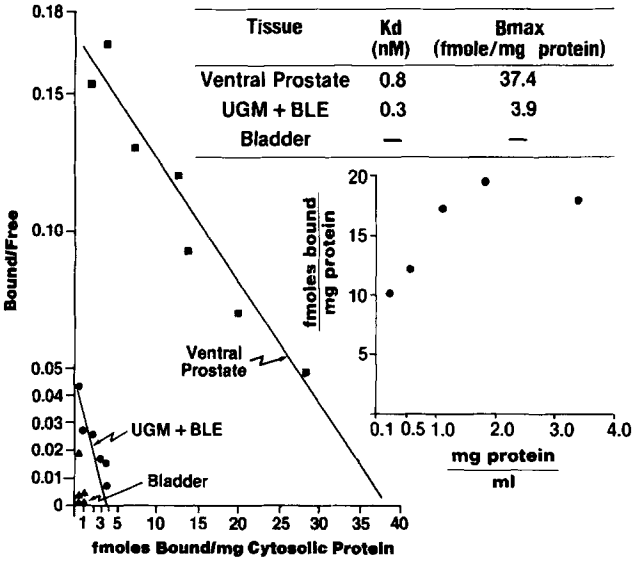

FIGURE 1 Scatchard analysis of R-1881 binding to androgen receptors in cytosol obtained from rat ventral prostate, bladder, and UGM plus BLE. Note the presence of high affinity androgen binding in ventral prostate and UGM plus BLE and the absence thereof in bladder. Inset represents the relationship between protein concentration and androgen receptor levels detected in the standard assay. B_{max}, maximum binding.

is an apparent decrease in cytoplasmic androgen receptor binding.

[^{3}H]DHT Autoradiography

Data from autoradiographic studies of [^{3}H]DHT binding sites in murine prostate, UGM plus BLE tissue recombinants, and bladder demonstrate that acinar cells of both host prostate (Fig. 2*a*) and UGM plus BLE tissue recombinants (Fig. 2*b*) are heavily labeled, with the majority of silver grains being localized over epithelial cell nuclei. In contrast, urothelial cells in the host bladder display low levels of silver grains without preferential localization over any cell organelles (Fig. 2*c*). Nuclear androgen localization in both the prostate and UGM plus BLE recombinants was abolished when the [^{3}H]DHT was incubated with a 600-fold excess of the radioinert steroid (data not shown).

Protein Synthesis Analyzed by Two-Dimensional PAGE

Total cellular proteins in host prostate, bladder, and 24 UGM plus BLE tissue recombinants carried in six hosts were labeled with [^{35}S]methionine and separated according to apparent isoelectric points as well as molecular weights (Fig. 3, *a*, *b*, and *c*). The overall intensity of protein labeling in the prostate (Fig. 3*a*) and tissue recombinant (Fig. 3*c*) was greater than in host bladder (Fig. 3*b*). Castration for 7 d decreased the intensity of labeling in the prostate and tissue recombinant but had no effect on the labeling in the bladder (data not shown). Numerous qualitative and quantitative differences were observed in the prostatic and bladder tissues. For example, individual proteins in regions P_1 and P_7 (Fig. 3*a*) are associated with the prostate, whereas the protein labeling associated with region B_1 (Fig. 3*b*) is bladder specific. UGM plus BLE tissue recombinants exhibited protein labeling in the prostate-specific regions, while the protein spot unique to bladder was not expressed (Fig. 3*c*).

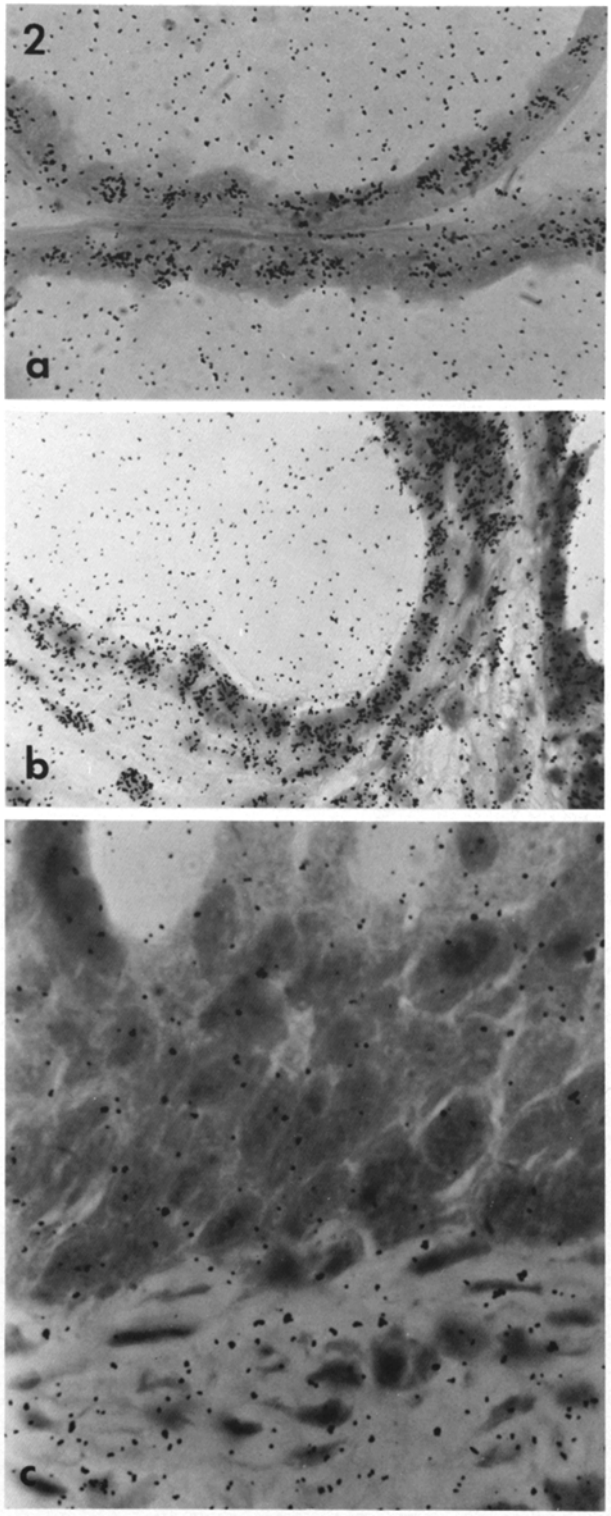

FIGURE 2 Autoradiograms of [^{3}H]DHT localization in murine (*a*) prostate (× 1,000), (*b*) UGM plus BLE recombinant (× 1,000), and (*c*) bladder tissues (× 1,600). Note the localization of silver grains in the epithelial nuclei of prostatic and UGM plus BLE specimens and the random distribution of labeling in bladder. The nuclear concentration of label observed in *a* and *b* was abolished when specimens were exposed simultaneously to [^{3}H]DHT and a 600-fold excess of radioinert steroid (not shown).

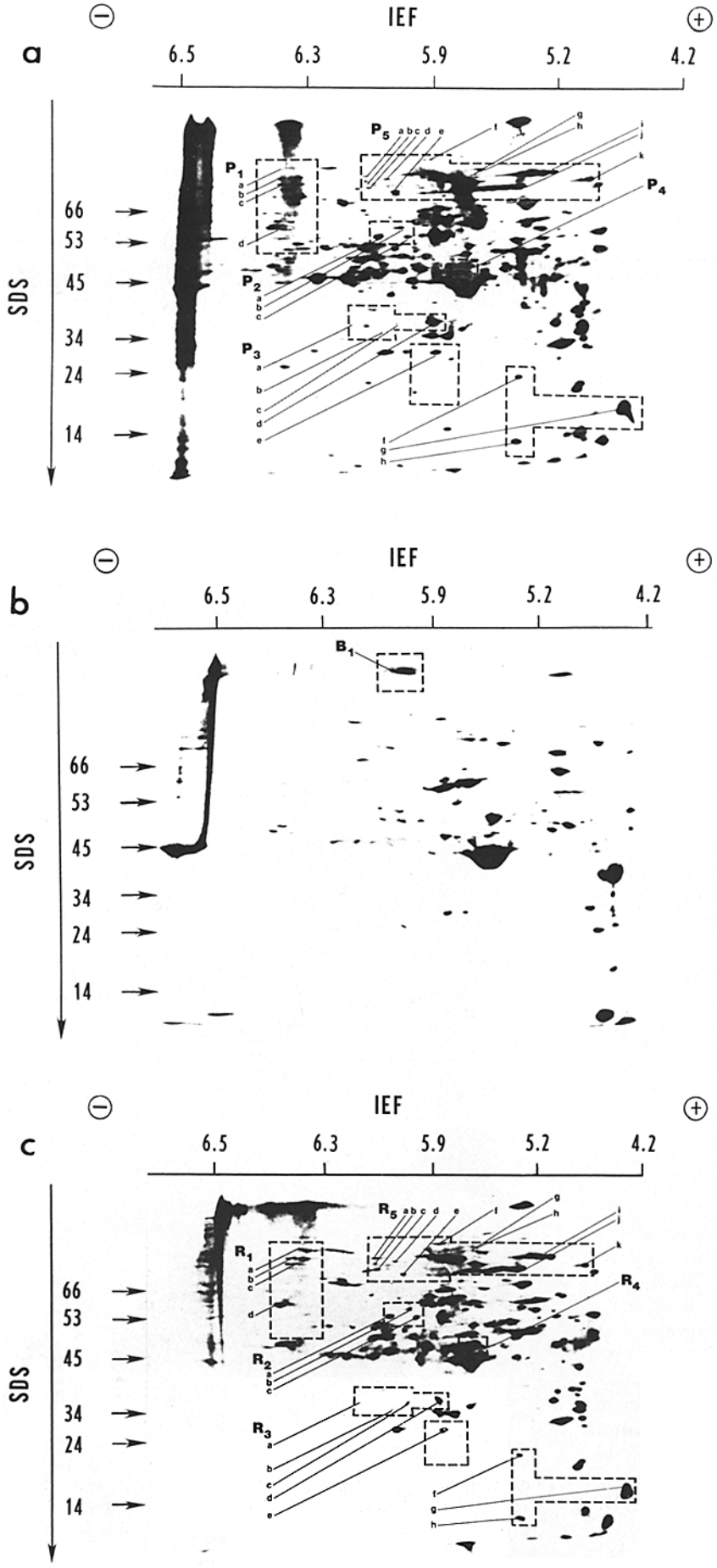

FIGURE 3 Autoradiograms of total cellular proteins separated by two-dimensional PAGE. Specimens ([*a*] murine prostate, [*b*]) murine urinary bladder, and [*c*] UGM plus BLE recombinants) were minced and labeled with [^{35}S]methionine in Krebs-Ringer phosphate buffer and then subjected to two-dimensional electrophoretic separation (see Materials and Methods). (*a*) Murine prostate. Proteins within areas P_1 to P_5 are prostate-specific. (*b*) Murine urinary bladder. The protein designated B_1 is bladder specific. (*c*) UGM plus BLE recombinant. Note the similarity between protein labeling in R_1 to R_5 and that obtained from prostatic tissue (P_1 to P_5).

Androgen-dependent DNA Synthesis Measured by [^{3}H]Thymidine Incorporation into Tissue DNA

Previous findings (6, 11) that maximal rates of prostatic DNA synthesis occurred 72 h after initiation of TP administration to castrated rats were confirmed using castrated male mice treated with TP (data not shown). DNA synthesis in prostate, bladder, and 24 UGM plus BLE tissue recombinants carried out in six hosts in the presence and absence of exogenously administered androgens (Fig. 4) shows that, at the period of maximal TP-induced cellular proliferation, there was a significant ($P < 0.05$) sixfold stimulation in the prostate. A significant ($P < 0.05$) sixfold stimulation of DNA synthesis in the tissue recombinants was also observed in the TP- vs. lactose-treated castrates at 72 h. The amount of TP-induced [^{3}H]thymidine incorporation into tissue DNA in the recombinants was lower than in prostates of TP-treated hosts. There were no significant alterations of [^{3}H]thymidine incorporation into the bladder DNA after TP treatment during the entire course of this study.

DISCUSSION

The influence of various mesenchymal tissues upon epithelial morphogenesis and differentiation has been analyzed in different experimental systems. Most studies have demonstrated that the mesenchyme may influence or modify the morphological organization of the epithelium, i.e. whether the epithelium is simple columnar, stratified, or organized into acinar configurations (12–16). Epithelial products characteristic of induced states are produced in experimental recombinations. Karkinen-Jaaskalainen (17) has demonstrated by immunofluorescence the expression of lens crystallins in epidermal cells of ventral trunk skin induced by the optic cup to form lentoids. Dhouailly et al. (18) showed the expression of scleroproteins possessing features of scale keratins in feather epidermis induced to form scales by association with scale dermis. However, morphogenetic inductions (changes in glandular branching patterns) may occur exclusive of biochemical reprogramming. Sakakura et al. (19) have demonstrated that while ductal arborization of mammary epithelium is modified by recombination with salivary mesenchyme to form salivarylike glands, casein continues to be produced by the tissue recombinants. The studies described herein illustrate that several biochemical activities of the UGM plus BLE recombinants exhibit features that are distinctly prostatic, the foremost being the expression of androgenic sensitivity.

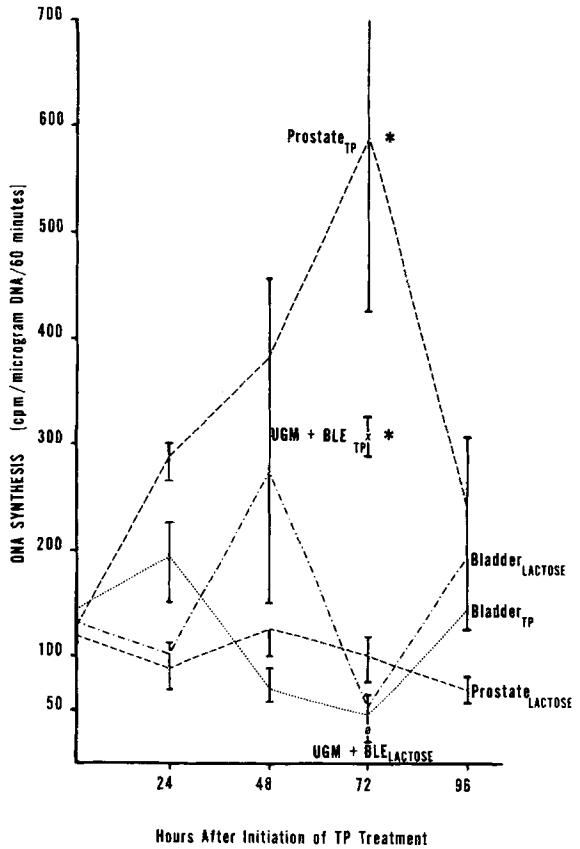

FIGURE 4 The effects of TP-induced DNA synthesis in murine prostate, bladder, and UGM plus BLE recombinants. Note the time-dependent TP-induced increase in DNA synthesis in prostate and an absence of this effect in bladder tissues. At the point of maximal TP-induced [^{3}H]thymidine uptake in the prostate, the UGM plus BLE tissue recombinants exhibited a comparable (six-fold) stimulation over lactose-treated control levels.

The effects of androgens in target organs such as the prostate are mediated via cytosolic androphiles (20). In nontarget organs such as the urinary bladder, levels of cytosolic androphiles are exceedingly low or undetectable. Autoradiographic analysis of nuclear [^{3}H]DHT binding sites indicates a complete absence of androgen receptor activity in urothelium. Induction by UGM of prostatic differentiation in adult bladder epithelium results in the expression of nuclear androgen binding sites within the normally androgen-receptor-deficient urothelium as measured by steroid autoradiography.

Scatchard plot analyses of [^{3}H]R-1881 binding supported the autoradiographical data that the high affinity androgen receptor was present in the host prostate and the UGM plus BLE tissue recombinant but absent in the host bladder. Differences in the observed affinities and number of binding sites between ventral prostate and the recombinants may be explained by the observation that the UGM plus BLE specimens are composed of all elements of the prostatic complex (i.e., acini from different lobes, renal capsular elements, urethral epithelia, and urethral glands). The ventral prostatic samples were, in contrast, representative of a singular accessory sex organ element and contained only the active acinar parts thereof. It has been documented that the cytosolic androgen receptor in the rat ventral prostate changes as a function of age and that the receptor content in the ventral prostate is 10-fold greater than in the dorsolateral lobes (21, 22). In addition, the lower androgen receptor content detected in the tissue recombinants may be underestimated, since somewhat lower amounts of protein were used in the assay. Lower protein content per assay may result in the destruction of binding activity during incubation (23). This finding was confirmed herein (Fig. 1). It is also probable that measurement of cytosolic androgen binding activity in UGM plus BLE recombinants reflects the relative contributions of tissue components possessing differing androgenic sensitivity. The biochemical analyses of androgen receptor activity in tissue homogenates are complimented by the steroid autoradiographic data that demonstrated that the bulk of androgen binding activity is associated with the induced urothelial cells per se. In this sense, adult urothelium is similar to embryonic bladder epithelium (24), since in both cases the inductive activity of UGM can elicit the expression of nuclear androgen localization.

Expression of androgen receptor activity within epithelial cells provides the basis for the regulation of many biochemical

activities. Protein synthesis in male accessory sex organs is dependent upon androgenic stimulation (25–27). Total cell protein [^{35}S]methionine labeling in the three tissues after two-dimensional gel electrophoretic separation was indicative of the induction of an androgen-sensitive, prostatelike profile in UGM plus BLE tissue recombinant. Labeling of cellular proteins with [^{35}S]methionine in the prostate and tissue recombinant was dependent upon the androgen status of the host (namely, castration decreased and TP administration increased the overall labeling on the gels), while the amount of labeling in the bladder was unaffected by such manipulations. In addition, a qualitative similarity in the protein synthetic profiles was observed for both the UGM plus BLE recombinant and the prostate, while the bladder exhibited a generally different profile.

Cells of prostate and UGM plus BLE recombinants also exhibit a marked stimulation of DNA synthesis after administration of TP to previously castrated hosts. By contrast, this treatment did not affect DNA synthesis in the host's bladder. In other studies, TP-induced epithelial DNA synthesis stimulation evaluated histologically in the prostate and UGM plus BLE recombinants could be blocked by the simultaneous injection of the antiandrogen, cyproterone acetate (11, 27). Urothelial DNA synthesis and morphology were not influenced by these manipulations.

Although none of the biochemical responses measured in the tissue recombinant were identical to those found in the prostate, it must be realized that the prostatic preparations contained only the acinar components of the gland, while the tissue recombinants were composed of all prostatic elements. These differences in the responses of the two tissues to androgenic stimulation are particularly evident in the studies of DNA synthesis in which the levels of [^{3}H]thymidine incorporation in the recombinants were consistently lower than in the prostate. Although the magnitude of the response was somewhat lower in the recombinant because of the presence of androgen-insensitive elements (urethral epithelium, urethral glands, and some possible minor contaminating elements from the graft sites), the difference in the magnitude of [^{3}H]thymidine incorporation at 72 h in TP vs. lactose-treated specimens was remarkably similar for the prostate and recombinant and distinctly different than in the bladder. These results are consistent with the suggestion that the UGM plus BLE recombinants acquired androgenic sensitivity and expressed characteristic androgen-induced proliferative responses.

Although biochemical data derived from homogenized specimens cannot specify the cell type responsible for a particular activity, morphological analysis corroborated the biochemical data. For instance, nuclear localization of [^{3}H]DHT in the acinar epithelium of the recombinant indicates that the androgen receptor activity measured biochemically is due to the presence of this activity within the induced bladder epithelium. This, in turn, lends strong circumstantial evidence to the idea that androgen-induced protein and DNA synthesis was primarily a function of the induced epithelium, with little contribution from the surrounding stroma. Finally, localization of prostate-specific antigens (2) in the induced epithelium provides further evidence for the expression of additional prostatic phenotype in UGM plus BLE recombinants.

It has been proposed that developmental inductive processes reexpressed in adulthood may be involved in the pathogenesis of human benign prostatic hyperplasia (28, 30). These studies demonstrate that an inductive mesenchyme has the potential to alter the functional phenotype of a responsive adult epithelia.

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