

Development and Characterization of Nontumorigenic and Tumorigenic Epithelial Cell Lines from Rat Dorsal-Lateral Prostate

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ABSTRACT

We have established two new epithelial cell lines (NRP-152, NRP-154), with markedly different properties, from the dorsal-lateral prostate of Lobund/Wistar rats treated with *N*-methyl-*N*-nitrosourea and testosterone propionate. NRP-152 cells do not form tumors in athymic mice and retain many of the properties of normal prostatic epithelial cells. They produce prostatic acid phosphatase, have functional androgen receptors, and require the combination of several growth factors in addition to serum for optimal growth. Their growth is stimulated by epidermal growth factor, insulin, dexamethasone, cholera toxin, dihydrotestosterone, and testosterone, and their growth is inhibited by transforming growth factor β s and retinoic acid. These cells also respond to 1,25-dihydroxyvitamin D₃ with an early growth stimulation followed by growth inhibition at later times. In contrast, tumorigenic NRP-154 cells lack detectable androgen receptor mRNA and have less stringent growth factor requirements for optimal growth. Growth of NRP-154 cells is stimulated by dexamethasone and insulin, inhibited by transforming growth factor β 1, but not significantly altered by epidermal growth factor, cholera toxin, dihydrotestosterone, retinoic acid, or 1 α ,25-dihydroxyvitamin D₃. Our data suggest that the NRP-152 and NRP-154 cell lines are suitable systems for analysis of normal prostate growth and prostatic carcinogenesis.

INTRODUCTION

Cultures of normal cells under hormonally defined conditions offer useful *in vitro* model systems for studying the mechanisms of normal cell function and regulation, as well as cellular transformation. However, this is possible only if the normal response patterns and function of cells are preserved when they are separated from their *in vivo* milieu. Although much effort has been expended in optimizing *in vitro* conditions for growth and viability of normal adult prostatic epithelial cells in rodents, these specialized cells have a particularly limited growth potential and undergo rapid senescence in culture (1-4). Thus, immortalization of normal prostatic cells has been an important undertaking; however, to date only a limited number of prostatic cell lines are reported to be nontumorigenic (5-7). One of these epithelial cell lines (derived from rat ventral prostate) forms tumors when coinoculated with a fibroblastic line (6). The tumorigenicity of several rat prostate cell lines (immortalized by SV40 large-T antigen) has not been determined (8, 9). Of these cell lines, only one was derived from the dorsal-lateral prostate (9), which is the region of the rat prostate most similar, functionally and structurally, to the human prostate (10-12); this cell line was not characterized with respect to expression of prostatic markers and responsiveness to growth factors and hormones.

Progress in the study of prostatic carcinogenesis has also been limited by the availability of appropriate animal model systems, since spontaneous prostatic cancers are rare in nonhuman mammals (13). One exception, however, is the Lobund/Wistar rat strain, which developed spontaneous prostate and seminal vesicle adenocarcinomas at an incidence of 26% in 26 months (14, 15). Treatment of these

animals with MNU² and TP increases the percentage of tumor incidence and decreases tumor latency (16).

To obtain premalignant as well as malignant prostatic cell lines, we have cultured cells and explants of dorsal-lateral prostates from Lobund/Wistar rats that have been treated with MNU and TP. We have derived and characterized nontumorigenic and tumorigenic epithelial lines; designated them NRP-152 and NRP-154, respectively; and then studied their responsiveness to several hormones and growth regulatory peptides. In this paper, we show that NRP-152 cells are highly responsive to several hormones and growth factors and possess functional ARs. In contrast, NRP-154 cells have less stringent requirements for growth, are less responsive to hormones and growth factors, and lack detectable levels of ARs. NRP-152 cells are exquisitely responsive to retinoic acid and 1,25(OH)₂D₃, whereas NRP-154 cells are unresponsive to these hormones. Such sensitivity to both retinoids and 1,25(OH)₂D₃ makes NRP-152 cells suitable for *in vitro* studies on the mechanism of action of these hormones the potential of which in the chemoprevention of breast and prostate cancer is currently under investigation.

MATERIALS AND METHODS

Materials. Sources were: Lobund/Wistar rats, Harlan-Sprague-Dawley, Inc. (Indianapolis, IN); anti-pan keratin rabbit polyclonal, anti-vimentin, and anti-desmin monoclonal antibodies, New Fuchsin substrate system, and phosphatase-linked rabbit anti-mouse IgG, Dako Laboratories (Carpinteria, CA); anti- α -actin monoclonal antibody, Boehringer Mannheim (Indianapolis, IN); TGF- β s 1, 2, and 3, R&D Systems, Inc. (Minneapolis, MN); antibodies against cytokeratins 1, 8, 10, 13, and 14, Drs. Stuart Yuspa and Adam Glick; monoclonal antibodies against cytokeratin 14 and 18, Drs. Ellen Lane and Timothy Thompson; [³H]R1881 (86.7 Ci/mmol) and R1881, NEN (Boston, MA); phosphatase-linked goat anti-rabbit and anti-mouse IgGs, Kirkegaard and Perry, Inc. (Gaithersburg, MD); MNU, Ash Stevens (Detroit, MI); AR-38 mouse fibrosarcoma cells, Dr. Umberto Saffiotti; keratinocyte growth factor, Dr. Jeffrey Rubin.

Development of NRP-152 Cell Line. Lobund/Wistar rats were treated with MNU (30 mg/kg body weight, injected into the intralingual vein). One week later they received a s.c. implant of TP (50 mg) encased in a Silastic capsule. The TP implants were replaced at 2.5-month intervals. Rats were killed by suffocation in carbon dioxide. The dorsal-lateral prostate was excised sterilely and washed extensively in Hanks' solution and then transferred to a dish containing 2 ml of Hanks' solution supplemented with 10% FBS, where it was minced to <1-mm fragments with fine scissors. Fragments were then transferred to ten 80-cm² flasks containing 15 ml of GM1. Cells appeared to attach within 1 week, at which time the medium was replaced with GM1 and then changed every 2 days thereafter. At 3-5-day intervals, fibroblasts were removed by a 2-10-min treatment with trypsin-EDTA at 37°C, and then the adherent monolayer was washed with PBS. Epithelial cells were allowed to

² The abbreviations used are: MNU, *N*-methyl-*N*-nitrosourea; AR, androgen receptor; [³H]R1881, [17 α -methyl-³H]methyltrienolone; CT, cholera toxin; Dex, dexamethasone; DHT, dihydrotestosterone; 1,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; EGF, epidermal growth factor; FBS, fetal bovine serum; Ins, bovine insulin; PAP, prostatic acid phosphatase; PBS, phosphate-buffered saline; RA, all-*trans*-retinoic acid; TGF- β , transforming growth factor- β ; TP, testosterone propionate; BSA, bovine serum albumin; RT, room temperature; cDNA, complementary DNA; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F-12 medium; GM1, DMEM/F12 supplemented with 10% FBS, 20 ng/ml EGF, 5 μ g/ml Ins, 0.1 μ M Dex, 10 ng/ml CT; trypsin-EDTA, 0.05% trypsin-0.53 mM EDTA in Hanks' solution; SFM, serum-free medium.

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grow until the flask was near-confluent, at which point epithelial monolayers were detached by treatment with trypsin-EDTA at 37°C. Cells that were detached by trypsinization and those few that remained attached to the culture flasks after trypsinization were washed once with 10 ml of GM1 and cultured separately with 15 ml of GM1. All detached epithelial cells that were passaged in this manner died within 1 week. However, one colony of epithelial cells that remained attached after trypsinization continued to grow. Once subconfluent, these cells were passaged by trypsinization with approximately 1–2% viability of the detached epithelial cells. Viability increased upon subsequent passaging; passages 2, 3, and 4 resulted in over 5, 30–50, and 80% viability, respectively. These cells passaged much better at higher density than at low density and several attempts at cloning these cells were unsuccessful.

Development of NRP-154 Cell Line. Development of the NRP-154 cell line was similar to that of NRP-152, with two modifications: (a) tissue was digested with collagenase (670 units/ml collagenase and 10% FBS in Hanks' solution) for 1 h at 37°C, passed through 1 mm and 250 μ m mesh filters, and after one wash with WAJC 404 (1), supplemented with 10% FBS, followed by two washes with WAJC 404 alone; (b) cells were cultured in a SFM (WAJC 404 supplemented with 200 μ g/ml BSA V, 80 μ g/ml reduced glutathione, 20 μ g/ml human transferrin, 10 μ g/ml Ins, 1 μ M Dex, 20 ng/ml EGF, 10 ng/ml CT, and 10 ng/ml keratinocyte growth factor), as modified from the system of McKeehan *et al.* (1) to bypass the problem of stroma growth. After 5 days in culture, medium was changed at 2-day intervals. After an additional 10 days, all epithelial colonies were essentially growth arrested and dying. To revive the remaining colonies, the medium was replaced with GM1. Although most epithelial colonies grew initially, only one colony, which gave rise to the NRP-154 cell line, survived past 3 weeks. Following a 30-min trypsinization, about 30% of the detached cells survived and grew as pure epithelial cells. This constituted passage 1 of NRP-154 cells.

Culturing Established Lines. Established cell lines were maintained in GM2 [HEPES-free, and antibiotic-free DMEM/F12 (1:1, v/v) supplemented with 5% FBS, 20 ng/ml EGF, 10 ng/ml CT, 0.1 μ M Dex, and 5 μ g/ml Ins] in 80-cm² Nunc tissue culture flasks and passaged every 3–4 days at subconfluence, seeding at 2–5 \times 10⁵ cells/flask. For passaging, a flask of cells was washed with 5 ml of trypsin-EDTA and treated for 5 to 7 min with trypsin-EDTA (3 ml at 37°C). Cells were dislodged by gentle tapping and then recovered following centrifugation (1000 \times g, 5 min) with 7 ml of 10% FBS in DMEM/F12.

Athymic Mouse Studies. Cell suspensions trypsinized off subconfluent monolayers were washed once with DMEM/F12 containing 10% FBS and twice with DMEM/F12 by centrifugation and then resuspended in DMEM/F12 or in DMEM/F12:Matrigel (1:1, v/v) at a density of 3–5 \times 10⁷ cells/ml. Six-week-old intact male athymic mice (five animals/group) were inoculated s.c. in a hindlimb with 0.1–0.2 ml of the cell suspension.

Cell Proliferation Assays. Cell growth was measured by the binding of crystal violet to formalin-fixed monolayers in Costar 6-well dishes (17) and cell enumeration in single-cell suspension with a Coulter Counter. DNA synthesis was also measured by the incorporation of [³H]thymidine (18), following a 24-h pulse of 50,000 cells (in 24-well dishes) with 0.25 μ Ci/well [³H]thymidine.

AR Assay. ARs were assayed using the modification of Chang *et al.* (19). Cells dispensed at either 100,000/well in Costar 24-well dishes or 500,000/well in 6-well dishes were cultured overnight in DMEM/F12 containing dialyzed and charcoal-treated FBS (10%), 20 ng/ml EGF, 5 μ g/ml Ins, and 10 ng/ml CT. The medium was replaced twice with DMEM/F12, 15 mM HEPES, 0.1% BSA V, and 0.1 μ M triamcinolone, after which various concentrations of [³H]R1881 \pm 100-fold excess R1881 were added. Cells were incubated 37°C for 2 h; the free label was removed by three washes with DMEM/F12, 15 mM HEPES, 0.1% BSA V, and 0.1 μ M triamcinolone at 4°C; and the bound ligand was extracted with 1.0 ml of 100% ethanol for 20 min at room temperature. The average number of receptors per cell and K_d values were derived by Scatchard analysis.

Prostatic Acid Phosphatase Assay. Prostatic acid phosphatase activity was measured as tartrate-sensitive acid phosphatase using the prostatic acid phosphatase kit from Sigma according to the protocol described in the kit. The presence of PAP was further confirmed by PAP mRNA expression on Northern blots.

RNA Purification and Northern Blot Analysis. Total RNA was purified using a modification (20) of the method of Chomczynski and Sacchi (21). Ten

μ g of total mRNA were electrophoresed through 1% agarose-0.66 M formaldehyde gels. Gels were treated with 60 mM NaOH for 20 min, neutralized with 50 mM Tris-HCl (pH 7.4)-10 mM NaCl, for 20 min, and then blotted onto Nytran (pore size, 0.45 μ m; Schleicher & Schuell, Keene, NH). After membranes were cross-linked by UV irradiation, they were prehybridized, hybridized, and washed at 65°C as described by Church and Gilbert (22). Hybridization was done with 3 \times 10⁶ cpm/ml (>10⁹ dpm/ μ g DNA) of [³²P]cDNA probes that were prepared by random priming (GIBCO BRL, Gaithersburg, MD).

cDNA Probes. For the rat AR probe, a 570-base pair cDNA insert from nucleotides 2067–2636 was obtained from Dr. Terry Timme. Rat PAP cDNA probe (411-base pair) was prepared by reverse transcription-polymerase chain reaction amplification from nucleotides 481–891, using rat ventral prostate polyadenylated mRNA as a template. The human TGF- β type II receptor cDNA probe (1.7-kilobase insert from the open reading frame) was provided by Drs. Andrew Geiser and Herbert Lin.

Immunocytochemistry. Cytoskeletal markers were assayed immunocytochemically using subconfluent monolayers grown in chamber slides. Cells were fixed on these slides for 10 min at –20°C with methanol:acetone (1:1, v/v). Slides were blocked with BB (PBS plus 1% BSA, plus 1% goat serum) for 30 min at RT, treated with primary antibodies or control IgG in BB1 (BB plus 0.05% Tween 20) for 1 h at RT, followed by 5 μ g/ml of phosphatase-conjugated secondary antibodies in BB1 for 1 h at RT, using extensive washes with PBS-0.05% Tween 20 between each step. Slides were developed using Dako's New Fuchsin Substrate System.

Karyotype and Isozyme Analysis. Karyotype and isozyme analyses were performed by Drs. Ward D. Peterson and Joseph Kaplan at the Cell Culture Laboratory, Children's Hospital of Michigan, Detroit.

Mycoplasma and Viral Testing. The absence of *Mycoplasma* contamination of NRP-152 and NRP-154 cells was confirmed by an indirect Hoechst method and by a direct agar method (American Type Culture Collection). The absence of eight viruses (Toolans H-1, GDVII, KRV, PRV, Reo, Sendai, RCV/SDA, and Hantaan) in NRP-152 and NRP-154 cells was confirmed by a rat antibody production test (Microbiological Associates).

RESULTS

We have established cell lines from the dorsal-lateral prostates of 2 (No. 152 rat and No. 154 rat) of a group of 8 rats, 5 to 8 months after initial carcinogen treatment. The prostates were transected bilaterally; one lobe was used for histology and the other for cell culture. Histological examination of No. 152 showed no microscopic lesion in the dorsal-lateral region, whereas No. 154 had atypical hyperplasia in this region. In culture, NRP-152 cell monolayers are cuboidal at low cell density, occasionally aligned in parallel curving bundles at high density (Fig. 1, A and B). NRP-154 cells show a different morphology: they are larger and flatter than NRP-152 cells (Fig. 1C)

Conditions for Optimal Growth of NRP-152 and NRP-154 Cells. Unlike NRP-154 cells, which were able to grow continuously in DMEM/F12 supplemented with only 10% FBS, NRP-152 cells at passages 3 through 11 were unable to grow in this medium, and additional growth factors and hormones (EGF, Ins, Dex, CT) were required for derivation and maintenance of this cell line. Under optimal growth conditions (GM1) NRP-152 and NRP-154 cells doubled approximately every 17 h. To define the supplements required for growth, we studied the contribution of each of the four supplements in GM1 to the overall growth of these cells after 6 days, using crystal violet staining as a convenient method of approximating cell growth (Fig. 2, A and B). At day 6, NRP-152 cells receiving all four supplements were confluent, whereas deletions of single components resulted in less growth. When added alone, EGF was significantly mitogenic, whereas single additions of other supplements stimulated little to no growth. The addition of 200 pM TGF- β 1 not only inhibited growth in the absence of exogenous growth factors but also caused a dramatic inhibition of the optimal cell growth induced by the combination of by EGF, Ins, Dex, and CT. At later passages (>p14), cells

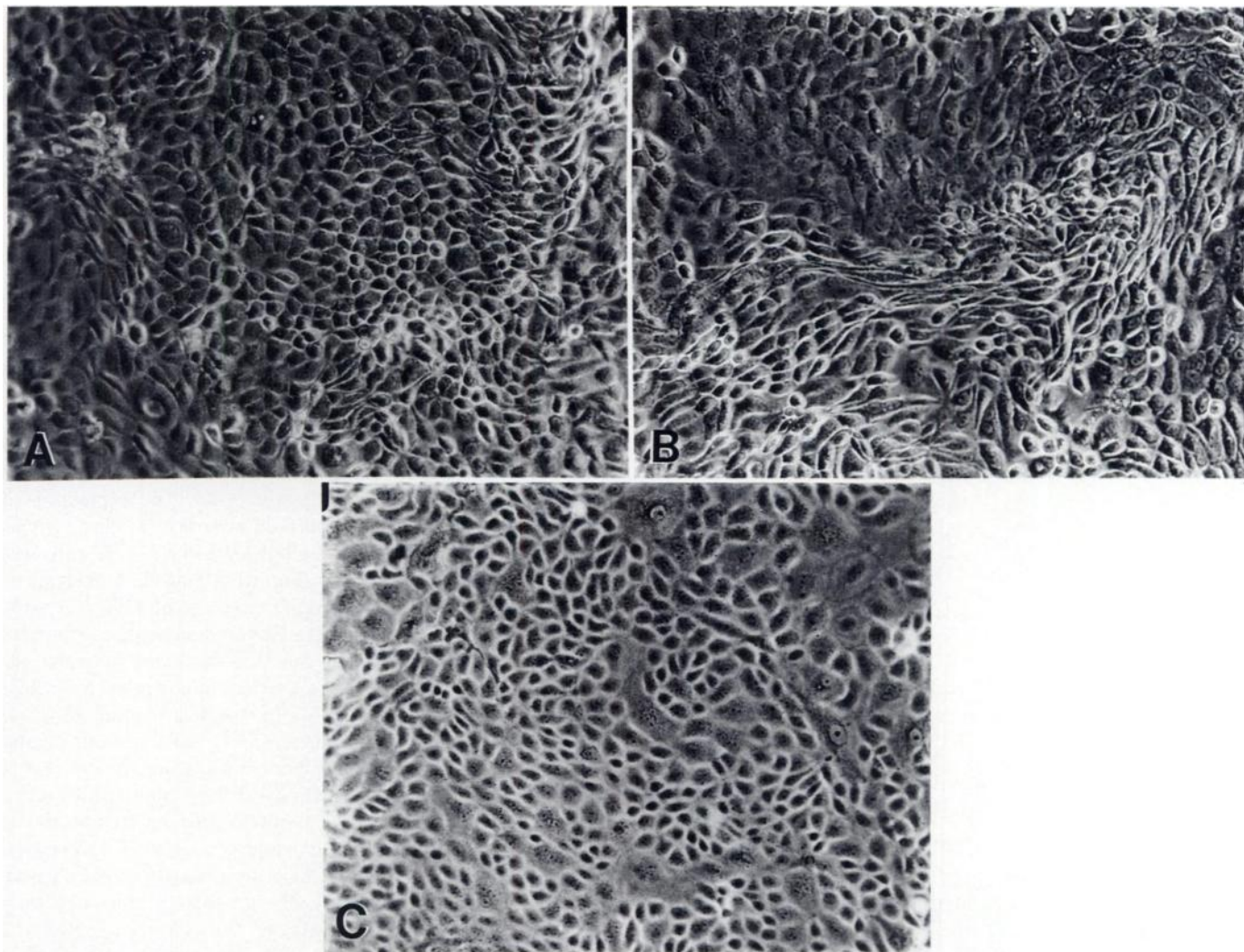


Fig. 1. Morphology of NRP-152 and NRP-154 cells grown on plastic. NRP-152 cells (passage 2) at near confluence (A) and confluence (B) and NRP-154 cells (passage 2) at near confluence (C) were grown on plastic in DMEM/F12 supplemented with 10% FBS, 20 ng/ml EGF, 5 μ g/ml Ins, 10 ng/ml CT, and 0.1 μ M Dex.

were no longer completely growth arrested in serum alone, exhibiting minimal growth from 4 to 6 days and reaching growth arrest by day 7 (Fig. 2C). In contrast, when NRP-154 cells (passage 7) were analyzed as above, growth in wells receiving basal medium alone was 44% of that in complete medium; under these conditions only Ins and Dex significantly stimulated growth over control (data not shown). The addition of 200 pM TGF- β 1 to NRP-154 cells caused marked inhibition of growth in both basal and supplemented medium (data not shown).

Tumor Growth in Athymic Male Mice. We first tested the tumorigenicity of NRP-152 and NRP-154 cells at passages 8 and 34, using a 3 million-cell inoculum. None of the 10 mice receiving either passage 8 or 34 NRP-152 cells had palpable tumors, even 11 months after inoculation. The positive control AR-38, a mouse fibrosarcoma cell line, formed tumors 1 week after inoculation; these tumors weighed an average of 2.2 ± 1.2 (SD) g by 2 weeks. After 6–7 weeks, palpable masses were observed at the site of inoculation with NRP-154 cells. All five mice in this group had sizable growths, averaging 0.50 ± 0.30 g, 10 weeks after inoculation, that were confirmed histologically as desmoplastic tumors. Because coinoculation of tumorigenic cells with Matrigel has been shown to shorten tumor latency and enhance tumor growth (23), we also tested the tumorigenicity of NRP-152 cells at passage 35, using a large inoculum of 10^7 cells/0.2 ml in both the presence and the absence of Matrigel. None of

the 10 mice in these groups formed tumors after 8 months. The positive control in this experiment, NRP-154 cells without Matrigel, formed tumors in all animals after 7 weeks.

Karyotype and Isozyme Analysis. The karyotype and isozyme analyses of NRP-152 cells were done at both passage 7 and passage 61 to detect any genotypic drift in culture (NRP-154 was done as passage 7). For both cell lines the electrophoretic mobilities of glucose-6-phosphate dehydrogenase, purine nucleoside phosphorylase, lactate dehydrogenase, and malate dehydrogenase were comparable to those of a normal rat cell control preparation. Karyotype analyses from 100 cells from each passage of NRP-152 demonstrated that the cell line is aneuploid (XXYY), with most chromosomal counts in the hypertriploid range. Karyotype analysis of NRP-154 demonstrated that the cell line is also aneuploid (XY), with 50% of the cells essentially diploid and the remainder in the tetraploid range. For both cell lines structural modification of chromosomes was infrequent.

Immunocytochemical Staining. In order to determine the cellular origin of the established cell lines, they were stained with various specific antibodies directed against cytoskeletal proteins. Pan anti-keratin polyclonal antibody stained 100% of the cells of both lines, confirming that they represent pure populations of epithelial cells. For NRP-152 cells, antibodies directed against cytokeratins 1, 10, 13, and 18 exhibited no specific staining, whereas anti-cytokeratin 14 stained 100% of the cells. In contrast, NRP-154 cells were positive for

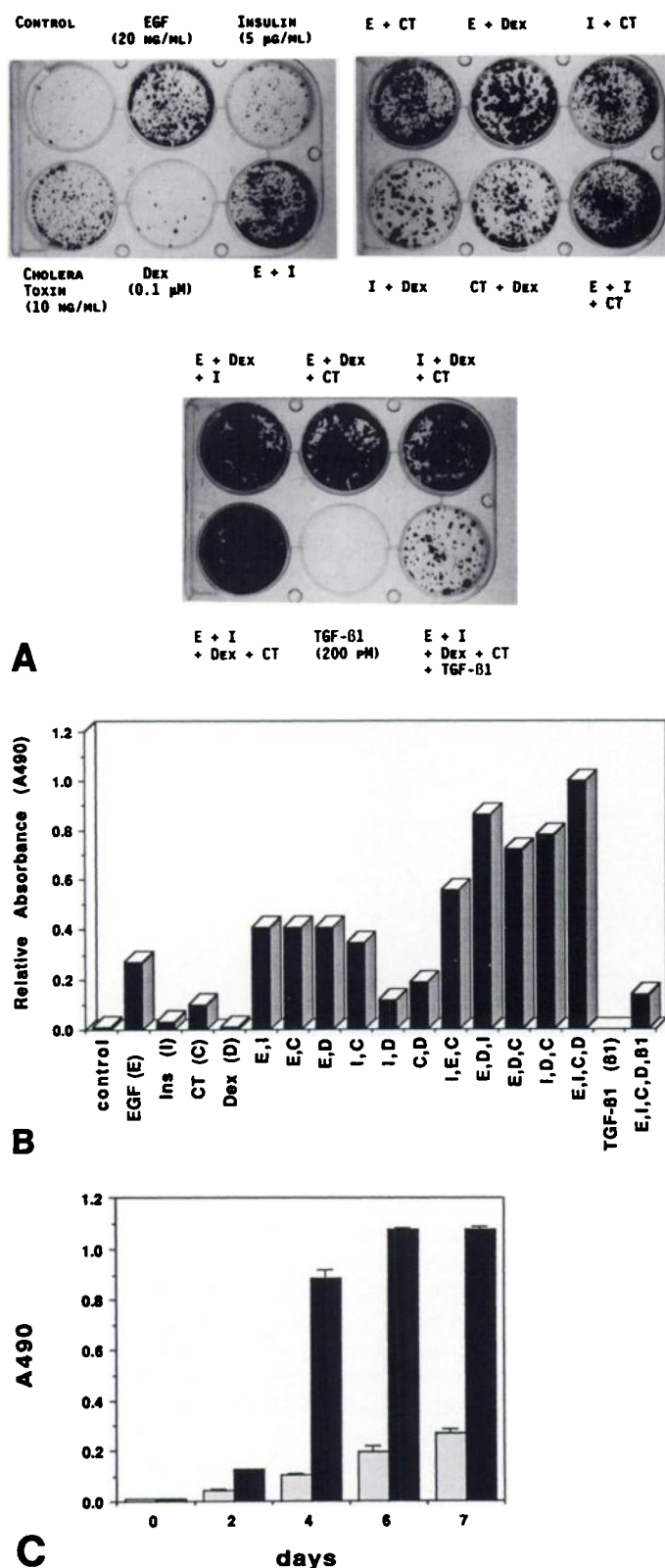


Fig. 2. Growth of NRP-152 in response to regulatory molecules. Growth of NRP-152 passage 7 cells was determined by combinations of 20 ng/ml EGF, 5 µg/ml Ins, 0.1 µM Dex, and 10 ng/ml CT in DMEM/F12 plus 10% FBS. The effect of 200 pM TGF-β1 was determined in basal serum medium alone and in the complete medium. (A) Cells were fixed with formalin after 6 days of treatment and then stained with crystal violet. (B) To quantitate growth, crystal violet was eluted with ethanol-citrate buffer and the absorbance at 490 nm was determined. (C) The growth kinetics of NRP-152 passage 24 cells was determined as above in DMEM/F12 plus 10% FBS alone (□) and supplemented with the combination of 20 ng/ml EGF, 5 µg/ml Ins, 0.1 µM Dex, and 10 ng/ml CT (■). Bars, SD.

cytokeratins 18, but negative for cytokeratins 1, 10, 13, and 14. Expression of cytokeratins 14 and 18 by NRP-152 and NRP-154 cells, respectively, suggests that NRP-152 cells are derived from basal epithelium and NRP-154 cells from luminal epithelium (24, 25). When stained for other intermediate filaments, both NRP-152 and NRP-154 cells were shown to express vimentin but not desmin and α-actin. The positive controls, BHK-21 baby hamster kidney cells and rat neonatal cardiac fibroblasts stained for desmin and α-actin, respectively.

AR Assay. AR was assayed in whole cells by the specific binding of [³H]R1881 (Fig. 3A). The specific binding of R1881 (2 nM) to NRP-152 cells was about 10-fold higher than that of NRP-154 cells. Northern blot hybridization, using a cDNA probe to rat AR, demonstrated that NRP-152 cells expressed AR mRNA at levels comparable to those in rat prostate *in vivo*, while no AR transcript was detected in NRP-154 cells (Fig. 3B). NRP-152 cells produced two similar-sized transcripts, one of which comigrated with AR in the seminal vesicle and all lobes of the rat prostate and the other of which migrated slightly faster. The expression of two such AR transcripts has also been observed in other systems (26, 27). The specific binding of [³H]R1881 to NRP-152 cells was saturable at about 1 nM ligand (Fig. 3C). Scatchard analysis of passage 17 NRP-152 cells showed an average of 5300 receptors/cell with a K_d of 53 pM (Fig. 3D). Neither number nor affinity of these sites changed at passage 56. One hundred-fold molar excesses of progesterone, Dex, and β-estradiol competed for <10%, <1%, and 45%, respectively, of the specific binding of 1 nM [³H]R1881 to NRP-152 cells, whereas DHT and testosterone competed for >95% of the specific binding (data not shown). These data confirm the androgen specificity of these high-affinity sites.

Effect of Androgens on Cell Growth. Attempts to demonstrate mitogenic effects of androgens on early passage NRP-152 cells in medium containing dialyzed and charcoal-extracted FBS were unsuccessful, since the stripped serum did not support growth of early passage NRP-152 cells over several days. At a later passage, NRP-152 cells (passage 67) exhibited sustained growth (doubling time, 67 h) in the above medium, and a single addition of DHT at 10 nM over 11 days stimulated a 2-fold increase in cell number (data not shown). Growth of NRP-154 cells was not stimulated by DHT under identical conditions. To test early (1–2 days) effects of DHT on cell growth, we assayed for DNA synthesis in a SFM consisting of DMEM/F12, 15 mM HEPES, 0.1% BSA V, 10 µg/ml human transferrin, 1× trace element mix, and 20 ng/ml EGF. Using this medium, we showed that as little as 1 nM DHT caused a 2-fold increase in DNA synthesis in NRP-152 cells; similar effects were seen with R1881 (Fig. 4A). These were not nonspecific steroid hormone effects since neither estradiol nor progesterone (10 nM) was mitogenic for these cells, whereas 10 nM DHT or testosterone stimulated DNA synthesis 2.5-fold over control (data not shown). The specificity of this effect was further confirmed by use of the anti-androgen hydroxyflutamide (1 µM), which blocked induction of DNA synthesis by 10 nM DHT (Fig. 4B).

Expression of Acid Phosphatase. Total acid phosphatase and PAP activity were measured in NRP-152 cells cultured in SFM for 4 days with and without 10 nM DHT. When the specific activity of phosphatase was expressed in units/mg protein and compared to that of rat male sex accessory organs, NRP-152 exhibited a substantially higher level of both acid phosphatase and PAP activity than normal prostatic tissue or seminal vesicle (Table 1). Furthermore, the PAP: non-PAP ratio was much higher in NRP-152 cells than in any normal prostatic tissue. DHT treatment of NRP-152 cells caused a 36% decrease in PAP activity, while not affecting that of the non-PAP. Under identical conditions without DHT, the specific activities of PAP and non-PAP in NRP-154 cells were 8.5 and 3.6 units/mg,

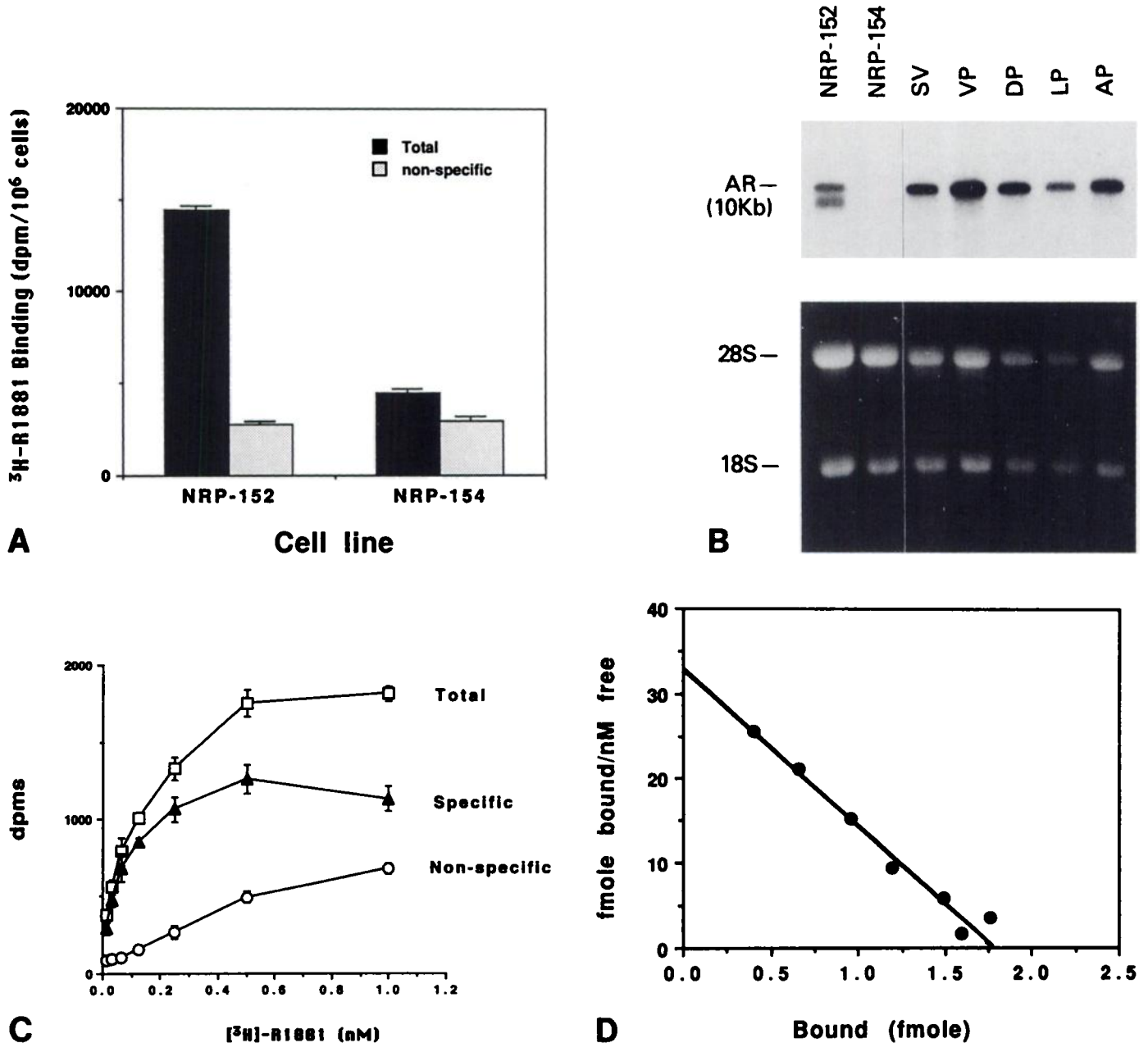


Fig. 3. ARs in prostatic cell lines. (A) Binding of 2 nM [³H]R1881 to NRP-152 (passage 34) and NRP-154 cells (passage 15) was done in the absence (specific) and presence (nonspecific) of 0.2 μM unlabeled ligand; (B) mRNA for AR in the above cell lines and in adult rat the seminal vesicle (SV), ventral prostate (VP), dorsal prostate (DP), lateral prostate (LP), and anterior prostate (AP) from adult rats were determined by Northern blot hybridization; (C) presence of high-affinity androgen binding to NRP-152 passage 17 cells was demonstrated by saturable specific binding of [³H]R1881; (D) Scatchard analysis is shown. All values represent the average of triplicate determinations ± SD (bars). Kb, kilobases.

respectively (Table 1). NRP-152 and NRP-154 cells also express high levels of the 4.9-kilobase PAP transcript (28) relative to rat dorsal and lateral prostates *in vivo*, with higher levels in NRP-152 cells than in NRP-154 cells (Fig. 5).

Inhibition of Growth by TGF-βs. Previous investigators have shown TGF-β1 to be a potent inhibitor of prostatic epithelial cell growth (29–31). Here we show that the single addition of 200 pM TGF-β1 over 2–5 days not only inhibits growth of NRP-152 and NRP-154 cells but also promotes death of NRP-154 cells (Fig. 6, A and B). Within 48 h of its addition, TGF-β1 inhibited DNA synthesis in both NRP-152 and NRP-154 cells by >95% in the presence of 10% FBS and a variety of mitogens (data not shown). In a dose-response experiment, TGF-β3 was more active than TGF-β1, with TGF-β2 being the least active in inhibiting DNA synthesis of NRP-152 cells (Fig. 6C).

Regulation of Cell Growth by RA and 1,25(OH)₂D₃. The effects of retinoids and vitamin D derivatives on inhibiting growth and promoting differentiation of epithelial cells is well documented (32, 33). The effects of RA and 1,25(OH)₂D₃ on growth of NRP-152 and NRP-154 cells were examined as a function of time. Growth of NRP-152 cells was inhibited by RA as measured by both changes in cell number (Fig. 7) and [³H]thymidine incorporation (Fig. 8) with 50% effective dose of about 1 nM (data not shown). 1,25(OH)₂D₃ stimulated growth of NRP-152 cells at early time points and inhibited growth at later times (Figs. 7, A and B, and 8). At early time points (24–48 h), 1,25(OH)₂D₃ also stimulated the incorporation of [³H]thymidine by severalfold with a 50% effective dose of 20 nM (data not shown). In contrast, growth of NRP-154 cells, under conditions identical to those in Fig. 7, was neither stimulated nor inhibited by either RA or 1,25(OH)₂D₃ (data not shown). Our preliminary results suggest

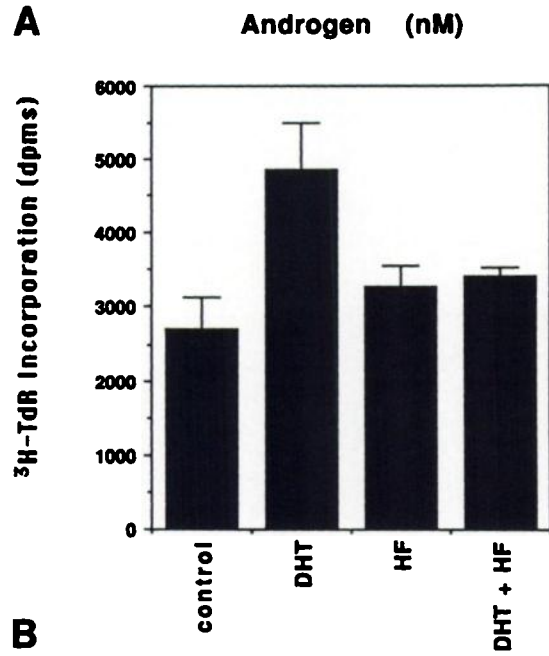
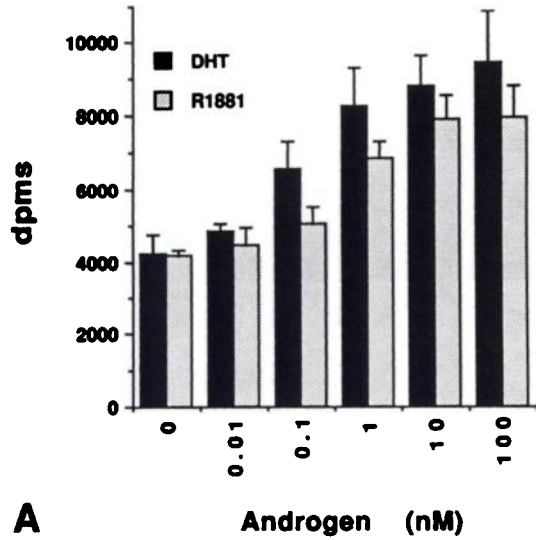


Fig. 4. Stimulation of the growth of NRP-152 cells by androgens. (A) Dose response of DHT and R1881 on DNA synthesis. (B) Effect of 1 μ M hydroxyflutamide (HF) on stimulation of DNA synthesis by 10 nM DHT. Cells were stimulated in SFM and the incorporation of [³H]thymidine (³H-TdR) was determined. All values represent the average of triplicate determinations \pm SD (bars).

that lack of response of NRP-154 cells to 1,25(OH)₂D₃ may be the result of vitamin D receptor deficiency, since these receptors are expressed at much lower levels in NRP-154 cells relative to NRP-152 cells (data not shown).

Recent evidence suggests that effects of RA and 1,25(OH)₂D₃ on inhibition of growth may be synergistic (34). We examined the potential synergism of 1 μ M RA and 10 nM 1,25(OH)₂D₃ on growth of NRP-152 cells in DMEM/F12 supplemented with 20 ng/ml of EGF and FBS as assayed by the incorporation of [³H]thymidine after a 6-day treatment (Fig. 8, A, B, and C). The concentration of FBS was varied to optimize the response to these agents. 1,25(OH)₂D₃ inhibited growth in the presence of either 5 and 2.5% FBS, whereas it was slightly stimulatory in the presence of 10% FBS (Fig. 8A). RA, on the other hand, was inhibitory under all conditions. When the data were normalized to the percentage of that of the serum control, the combined effect of RA and 1,25(OH)₂D₃ was clearly synergistic in 5 and 10% FBS.

DISCUSSION

We have developed nontumorigenic and tumorigenic epithelial cell lines from Lobund/Wistar rat dorsal-lateral prostate. Corresponding with the differentiated phenotype of prostatic epithelium, both cell lines produce relatively high levels of PAP, and NRP-152 cells express AR mRNA and possess functional ARs. In contrast, NRP-154 cells show no detectable AR mRNA and do not respond to androgens under identical conditions. Furthermore, similar to primary cultures of prostatic epithelium (1-4, 35, 36), NRP-152 cells require the combination of EGF, Ins, Dex, and CT for optimal growth. NRP-154 cells, however, grow much more autonomously in culture, which is consistent with their tumorigenic phenotype. Although *K_r-ras* mutation at codon 12 is common in prostatic tumors induced by *N*-methyl-*N*-nitrosourea (37, 38), neither cell line has this mutation (data not shown).

Table 1 Levels of prostatic acid phosphatase in NRP-152 cells and in rat prostate in vivo

TAP^a and PAP were measured in lysates of NRP-152 cells treated with and without 10 nM DHT for 4 days in SFM, in extracts of NRP-154 cells maintained in SFM for 4 days, and in extracts of adult Sprague-Dawley rat prostate. Values shown for the NRP-152 and NRP-154 cells represent the average of two treatments \pm SD.

	Sigma units/mg protein		
	TAP	PAP	nonPAP
NRP-152 cells			
Control	34.3 \pm 0.5	28.3 \pm 0.8	6.0 \pm 0.3
10 nM DHT	23.8 \pm 1.5	18.1 \pm 0.4	5.7 \pm 1.1
NRP-154 cells	12.1 \pm 1.3	8.5 \pm 0.9	3.6 \pm 1.6
Anterior prostate	0.96	0.52	0.44
Lateral prostate	1.79	0.63	1.15
Dorsal prostate	1.42	0.71	0.71
Ventral prostate	2.45	1.07	1.38
Seminal vesicle	3.39	1.35	2.04

^a TAP, total acid phosphatase.

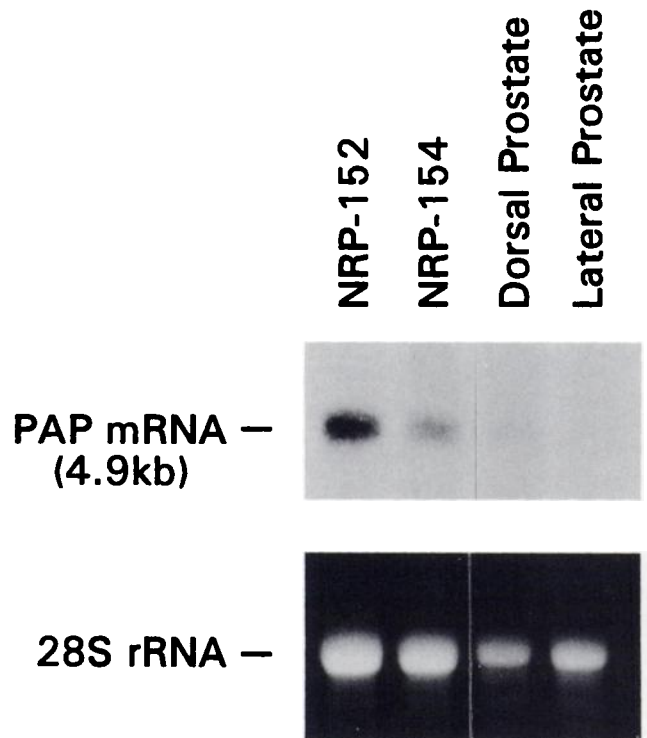
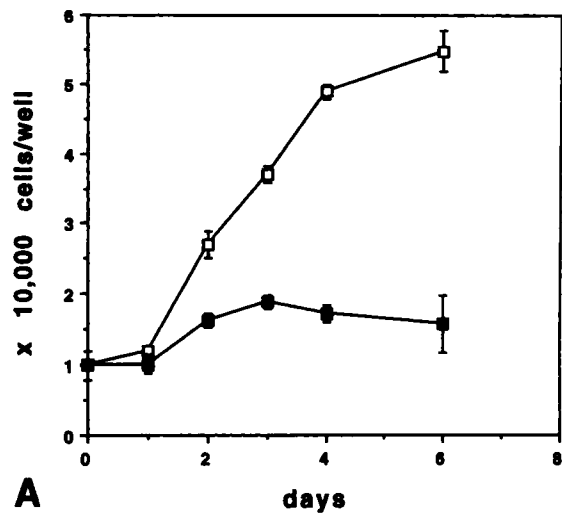
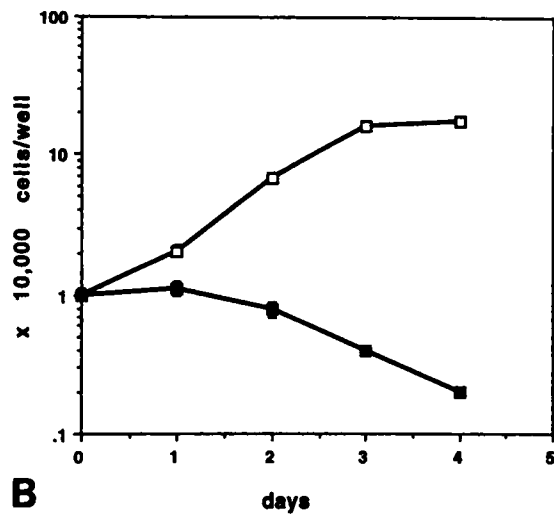


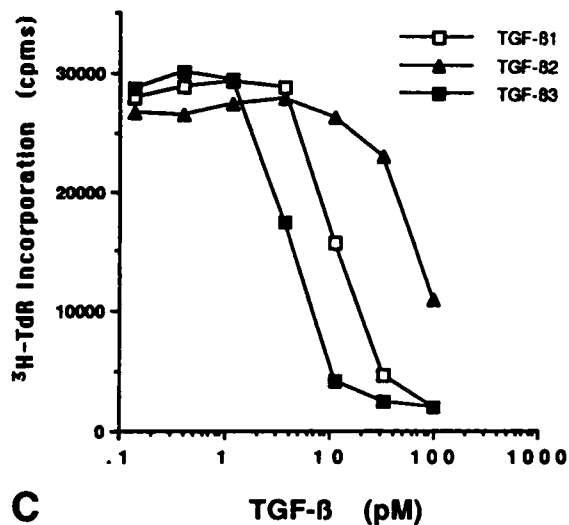
Fig. 5. Northern blot analysis of PAP mRNA. Expression of the 4.9-kilobase (kb) PAP mRNA in NRP-152 and NRP-154 cells grown in GM1 and in dorsal and lateral prostates of adult rats was determined by Northern blot hybridization using 10 μ g of total RNA/lane.



A

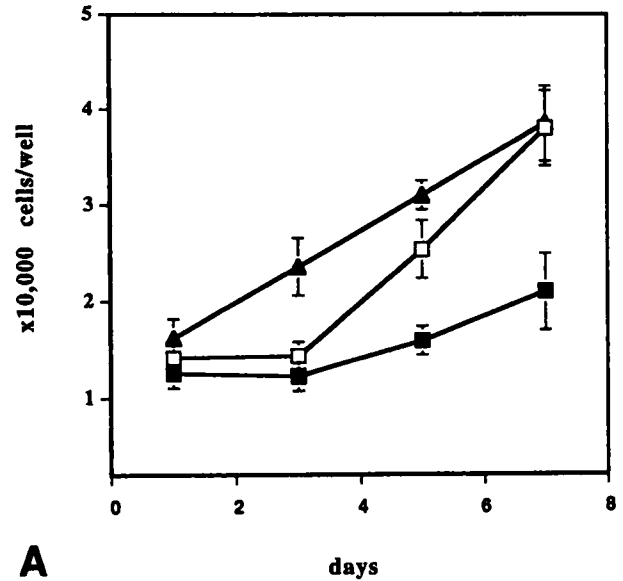


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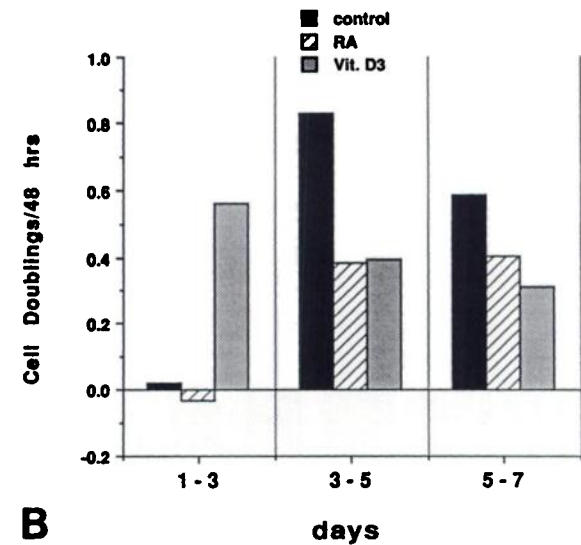


C

Fig. 6. Effect of TGF- β s on growth of NRP-152 and NRP-154 cells. Inhibition of cell growth by 200 pM TGF- β 1 (■) on NRP-152 (passage 26) cells (A) and NRP-154 (passage 10) cells (B) was determined by changes in cell number as a function of time (□, control). Cells were plated in DMEM/F12 supplemented with 10% FBS plus 20 ng/ml EGF. (C) Dose response of TGF- β s 1, 2, and 3 on growth of NRP-152 cells (passage 12) in DMEM/F12 supplemented with 10% FBS plus 20 ng/ml EGF, as measured by the incorporation of [3 H]thymidine. All values represent the average of triplicate determinations \pm SD (bars).



A



B

Fig. 7. Effects of RA and 1,25(OH) $_2$ D $_3$ on NRP-152 cell growth. (A) Effects of 0.1 μ M RA (■) and 1,25(OH) $_2$ D $_3$ (▲) or ethanol vehicle (□) on growth of NRP-152 cells (passage 12) in DMEM/F12 supplemented with 5% FBS and 20 ng/ml EGF were measured as a function of time. (B) Data from A was divided into three 48-h segments and expressed as cell population doubling/48 h.

The NRP-152 cell line offers a suitable and unique system for studying early events during carcinogenesis: (a) the dorsal-lateral prostate is believed to be functionally and structurally more similar to human prostate than rat ventral prostate (10–12); (b) carcinomas can be induced in the dorsal-lateral prostate in several rat models (38–40); (c) NRP-152 is the only nontumorigenic prostatic cell line reported to have functional ARs (5–7); (d) previously described human and rat dorsal-lateral prostatic cell lines that are nontumorigenic have often been immortalized by overexpression of SV40 large-T antigen (5, 7–12), a viral product that has not been reported to be involved in the etiology of prostate cancer in any system. In contrast, the NRP-152 cell line has been derived from animals that undergo spontaneous prostate adenocarcinoma. Thus, although the immortalizing gene alteration(s) of this cell line is as yet undefined (in contrast to the SV40 immortalized lines), NRP-152 cells are more likely to be representative of a preneoplastic state than any of the immortalized prostatic cell lines.

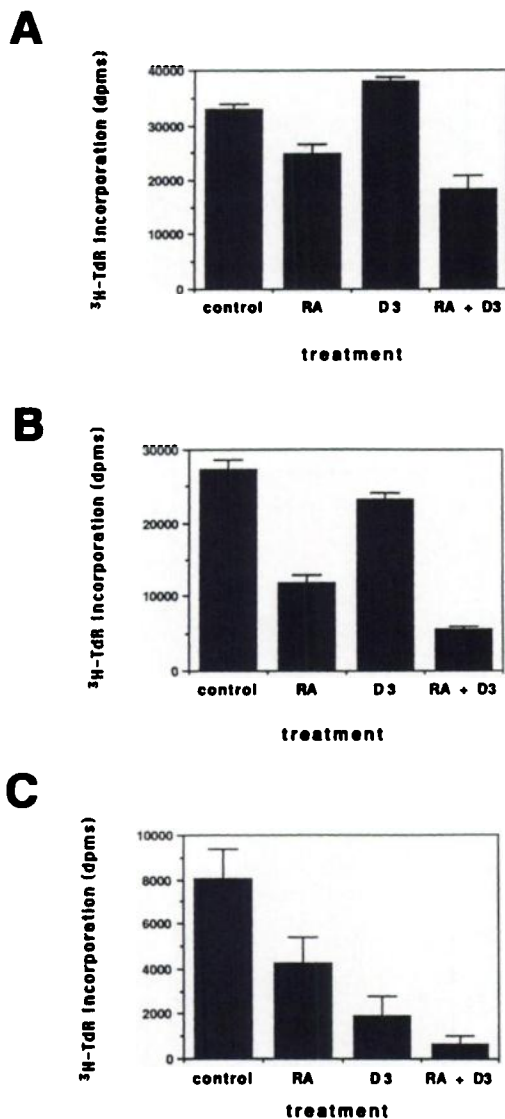


Fig. 8. Interaction of RA and 1,25(OH)₂D₃ on growth of NRP-152 cells. The effects of 1 μ M RA and 10 nM 1,25(OH)₂D₃ (D3) on NRP-152, passage 6 cell growth were determined in DMEM/F12 plus 20 ng/ml EGF supplemented with 10% FBS (A), 5% FBS (B), and 2.5% FBS (C). [³H]Thymidine was added 5 days after hormone treatment. All values represent the average of triplicate determinations \pm SD (bars).

Stimulation of NRP-152 growth by DHT is in contrast to the absence of a direct mitogenic effect of this steroid on primary prostatic epithelial cells reported by other investigators (1, 19, 35). The reason for this apparent discrepancy is not known. Variables such as growth conditions, state of differentiation, and origin of cell type may be critical for such androgenic effects. In support of the latter two possibilities, androgen receptors are not detectable in the prostatic epithelium of fetal and neonatal stages of development (41) and are not present in most basal and some luminal epithelial cells of adult prostate (42). The absence of direct mitogenic effects of androgens on primary cultures of prostatic epithelium has led to the investigation of a paracrine mechanism of androgen action mediated by the release of growth factor(s) (19), such as keratinocyte growth factor (43)³ by prostatic stroma. However, the absence of stroma in NRP-152 cells, in LNCaP human prostatic carcinoma cells (44), and in Dunning R3327G cells (45) for which growth in all is stimulated by androgens,

³ J. Rubin, personal communication.

suggests that certain epithelial cells can also respond to androgen directly.

It has been shown recently that although prostatic tumors initially express elevated levels of PAP, such levels drop with tumor progression toward a more malignant state (46). Our demonstration of elevated levels of PAP in NRP-152 cells relative to the whole prostate and in NRP-154 cells is consistent with increased PAP expression occurring as an early event in carcinogenesis. The increased growth potential of more malignant prostate tumors may be explained by reduced cellular PAP, which has been shown to be the major phosphotyrosine phosphatase in human prostate (47), and is believed to dephosphorylate phosphotyrosine residues that are essential for growth factor signaling (48). This model has recently been tested by transfection of malignant prostatic carcinoma cells with a PAP expression construct, which elevated PAP levels and inhibited growth of the transfectants (46). By this mechanism, reduction of PAP levels by DHT in NRP-152 (Table 1) and LNCaP (49) cells may stimulate growth and thereby account for the mitogenic action of androgens on these cells.

There is strong evidence that TGF- β s play an important role in prostate development and function. TGF- β 1 not only inhibits growth of prostatic epithelial cells (29–31) but may also be responsible for apoptosis in the prostate since its mRNA expression is dramatically elevated in regressing rat prostates following castration (50). Here, we show that TGF- β 1 not only inhibits growth of NRP-152 and NRP-154 cells but also induces death of NRP-154 cells. The apparent greater sensitivity of NRP-154 cells than of NRP-152 cells to TGF- β 1, which is consistent with the selective loss of luminal epithelium during apoptosis of the prostate (24), may be explained by the greater abundance of mRNA for TGF- β type II receptors in NRP-154 cells (data not shown), as recent evidence supports that this class of TGF- β receptors mediate inhibition of growth by TGF- β s (51). The great sensitivity of NRP-154 cells to TGF- β further demonstrates that loss of sensitivity to TGF- β is not an early event of carcinogenesis in this system.

The use of steroids and retinoids in the chemoprevention of cancer has recently attracted much attention. The high sensitivity of NRP-152 cells to both RA and 1,25(OH)₂D₃ makes these cells suitable for studying the mechanism of action of these hormones. We have shown here that inhibitory effects of RA and 1,25-(OH)₂D₃ can be synergistic, rather than additive, which is consistent with recent findings in human breast cancer cells (34). Recently, we have also demonstrated that inhibition of growth by RA in NRP-152 cells is mediated via induction of TGF- β s (52), as reported for neonatal mouse keratinocytes (53). These new data suggest that TGF- β s may play a major role in prostatic responsiveness to retinoids and the potential ability of retinoids to function in the chemoprevention of prostatic cancer.

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