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Terminal neuroendocrine differentiation of human prostate carcinoma cells in response to increased intracellular cyclic AMP

(hormone refractory/pp60$^{c\text{-}src}$/multipotential)

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ABSTRACT **Recent clinicopathologic studies have shown that many prostatic adenocarcinomas express focal neuroendocrine differentiation and that neuroendocrine differentiation is most apparent in advanced anaplastic tumors. While studying growth-regulatory signal transduction events in human prostate carcinoma cell lines, we found that in two of four cell lines, the androgen-sensitive line LNCaP and the highly metastatic androgen-independent line PC-3-M, elevation of cAMP through addition of cAMP analogues or phosphodiesterase inhibitors induced a markedly neuronal morphology. Also in LNCaP cells ultrastructural analysis showed that cAMP induced the appearance of neurosecretory cell-like dense-core granules. Phenotypic analysis of untreated LNCaP and PC-3-M cells showed that both cell lines express markers of the neural crest including S-100, chromogranin A, pp60$^{c\text{-}src}$, and neuron-specific enolase as well as the epithelial marker KS1/4 and stage-specific embryonic antigen 4. In PC-3-M cells, cAMP markedly elevated neuron-specific enolase protein and caused an increase in the specific activity of the neuroendocrine marker pp60$^{c\text{-}src}$, and in both cell lines expression of KS1/4 and stage-specific embryonic antigen 4 was down-regulated. In addition to effects on lineage markers, cAMP treatment induced G_1 synchronization, growth arrest, and loss of clonogenicity, indicating terminal differentiation. Our data provide direct evidence of plasticity in the lineage commitment of adenocarcinoma of the prostate. We have shown that cell-permeant cAMP analogues can induce terminal differentiation, suggesting that hydrolysis-resistant cyclic nucleotides may present an additional approach to the treatment of advanced prostate cancer.**

In an effort to develop new anticancer drugs directed at unique aspects of prostate cancer biology, we have been studying the signal transduction pathways regulating the growth of human prostate adenocarcinoma cells *in vitro*. As reported previously, we found that addition of dibutyryl (db) cAMP to the androgen-independent prostate carcinoma cell line PC-3 causes induction of type β2 transforming growth factor (TGF-β2) mRNA, production of bioactive TGF-β2, and growth arrest (1).

We have subsequently studied the effect of cAMP derivatives and phosphodiesterase inhibitors on the other two commonly available prostate carcinoma cell lines, DU 145 and LNCaP, as well as the highly metastatic variant of PC-3, PC-3-M, and found that all lines were growth inhibited by elevation of intracellular cAMP (data not shown). Data presented here demonstrate that in two of these lines, LNCaP and PC-3-M, elevation of intracellular cAMP induces permanent conversion from an epithelial to a neuronal morphology and that these cells express markers of the neuroendocrine phenotype. These data suggest that these cell lines, which are derived from metastatic adenocarcinoma of the prostate, contain or consist of multipotent cells capable of both neuroendocrine and epithelial differentiation.

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MATERIALS AND METHODS

Cell Culture. The human prostate carcinoma cell lines PC-3, DU 145, and LNCaP were obtained from the American Type Culture Collection. PC-3-M, a highly metastatic variant of PC-3 cells (2), was a kind gift of James Kozlowski (Department of Urology, Northwestern University). LNCaP and PC-3-M cells were maintained in RPMI 1640 medium with 10% fetal bovine serum. Treatment with db-cAMP or isobutylmethylxanthine (IBMX) was started 24 hr after subculturing cells and continued every other day. SKNAS neuroblastoma cells (3) were kindly provided by Lee Helman (Pediatric Branch, National Cancer Institute).

Growth Studies. Cells were seeded at an initial density of 5000 cells per cm^2, incubated for 24 hr, and treated as indicated. Viable cell number was determined by hemacytometer counts of trypan blue-excluding cells. For clonogenic assays, cells were treated for 6 days, harvested, washed twice, replated in fresh medium at the same viable cell number per plate, and grown in the absence of additional drug. After 7 days, the cells were stained with methylene blue and colonies were counted using an Artek 880 automated colony counter.

Electron Microscopy. For transmission electron microscopy, the cells were fixed *in situ* with glutaraldehyde, embedded in agar, postfixed in osmium tetroxide, stained *en bloc* with uranyl acetate, dehydrated, infiltrated with plastic, embedded and polymerized, sectioned 70 nm thick, poststained with lead citrate, and examined in a Zeiss EM 10/C electron microscope. For scanning electron microscopy, the cells were fixed *in situ* with glutaraldehyde, dehydrated, critical-point dried, coated with gold palladium, and examined in an Amray 1820 scanning electron microscope.

Immunocytochemistry. Control and cAMP-treated cells were fixed with acetone at 4°C for 10 min. Rabbit antibodies recognizing human neuron-specific enolase (NSE) and S-100 protein were obtained from Dako. Antibody reactivity was detected by using biotinylated F(ab')$_2$ anti-rabbit immunoglobulin and streptavidin-conjugated horseradish peroxidase,

Abbreviations: db, dibutyryl; TGF, transforming growth factor; IBMX, isobutylmethylxanthine; NSE, neuron-specific enolase.

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followed by incubation with hydrogen peroxide and the chromogen aminoethylcarbazole (Tago).

Flow Cytometry. The epithelial antigen KS1/4 and stage-specific embryonic antigen expression were analyzed by flow cytometry (4). For cell cycle analysis, cells were harvested, fixed in 50% ethanol, and incubated with the DNA intercalating dye chromomycin A (Sigma).

Western Blot Analysis. Cell lysates were subjected to electrophoresis in a SDS/7.5% polyacrylamide gel; proteins were transferred onto nitrocellulose paper, blocked, and incubated with monoclonal anti-human NSE antibody or monoclonal anti-chromogranin A antibody (Boehringer Mannheim). NSE immunoblots were washed and incubated with ^{125}I-conjugated goat anti-mouse immunoglobulin, washed, dried, and subjected to autoradiography. Chromogranin A immunoblots were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin and developed with a chemiluminescent substrate (ECL, Amersham).

Immune Complex Kinase Assay of pp60$^{c\text{-}src}$ Activity. pp60$^{c\text{-}src}$ was immunoprecipitated with mouse monoclonal antibody 327 (5) and protein A-Sepharose beads coated with rabbit anti-mouse immunoglobulin and incubated with [γ-^{32}P]ATP (DuPont/NEN) and the exogenous substrate rabbit muscle enolase (Sigma). Proteins were analyzed by SDS/PAGE followed by autoradiography. Monoclonal antibody 327 was a kind gift of Joan Brugge (ARIAD Pharmaceuticals, Cambridge, MA).

RESULTS

Effects of Elevating cAMP on Prostate Carcinoma Morphology. After addition of db-cAMP or IBMX to each of the four prostate carcinoma cell lines studied two of the cell lines, DU 145 and PC-3, showed little change in morphology (data not shown). In contrast, LNCaP and PC-3-M cells developed features of neuronal morphology after addition of either db-cAMP or IBMX. Fig. 1 *A* and *B* shows the effect of addition of db-cAMP plus IBMX on PC-3-M cells and Fig. 1 *C* and *D* shows the effect of this treatment on LNCaP cells. Untreated cells had an epithelial morphology and tended to grow in clusters with a somewhat acinar appearance. In contrast, db-cAMP- or IBMX-treated cells had a neuronal appearance, characterized by bipolar or multipolar cells with small cell bodies, long processes, and beaded varicosities. Equivalent morphologic effects were seen with a variety of phosphodiesterase inhibitors, including pentoxiphylline and theophylline, and with other cell-permeant cAMP analogs, including phosphorothioate-modified (Sp isomer) cAMP (data not shown). Thus, the effects of db-cAMP did not require the presence of the butyrate moiety. Treatment with db-cAMP alone was a sufficient stimulus to induce all or most of the effects we report (data not shown). The ability of db-cAMP to elevate intracellular cAMP levels in LNCaP and PC-3-M cells was verified by using a ^{125}I-labeled cAMP radioimmunoassay (DuPont) (data not shown).

As shown in the scanning electron micrograph of LNCaP cells treated with db-cAMP plus IBMX (Fig. 2*A*), rather than growing in glandular-appearing clusters, the cells developed a neuronal morphology in which each cell body is isolated, and multipolar processes form contacts with processes from neighboring cells.

The cell lines were further characterized by transmission electron microscopy. Untreated cells were flat and elongated without evidence of neural features except that myelin-like structures were weakly evident in PC-3-M and LNCaP (data not shown). After treatment with db-cAMP and IBMX, PC-3-M and LNCaP were more rounded and, as shown in Fig. 2*B*, LNCaP developed numerous double membrane-bound dense-core granules, characteristic of well-differentiated neurosecretory cells. Elevation of intracellular cAMP was not a sufficient signal for induction of dense-core vesicles in PC-3-M cells. Desmosomes and intracytoplasmic lumina,

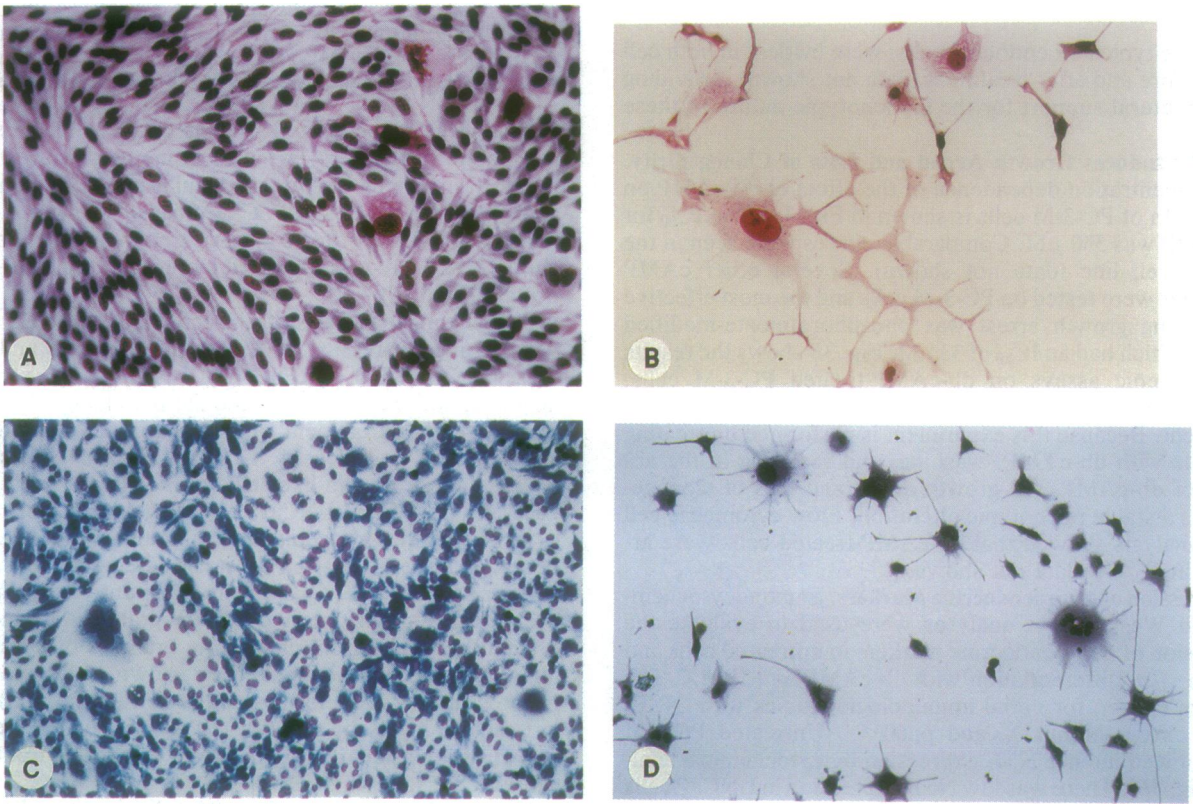

FIG. 1. Wright-stained PC-3-M and LNCaP cells. (*A*) Untreated PC-3-M cells. (*B*) PC-3-M cells treated for 5 days with 1 mM db-cAMP/500 μM IBMX. (*C*) Untreated LNCaP cells. (*D*) LNCaP cells treated for 5 days with 1 mM db-cAMP/500 μM IBMX.

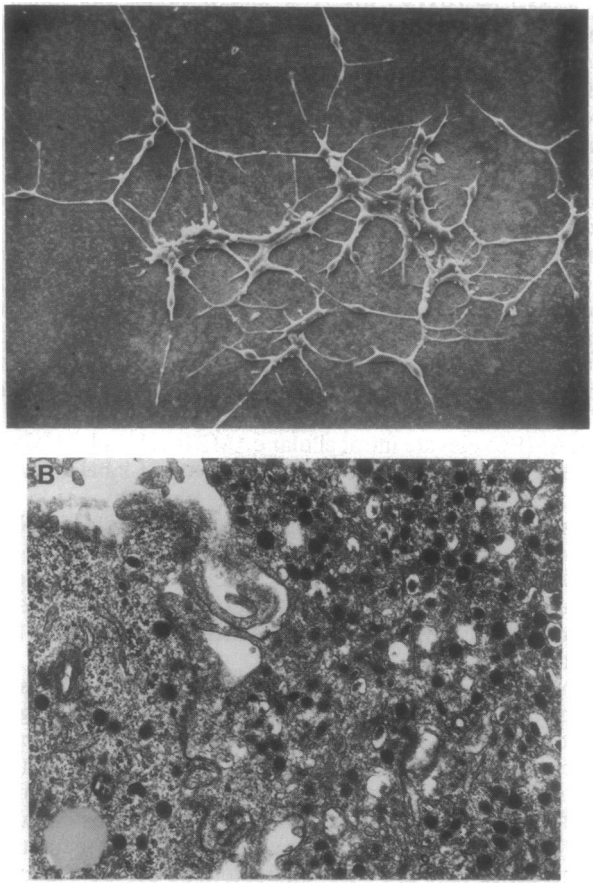

FIG. 2. (*A*) Scanning electron micrograph of LNCaP cells treated with 1 mM db-cAMP/500 mM IBMX. (*B*) Transmission electron micrograph of LNCaP cells treated with 1 mM db-cAMP/500 μM IBMX for 6 days. (×22,500.)

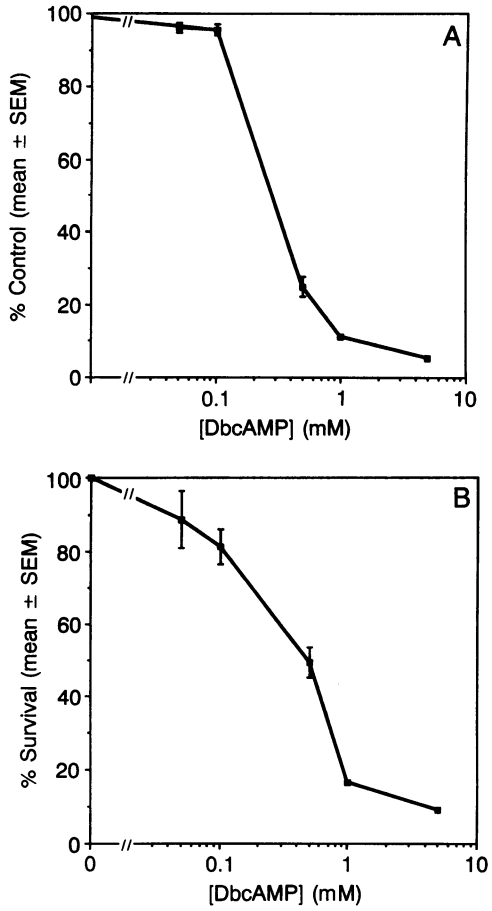

FIG. 3. (*A*) Concentration dependence of db-cAMP-induced growth inhibition in PC-3-M cells. Data are expressed as percentage of the number of viable cells in untreated control wells. Data are representative of three different experiments and values are means ± SEM ($n = 3$). (*B*) cAMP-induced loss of clonogenicity in PC-3-M cells. Data represent means ± SEM of three different experiments.

which are typical of epithelial cells, were evident in both cell lines before and after treatment (data not shown), providing ultrastructural support for the biphenotypic nature of these cells.

cAMP Induces Growth Arrest and Loss of Clonogenicity. The concentration dependence of the effect of db-cAMP on the growth of PC-3-M cells is shown in Fig. 3*A*. The IC_{50} for db-cAMP was 500 μM. Comparable curves were seen in the LNCaP cell line (data not shown). Several other cAMP analogues were tested on PC-3-M cells and the most effective in inducing growth arrest was phosphorothioate-modified cAMP, which had an IC_{50} of 33 μM. Fig. 3*B* shows the results of clonogenic assays on db-cAMP-treated PC-3-M cells. There was a marked decrease in clonogenicity after db-cAMP treatment. Because this experiment is performed by treating the cells with db-cAMP, washing, and replating in the absence of db-cAMP, the growth arrest and loss of clonogenicity is a stable phenotypic alteration. Flow cytometric cell cycle analysis showed that db-cAMP-treated cells were arrested in G_0/G_1 (data not shown).

Expression of Neuroendocrine Markers. Immunocytochemical and Western blot analyses were used to evaluate the expression of neuroendocrine markers in untreated cells and after *in vitro* differentiation with db-cAMP and IBMX. The markers chosen for initial immunologic studies were NSE, S-100, chromogranin A, and $pp60^{c\text{-}src}$. Untreated PC-3-M cells showed intense NSE expression by cytochemical analysis (Fig. 4*B*). There was low NSE expression in LNCaP cells (data not shown). An advantage of NSE as a marker is that monoclonal anti-NSE antibody can be used to detect NSE on Western blots, allowing identification of the molecular mass of the protein recognized cytochemically. NSE is a homodimeric protein composed of monomers with a relative mobility corresponding to a molecular mass of ≈46 kDa (6). In Western blot analysis of PC-3-M cells, NSE ran as a single band of ≈47 kDa. After 6 days of treatment with db-cAMP and IBMX, there was a marked increase in the level of NSE protein (Fig. 5*A*).

Cytochemical analysis of untreated PC-3-M cells showed very strong expression of the neuroendocrine and neural crest marker S-100 (7) (Fig. 4*C*). LNCaP cells were S-100 negative, and the levels of S-100 did not change discernably after db-cAMP treatment of PC-3-M or LNCaP (data not shown). The presence of S-100 in PC-3-M cells and the observation of myelin-like structures, characteristic of glial cells, in PC-3-M and LNCaP cells suggest that there may be evidence of multipotential differentiation (glial as well as neuronal and epithelial) in prostatic carcinoma cells.

Both LNCaP and PC-3-M cells when untreated expressed the neuroendocrine marker chromogranin A, which was originally isolated from chromaffin granules of adrenal medulla, and has subsequently been shown to be widely distributed in endocrine tissues and their tumors (8, 9). Chromogranin A levels did not vary greatly with cAMP treatment and are shown relative to a positive control neuroblastoma cell line SKNAS (Fig. 5*B*).

Untreated LNCaP and PC-3-M cells expressed the pan-epithelial surface membrane antigen identified by the monoclonal antibody KS1/4 (10) (data not shown) and the level of

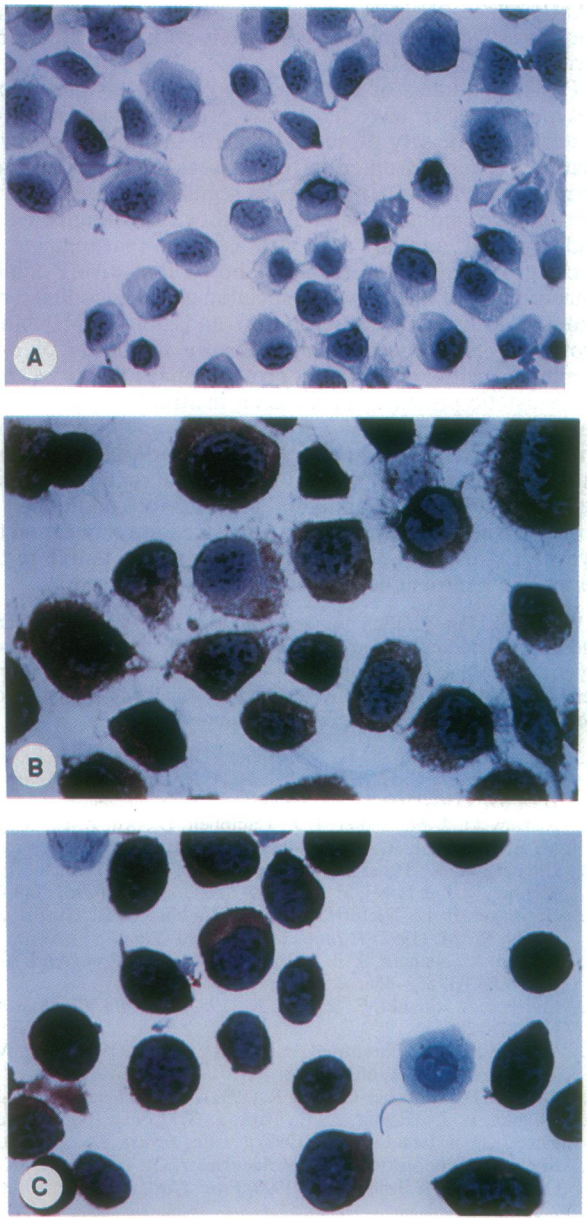

FIG. 4. Immunocytochemical analysis of PC-3-M cytospin preparations. (*A*) Isotype control antibody. (*B*) Antibody to NSE. (*C*) Antibody to S-100.

KS1/4 expression was down-regulated by treatment with db-cAMP and IBMX (30% decrease in LNCaP and 74% decrease in PC-3-M cells).

In data not shown, the intermediate filament phenotype of the cells was examined by cytochemical analysis. Both cell types were found to express cytokeratin (monoclonal anti-human epithelial keratin; Boehringer Mannheim) and low molecular weight neurofilament protein (Boehringer Mannheim), further supporting the biphenotypic nature of the cells.

LNCaP and PC-3-M cells were examined for expression of stage-specific embryonic antigens using anti-SSEA-1, -3, and -4 antibodies and were found to express SSEA-4 (data not shown). Treatment of PC-3-M cells for 5 days with db-cAMP and IBMX down-regulated SSEA-4 expression by 75%. Although not a specific stem cell marker, SSEA expression is characteristic of embryonic cells and embryonal carcinoma cells (11). It has been reported that db-cAMP treatment induces neuronal differentiation and down-regulation of SSEA expression in the teratocarcinoma cell line F9 (12). These preliminary data suggest that prostate carcinoma cells

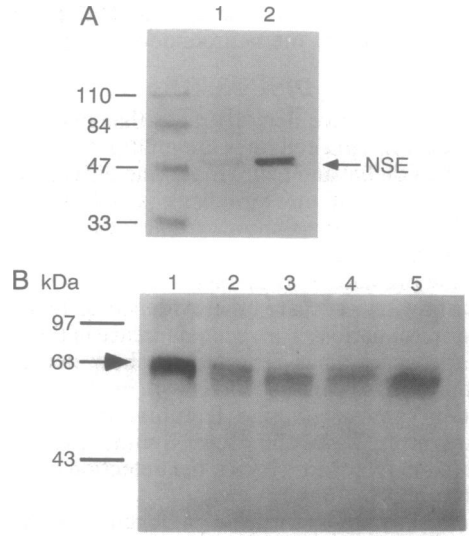

FIG. 5. Western blot analysis of NSE (*A*) and chromogranin A (*B*). (*A*) PC-3-M cells were untreated (lane 1) or treated with 1 mM db-cAMP/500 mM IBMX for 6 days. Western blot analysis was performed with anti-NSE antibody. (*B*) Chromogranin A levels were examined by Western blot using anti-chromogranin A antibody in positive control SKNAS cells (lane 1), LNCaP cells (lanes 2 and 3), and PC-3-M cells (lanes 4 and 5). Cells in lanes 3 and 5 were treated for 6 days with 1 mM db-cAMP/500 μM IBMX.

may express some stem cell markers and that these markers can be down-regulated by cytodifferentiation.

Both cell lines were examined for expression of the non-receptor-linked protein tyrosine kinase pp60$^{c\text{-}src}$, which has been reported to be a marker of neuroendocrine differentiation (13). The expression of pp60$^{c\text{-}src}$ activity was low in LNCaP cells (data not shown), while pp60$^{c\text{-}src}$ activity was readily detected in PC-3-M cells (Fig. 6). After treatment of PC-3-M cells for 6 days with db-cAMP/IBMX there was a modest increase in pp60$^{c\text{-}src}$ kinase activity as detected by autophosphorylation and phosphorylation of the exogenous substrate enolase. In contrast, treatment with TGF-β1, which is highly growth inhibitory to these cells but does not induce evidence of neuroendocrine differentiation, caused a decrease in pp60$^{c\text{-}src}$ activity. This suggests that the increase in pp60$^{c\text{-}src}$ activity with agents that elevate cAMP is related to cAMP-induced differentiation rather than growth arrest. In an experiment not shown, when cell lysates were used for both Western blot analysis and immune complex kinase assay and the radioactivity incorporated into the pp60$^{c\text{-}src}$ and enolase bands was compared to the amount of pp60$^{c\text{-}src}$ protein, it was found that cAMP treatment caused a 50%

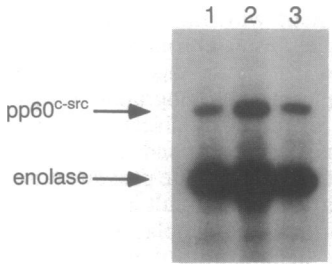

FIG. 6. *In vitro* kinase activity of pp60$^{c\text{-}src}$ in PC-3-M cells. Cells were treated with vehicle only (lane 1), db-cAMP/IBMX (lane 2), or TGF-β1 (10 ng/ml) (lane 3) for 6 days. Cells were immunoprecipitated with anti-pp60$^{c\text{-}src}$ antibody 327 and immunoprecipitates were incubated with [γ-^{32}P]ATP and rabbit enolase. Proteins were analyzed by SDS/PAGE and ^{32}P-labeled proteins were visualized by autoradiography.

increase in the $pp60^{c\text{-}src}$ kinase specific activity, consistent with the expression of the neuroendocrine phenotype (13).

DISCUSSION

In this study, we have demonstrated that two human prostatic adenocarcinoma cell lines are multipotential neoplasias capable of epithelial and neuroendocrine differentiation. The clinical relevance of these findings is indicated by recent clinicopathologic studies showing that focal neuroendocrine differentiation is a relatively common feature of adenocarcinoma of the prostate with as many as 47% (14) to 100% (15) of cases showing at least some evidence of focal neuroendocrine differentiation. The recent increase in the percentage of prostatic carcinomas reported to show neuroendocrine differentiation has been associated with improved methods of detection of neuroendocrine features, including the Grimelius argyrophil stain, the use of Bouin's fixative, and the use of immunocytochemistry for the detection of eutopic and ectopic hormones (16).

In an effort to understand the pathogenetic significance of neuroendocrine differentiation in prostatic carcinomas, Abrahamsson *et al.* (17) studied the incidence of neuroendocrine differentiation during tumor progression by analyzing repeat biopsies in the course of disease and correlating conventional histopathologic grading of prostatic tumors with assessment of neuroendocrine cells on the basis of the Grimelius silver-staining technique and chromogranin A immunoreactivity (17). The authors found a clearly positive relationship between the degree of neuroendocrine differentiation and tumor progression, confirming their earlier observation that the more anaplastic the prostatic carcinoma, the more numerous were its neuroendocrine cells (15). As suggested by Abrahamsson *et al.*, neuroendocrine differentiation of prostatic adenocarcinoma may represent malignant transformation of a prostatic stem cell, transformation of a prostatic neuroendocrine cell, or dedifferentiation accompanied by a new program of gene expression in a transformed prostatic epithelial cell. Regardless of the cell of origin, our data suggest that although neuroendocrine features may be a poor prognostic sign if untreated or if treated with androgen ablation or cytotoxic therapy, the expression of the neuroendocrine differentiation program presents a therapeutic opportunity to induce growth arrest and terminal differentiation.

The prostate gland contains the largest number of endocrine/paracrine cells of any genitourinary organ. Neuroendocrine secretory products associated with normal prostate and prostatic carcinoma including chromogranin A, serotonin, and thyroid-stimulating hormone-like and bombesin-like peptides may be valuable in screening for or in following prostatic carcinoma, especially poorly differentiated tumors that are most likely to strongly express neuroendocrine markers and that frequently have low levels of prostate-specific antigen and prostatic acid phosphatase secretion (17). In agreement with this, Kadmon *et al.* (18) recently reported that the plasma chromogranin A level was elevated in 48% of 25 patients with stage D2 prostate cancer and that in 2 patients plasma chromogranin A was the only useful blood marker for following disease progression.

It has been suggested that endocrine/paracrine cells, basal cells, and secretory cells of the prostate arise from a common precursor cell, and that multilineage differentiation is a potential of virtually all neoplasms (19). The data we have presented here demonstrate that this plasticity of prostate carcinoma cells can be observed and studied *in vitro* and that cAMP is sufficient to trigger a series of events (i.e., morphological alteration, new organelle formation, changes in enzyme synthesis and activity) associated with the emergence of the neuroendocrine phenotype. We have shown that cAMP induces these events in conjunction with growth arrest and loss of clonogenicity, consistent with the induction of terminal differentiation.

Previous studies of the reversal of the transformed phenotype by cAMP have led to the proposal that cAMP can restore the integrity of normal growth regulatory pathways that have been disrupted in the process of malignant transformation (20). The critical genes regulated by cAMP in the prostate carcinoma cells could be those that induce neuroendocrine differentiation as well as those that regulate cell cycle progression. It has been shown that cAMP inhibits transcription of the growth-associated genes encoding c-myc and the transferrin receptor (21) and that cAMP blocks growth factor-induced mitogenesis by inhibition of c-raf-stimulated mitogen-activated protein kinase activity (22).

Metastatic prostate cancer is an incurable neoplasm. We have shown that elevation of cAMP induces terminal differentiation in human adenocarcinoma cells derived from metastatic sites. Our data suggest that the induction of terminal neuroendocrine differentiation presents an additional approach to the treatment of metastatic prostate cancer and that hydrolysis-resistant cAMP analogues may be active in triggering the neuroendocrine differentiation program.

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1. Bang, Y.-J., Kim, S.-J., Danielpour, D., O'Reilly, M. A., Kim, K. Y., Myers, C. E. & Trepel, J. B. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 3556–3560.
2. Kozlowski, J. M., Fidler, I. J., Campbell, D., Xu, Z.-L., Kaighn, M. E. & Hart, I. R. (1984) *Cancer Res.* **44**, 3522–3529.
3. Cooper, M. J., Hutchins, G. M., Cohen, P. S., Helman, L. J., Mennie, R. J. & Israel, M. A. (1990) *Cell Growth Differ.* **1**, 149–159.
4. Bergsagel, P. L., Victor-Kobrin, C., Timblin, C. R., Trepel, J. & Kuehl, W. M. (1992) *J. Immunol.* **148**, 590–596.
5. Golden, A., Nemeth, S. P. & Brugge, J. S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 852–856.
6. Shimizu, A., Suzuki, F. & Kato, K. (1983) *Biochim. Biophys. Acta* **748**, 278–284.
7. Van Eldik, L. J., Ehrenfried, B. & Jensen, R. A. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6034–6038.
8. Winkler, H. & Fischer-Colbrie, R. (1992) *Neuroscience* **49**, 497–528.
9. Helman, L. J., Gazdar, A. F., Park, J. G., Cohen, P. S., Cotelingam, J. D. & Israel, M. A. (1988) *J. Clin. Invest.* **82**, 686–690.
10. Simon, B., Podolsky, D. K., Moldenhauer, G., Isselbacher, K. J., Gattoni, C. S. & Brand, S. J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2755–2759.
11. Kannagi, R., Cochran, N. A., Ishigami, F., Hakomori, S., Andrews, P. W., Knowles, B. B. & Solter, D. (1983) *EMBO J.* **2**, 2355–2361.
12. Andrews, P. W., Gonczol, E., Plotkin, S. A., Dignazio, M. & Oosterhuis, J. W. (1986) *Differentiation (Berlin)* **31**, 119–126.
13. Brugge, J. S., Cotton, P. C., Queral, A. E., Barret, J. N., Nonner, D. & Keane, R. W. (1985) *Nature (London)* **316**, 554–557.
14. di Sant'Agnese, P. A. & de Mesy Jensen, K. L. (1987) *Hum. Pathol.* **18**, 849–856.
15. Abrahamsson, P.-A., Wadstrom, L. B., Alumets, J., Falkmer, S. & Grimelius, L. (1987) *Pathol. Res. Pract.* **182**, 298–307.
16. di Sant'Agnase, P. (1992) *Hum. Pathol.* **23**, 287–296.
17. Abrahamsson, P.-A., Falkmer, S., Falt, K. & Grimelius, L. (1989) *Pathol. Res. Pract.* **185**, 373–380.
18. Kadmon, D., Thompson, T. C., Lynch, G. R. & Scardino, P. T. (1991) *J. Urol.* **146**, 358–361.
19. Mendelsohn, G. & Maksem, J. A. (1986) in *Pathology Annual*, eds. Sommers, S. C., Rosen, P. P. & Fechner, R. E. (Appleton–Century–Crofts, Norwalk, CT), Part 1, Vol. 21, pp. 91–119.
20. Ashall, F., Sullivan, N. & Puck, T. T. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3908–3912.
21. Trepel, J. B., Colamonici, O. R., Kelly, K., Schwab, G., Watt, R. A., Sausville, E. A., Jaffe, E. S. & Neckers, L. M. (1987) *Mol. Cell. Biol.* **7**, 2644–2648.
22. Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J. & Sturgill, T. W. (1993) *Science* **262**, 988–990.