**Endocrine,** *vol. 17, no. 2, 119–127, March 2002* 0969–711X/02/17:119–127/$12.25 

# Glucocorticoid Inhibition of 235-1 Rat Pituitary Tumor Cell Cycle Progression

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**Control of the cell cycle is accomplished by sequentially activated cyclin-dependent kinases and the action of inhibitory proteins. We have shown that exposure of 235-1 rat pituitary tumor cells to dexamethasone (DEX) leads to a 50% reduction in growth rate. We examined the mechanism by which DEX affects 235-1 cell proliferation by determining the expression levels of proteins involved in cell-cycle progression. The expression of the G1 markers c-Myc and cyclin D3 were unaffected by DEX treatment. Levels of retinoblastoma family proteins p107 and p116 Rb were not altered. The levels of p130/Rb2 were increased by DEX within 36 h of initiating treatment. Additionally, a higher-mobility Rb2-related protein appeared within 24 h and was further increased in DEX-treated cells by 36 h. We also observed reduced levels of M-phase proteins, Cdc2 kinase, and cyclin B in DEX-treated cells. These changes occurred prior to the reduction in cell numbers and thus may represent causative factors. Our data suggest that DEX induces a late G1 block in 235-1 cell-cycle passage, accompanied by a reduction in the levels of the regulatory proteins required for passage through subsequent phases of the cell cycle.**

**Key Words:** Cell cycle; cyclin; cyclin-dependent kinases; glucocorticoid; pituitary; retinoblastoma.

## Introduction

Prolactin (PRL)-secreting adenomas are among the more common forms of pituitary tumors. Thus, controlling their proliferation has been of clinical interest for some time. A number of hormonal and pharmacologic agents affect the growth of these tumors. It has been well established that estrogen stimulates and dopamine agonists inhibit the growth of lactotrophs and pituitary tumor cells *(1,2)*. The effects of peptides and growth factors have been investigated as well. The neuropeptide galanin induces lactotroph proliferation and PRL production *(3,4)*. Fibroblast growth factor and an antisense product, GFG, have been implicated in PRL regulation and growth control in human pituitary tumors and in cultured cell lines *(5)*. Transforming growth factor-β (TGF-β) inhibits pituitary tumor cell growth *(6)* and PRL gene transcription *(7)*. We have recently shown that the synthetic glucocorticoid dexamethasone (DEX) also inhibits cell proliferation and PRL gene expression in 235-1 rat pituitary tumor cells *(8)*. In addition, PRL itself is a growth factor for several pituitary tumor cell lines *(9,10)*.

The effects of glucocorticoid on several pituitary cell types have been described. In AtT20 corticotrophs, cortisol inhibits both adrenocorticotropic hormone secretion and cell growth *(11)*. In lactotrophs and somatotrophs, the effects of glucocorticoid include inhibition of PRL gene expression *(8,12,13)* and induction of growth hormone (GH) gene expression in cells secreting both GH and PRL *(7,14)*. Our investigation has shown that exposure to glucocorticoid also inhibits cell–cell aggregation and cell proliferation in 235-1 rat pituitary tumor cells *(8)*. This cell growth inhibition was not accompanied by a loss in cell number, nor any signs of apoptosis, although glucocorticoids are known to induce cell death in several cell types *(15)*. DEX-treated cells remain healthy and viable. The mechanism of this growth inhibition is not known.

Progression through the cell cycle requires the action of a series of kinase complexes consisting of members of the cyclin and cyclin-dependent kinase (CDK) families *(16)*. Specific CDKs pair with their cyclin partners at given points in the cell cycle, serving both as regulators of the cell-cycle machinery and as regulatory targets for factors that may influence cell growth. The G1 phase of the cell cycle is the longest and most variable phase. Cells in G1 grow; assess their environment; and at the restriction point, "R," make the commitment to the next round of DNA synthesis. In early G1, several proteins are induced in the presence of growth factors that contribute to cell-cycle passage. Principal among these is the transcription factor c-Myc, an immediate early gene, induced within minutes of receipt of a growth stimulus *(17–19)*. Myc protein pairs with other members of its family (Max, Mad) to induce genes required for progression through G1, including the genes for D-type cyclins. In a number of cell types, interference with c-Myc expression leads to growth arrest *(17,20)*.

Received January 17, 2002; Revised February 8, 2002; Accepted February 8, 2002.

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Passage through late G1 and the R point is believed to depend, at least partly, on the action of CDK4/6, paired with cyclin D, and of CDK2-cyclin E to phosphorylate a family of proteins related to the retinoblastoma gene product, Rb *(21,22)*. Rb family members are so-called pocket proteins because they contain a binding site in which they can sequester a protein member of the E2F family of transcription factors. Rb proteins exist in two functional states: hypo- and hyperphosphorylated. In the hypophosphorylated state, which predominates in early G1, Rb binds the E2F protein tightly *(21)*. In late G1, CDK4/6–cyclin D becomes active, followed by CDK2–cyclin E *(16,23)*. Both complexes phosphorylate Rb. As Rb becomes hyperphosphorylated, it releases E2F, which then pairs with a heterodimerization partner and activates transcription of genes required for DNA synthesis. This event is believed to be a central facet of the R point. Once cells leave G1 and enter S, the next active kinase complex is CDK2–cyclin A, followed in late G2 by Cdc2-cyclin B, the mitotic kinase complex.

An additional regulatory feature of the cell cycle is the action of two families of CDK inhibitors *(24)*. The p21 family members inhibit all CDK/cyclin complexes by directly inhibiting kinase activity. Members of the p27Kip1 family are most effective against the G1 CDKs and act to disrupt the CDK/cyclin complex, which drastically reduces kinase activity. Factors that block cell growth, such as general kinase inhibitors *(25)*, DNA damage *(26)* or inhibitory paracrine signals such as TGF-β *(27,28)*, can induce expression of these inhibitors.

To determine the mechanism of DEX-induced growth inhibition, we examined the expression of proteins associated with the early cell cycle (c-Myc, cyclin D3, the Rb-related proteins), those associated with later events in the cycle (Cdc2 and cyclin B1), and the CDK inhibitor p27Kip1 in DEX-treated 235-1 cells.

## Results

### *Time Course of Glucocorticoid-Induced Growth Inhibition*

We have previously shown that treatment of 235-1 cells with 100 n*M* DEX for 6 d leads to a 50% reduction in cell numbers *(8)*. To determine the time course of growth inhibition, replicate cultures of 235-1 cells were established and treated with 100 n*M* DEX for 0 h to 6 d. Cells were collected and counted to determine the point at which reduced growth was first detectable. To ensure that DEX-treated cells were not simply sloughing into the medium, spent media from control and treated cultures were subjected to centrifugation to collect unattached cells, and the pellets were resuspended in phosphate-buffered saline (PBS) and counted. Few cells were found under either condition, and in no instance were more cells lost in DEX-treated than in control cultures (data not shown). In addition, no morphologic evidence of cell death was ever noted in repeated observation of DEX-treated cultures, regardless of the treatment time. This suggests that apoptosis did not occur.

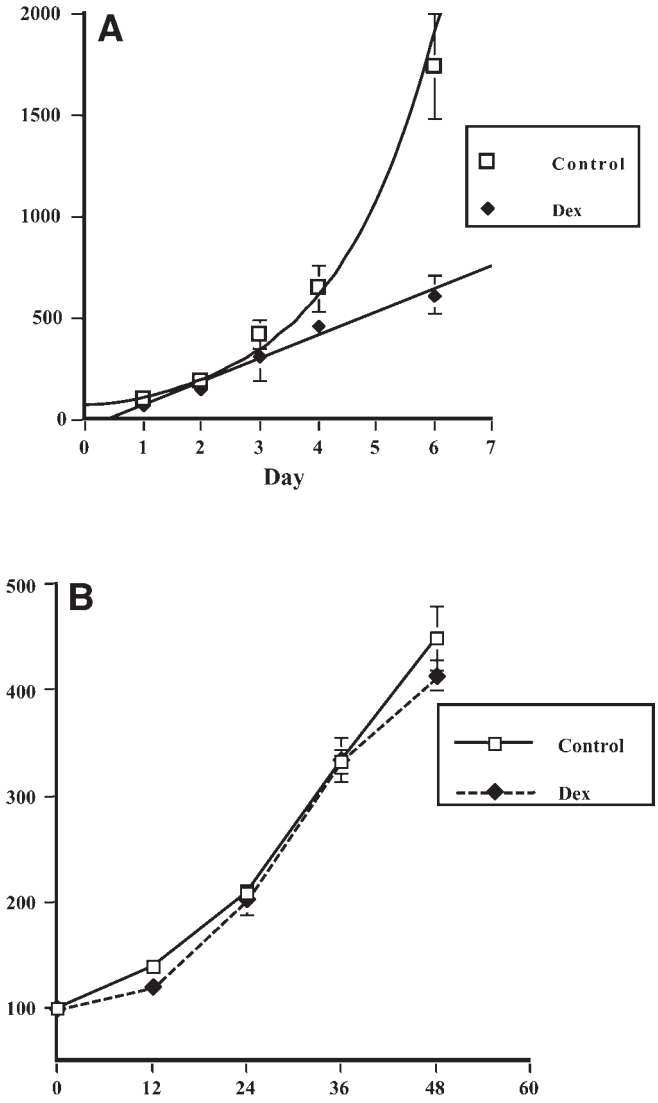


**Fig. 1.** Cell growth time courses for DEX-treated and control 235-1 cells. (**A**) Numbers of 235-1 cells during 6-d continuous time courses. To determine how quickly DEX was able to reduce 235-1 cell numbers, cells were cultured in the absence or presence of 100 n*M* DEX and collected in duplicate after 1, 2, 3, 4, and 6 d. Counts were performed using a hemocytometer. Cell counts from two experiments are plotted as a percentage of the d 1 control; $n = 4$–13. d 6, $p < 0.01$ vs same-day controls. Curve fitting was used to determine the relationship between cell number and days in culture. Controls: $y = 62.7 \times 10^{0.247x}$, $r^2 = 0.988$; DEX: $y = 114.2x - 52.2$, $r^2 = 0.979$. (**B**) Cell numbers at 12-h intervals for 2 d. To characterize closely the early part of this time course, cultures were established, treated, and collected in duplicate or triplicate at 0, 12, 24, 36, and 48 h. Cells were counted as in (A). Control and treated cell numbers are plotted as the percentage of the time 0 control value. 0–24 h, $n = 10$; 36 h, $n = 8$; 48 h, $n = 6$. No significant differences were noted.

As shown in Fig. 1A, cell numbers increased gradually in both groups, with numbers in control and DEX-treated cultures diverging after 3 d. By 6 d there was a 60% difference

in the numbers of control and treated cells. The increase in the numbers of DEX-treated cells again suggests that these cells were not dying. This was confirmed by trypan blue examination of DEX-treated and control cells: both were more than 95% viable. To determine whether any difference in the growth rate of control and DEX-treated cells was detectable at earlier times, a detailed time course was conducted, collecting cells every 12 h for 2 d (Fig. 1B). Note that at these early times, the curves are virtually identical in both treated and control cells the doubling time is approx 24 h. It is after this initial period that the curves diverge: the DEX-treated cells grow at the same rate in the last 3 d, as they did in the first three (Fig. 1A), and the growth curve can be fitted to a linear equation. The control cells grew much more quickly during the second 3-d period, exhibiting almost exponential growth. This suggests that the effect of DEX on cell growth was gradual and did not involve cell death. It also suggests that DEX inhibited entry into the exponential phase of cell growth that produced a rapid rise in control cell numbers between d 3 and 6.

### *Expression of G1 Proteins in DEX-Treated 235-1 Cells: c-Myc, Cyclin D3, and Retinoblastoma-Related Protein 2*

The c-Myc protooncoprotein is often downregulated during growth inhibition *(18,19,29)*, including during inhibition by steroids *(11,30)*. Thus, we used Western and Northern blot analyses to examine *c-myc* expression in control and DEX-inhibited cells. The levels of *c-myc* mRNA after 1 to 2 d of DEX treatment are shown in Fig. 2A. In this and repeated other experiments of 1- to 6-d duration, we were unable to demonstrate any consistent effect of DEX treatment on the level of *c-myc* mRNA.

Because *c-myc* expression is also regulated at the protein level *(31,32)*, we also investigated the possibility that DEX altered the amount of c-Myc protein. Figure 2B is one example of numerous experiments in which c-Myc protein levels were measured at various times during DEX treatment. Treatment for 6 h to 4 d with 100 n*M* DEX followed by analysis of c-Myc by Western blotting revealed no alteration in c-Myc protein levels compared to control cells. In one experiment, cells were treated for 4 d, and then DEX was withdrawn, in an attempt to induce rebound changes in c-Myc protein levels; again, c-Myc levels were unchanged (Fig. 2B, far right). These results show that in contrast to effects in other cell types *(30,33–35)*, glucocorticoid induces no alteration in *c-myc* mRNA or protein levels in 235-1 cells under normal growth conditions.

In light of these results, we examined whether expression of *c-myc* in 235-1 cells could be regulated under serum-free conditions. Two sets of duplicate cultures of 235-1 cells were established, then cultured either in growth RPMI, a serum-free medium, or RPMI plus 10% calf serum. Duplicate cultures in each medium were treated ± 100 n*M* DEX for 2 d. The cells were then harvested and protein extracts were analyzed for *c-myc* expression. The results (Fig. 2C)

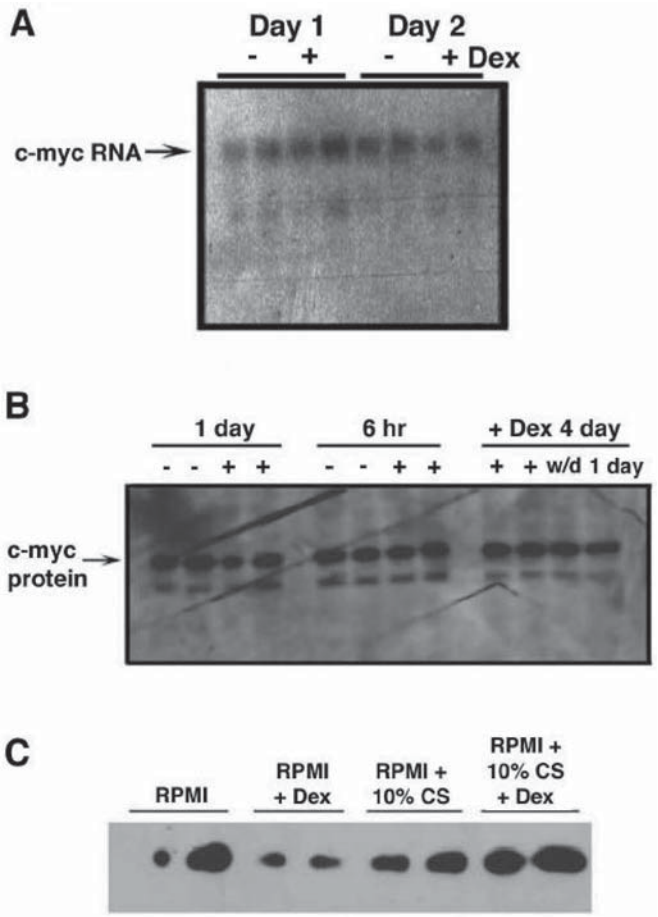


**Fig. 2.** Expression of c-myc. (**A**) Northern blot analysis of c-myc mRNA in control and treated cells. Duplicate cultures of 235-1 cells were treated for 1 to 2 d with 100 n*M* DEX, or kept as controls, then collected for isolation of total RNA. RNA was analyzed for c-*myc* expression by Northern blot hybridization. The signals were normalized to GAPDH. (**B**) Western blot analysis of c-Myc protein in control and treated 235-1 cells. Cells were treated for 6 h to 4 d with 100 n*M* DEX, or kept as controls. In one experiment, cells were with DEX treated for 4 d, then the treatment was withdrawn for 1 d (w/d), in an attempt to induce rebound changes in Myc protein levels. Cell lysates were analyzed for Myc protein by Western blotting. Signals were visualized by enhanced chemiluminescence. (**C**) Demonstration that c-myc is not deregulated in 235-1 cells. Two sets of duplicate cultures of 235-1 cells were established, then cultured either in their RPMI, a serum-free medium, ± 10% calf serum (CS). Duplicates in each medium were kept as controls or treated with 100 n*M* DEX for 2 d. The cells were then collected, and protein extracts were analyzed for c-myc expression by Western blotting. The results show that neither DEX treatment nor serum deprivation alone inhibited Myc protein levels. However, exposure to DEX in the absence of serum reduced Myc levels by approximately half.

show that while neither DEX treatment nor serum deprivation alone inhibited c-Myc protein levels, exposure to DEX in the absence of serum reduced c-Myc levels to approximately half the control level. This suggests that *c-myc* expression is not completely deregulated in 235-1 cells, but that

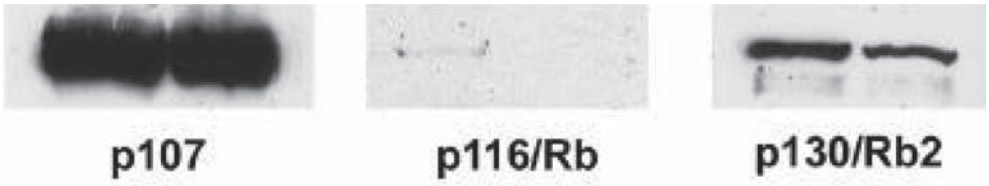


**Fig. 3.** Expression of retinoblastoma family proteins in 235-1 cells. Extracts from stocks of growing 235-1 cells were analyzed by Western blotting for the three known members of the Rb family: p107 (**left**), p116/Rb (**center**), and p130/Rb2 (**right**). Exposure time was 2 min for p107 and p130/Rb2 and 10 min for p116/Rb.

it is not part of a mechanism for glucocorticoid inhibition of growth in the presence of serum.

*G1 D Cyclins*

We also analyzed expression of the early G1 cyclins, D1 and D3, in control and DEX-treated cells. Cyclin D1 was not detectable in the 235-1 cells. Cyclin D3 levels were unaffected by DEX treatment at any time up to 6 d (data not shown). These data are similar to the cyclin D expression profile reported for $GH_3$ cells, another lactotrophic rat pituitary tumor cell line *(36)*.

*Restriction Point: Rb Family Proteins*

Pocket proteins of the retinoblastoma family are believed to act as "gate keepers" at the restriction point of the cell cycle. Regulation of both the level and the phosphorylation state of these proteins helps to control cell-cycle passage *(37)*, with expression levels often increasing as growth is inhibited. The underphosphorylated forms of these proteins inhibit cell-cycle progression by sequestering E2F transcription factors. Phosphorylation of Rb family members by active CDK/cyclin complexes causes the release of the E2F, which then induces other growth-related genes. We examined expression of the Rb-related proteins p107, p116/Rb, and p130/Rb2 in DEX-treated and control 235-1 cells. All three of these proteins were detectable in 235-1 cells by Western blotting (Fig. 3). The p130 and p107 proteins were expressed abundantly; however, the levels of p116/Rb were extremely low. The levels of p116 and of p107 were not consistently altered in cells treated with glucocorticoid (data not shown).

The levels of p130/Rb2 were increased modestly in 235-1 cell cultures as they became confluent over a 4-d treatment period (Fig. 4A). In two separate experiments (Fig. 4A,B), Rb2 expression was slightly increased over time in both control and DEX-treated cultures. To determine when a change in Rb2-related proteins was first detectable, experiments were conducted to collect cells every 12 h for the first 2 d of DEX treatment (Fig. 4B). In the presence of DEX there was an increase in p130 levels over controls at 48 h (Fig. 4B). A lower molecular weight band (approx 120 kDa; line ?Rb2) appeared in samples from both DEX-treated and control cells. In DEX-treated cultures, this band appeared earlier (24–36 h vs 36–48 h; Fig. 4A,B) and was increased over control levels at 24–48 h. DEX withdrawal for 2 d had no apparent effect on the levels of either p130 Rb2 or the faster migrating protein (Fig. 4A, last two lanes).

Based on data published for the Rb protein *(21)* and for Rb2 *(38,39)*, we tested the possibility that the two bands detected by Rb2 antibody represent the hyper- (p130 Rb2, Fig. 4A,B) and hypophosphorylated (?Rb2, Fig. 4A,B) forms of the p130 protein. Equal aliquots (200 µg) of sample protein were incubated with buffer alone (no addition) or with λ-phosphatase for 0–30 min, followed by Western blotting for Rb2 as before. Contrary to expectation, both bands shifted downward, rather than the upper band collapsing into the lower (Fig. 4C). This suggests that the 120-kDa band is also a phosphoprotein and that it represents either a fragment of Rb2 or a novel crossreacting protein. We do not believe that it is one of the other known family members because it is larger and does not crossreact with the p107 antiserum and exhibits different behavior than either p107 or p116 Rb.

These results were obtained using an antibody to an epitope in the N-terminus of Rb2. Use of an antibody to a C-terminal epitope detects only the upper band (data not shown), suggesting that the C-terminal epitope is missing or altered in the smaller protein. This could indicate that the p120 band is a proteolytic cleavage product. However, production of a cleavage product would be expected to decrease the levels of the p130 band, which does not occur (Fig. 4A,B). The reported cleavage sites in Rb-related proteins are in the C-terminus *(40–42)*, producing fragments of 5 or 48 kDa. Neither of these is consistent with our data, since the size shift in the lower band is about 10 kDa. An alternative explanation is that the lower band represents an alternate form of Rb2 or a related protein. We are currently analyzing both the p130 and p120 bands by mass spectrometry. Preliminary indications are that the proteins are at least related (Blair, Frost, and Delidow, unpublished observation).

***Regulation of M-Phase Proteins: Cdc2 and Cyclin B***

The Cdc2–cyclin B complex is the active kinase complex of mitosis and therefore might be expected to decrease in activity in growth-inhibited cells. Cyclins are known to exhibit cell-cycle-dependent changes in their levels, regulated by cyclic protein synthesis and degradation *(23,43)*. There are also several reports of altered CDK levels with changes in growth state *(44,45)*. We examined the levels of

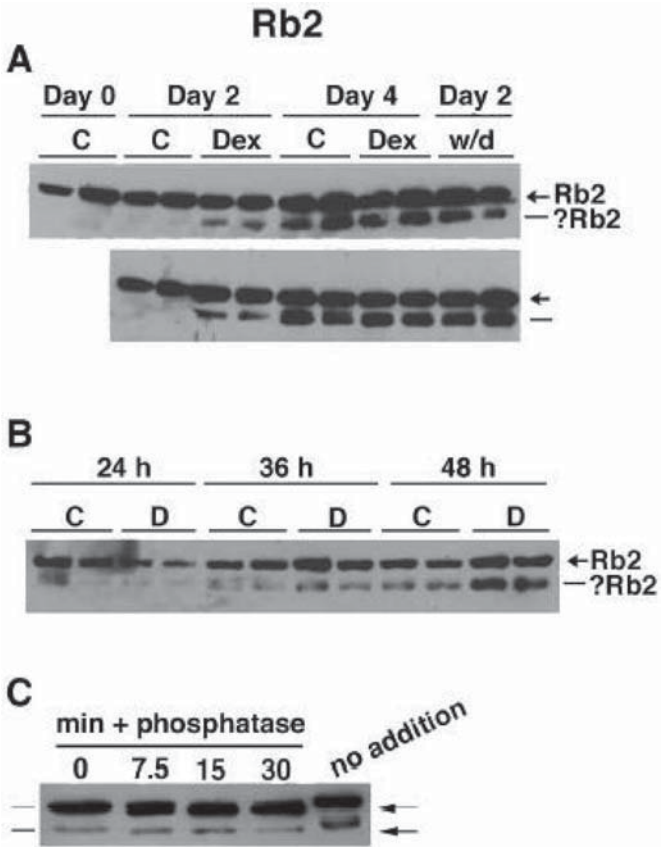


**Fig. 4.** Expression of Rb2 and a related protein during growth inhibition. (**A**) Rb2 protein is expressed in two forms in 235-1 cells and their levels are DEX and growth responsive. Replicate cultures were established and grown as controls or treated with 100 n*M* DEX. Duplicates were collected at 0, 2 d, and 4 d. One set of replicates was treated for 2 d, and then DEX was removed for 2 d (w/d). The cells were extracted and analyzed for Rb2 by Western blotting. A portion of this gel is shown. The solid arrow indicates the p130 band. The dash indicates a second crossreacting protein at approx 120 kDa. (**B**) Twelve-hour interval time course of Rb2 expression. As in (A), cultures were established and grown in the presence or absence of 100 n*M* DEX. Duplicates were collected every 12 h for 48 h, and protein extracts were analyzed for Rb2 expression. These were the cells represented in Fig. 1B. The arrow indicates the p130 band, and the dash indicates the 120- kDa crossreacting band. (**C**) Phosphatase treatment of cellular extracts does not collapse the Rb2-reactive bands. Aliquots of cellular lysates containing 200 µg of protein were incubated in λ-phosphatase buffer in the absence (control, no addition) or presence of 1000 U of λ-phosphatase for 0, 7.5, 15, or 30 min. An equal volume of 2X sample buffer was then added and the samples were denatured, run on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred to nitrocellulose for Western blot analysis of Rb2. The dashes on the left side represent the positions of the unaltered bands (upper dash: p130; lower dash: 120-kDa crossreacting protein); the arrows on the right indicate the shifted positions of these bands after removal of phosphate groups.

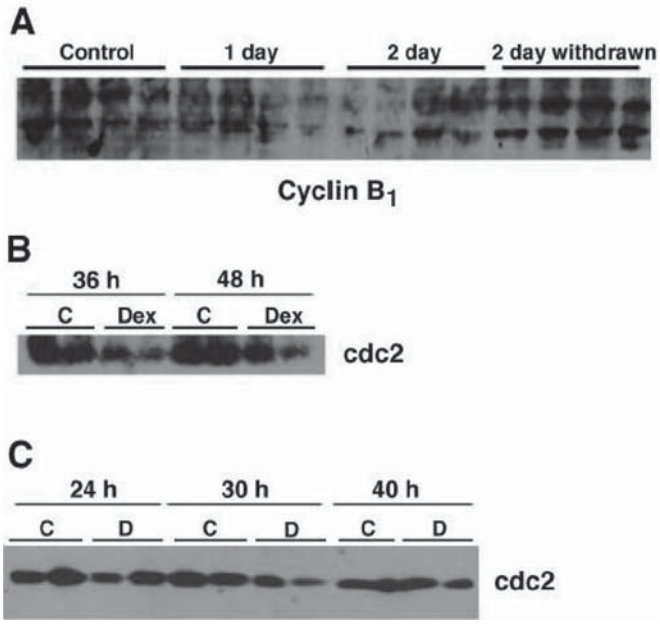


**Fig. 5.** Expression of the G2/M markers cyclin B and Cdc2 in treated and control 235-1 cells. (**A**) Cyclin B expression in control and DEX-treated 235-1 cells. Quadruplicate cultures of 235-1 cells were established and treated with DEX for 0, 1, or 2 d, or treated for 2 d, then withdrawn from treatment for 2 d. Protein extracts were analyzed for cyclin B expression by Western blotting. A representative blot is shown. (**B**) Cdc2 kinase levels in control and DEX-treated cells. Duplicate cultures were established and grown in the presence or absence of 100 n*M* DEX. At 12-h intervals, up to 48 h, duplicate dishes were collected and protein extracts were prepared. A representative experiment is shown. (**C**) Cdc2 kinase levels in control and Dex treated cells between 24 and 40 h of culture. Duplicate cultures were established and treated as in B for 24, 30, or 40 h before preparation of extracts for protein analysis. A representative blot is shown.

both proteins in DEX-treated and control cells. Quadruplicate cultures of 235-1 cells were treated with DEX for 0, 1, or 2 d, or treated for 2 d before DEX was withdrawn. As shown in Fig. 5A, levels of cyclin B were reduced after 1 and 2 d of DEX treatment and returned toward control levels 2 d after withdrawal of DEX.

We also measured the levels of Cdc2 kinase by Western blotting. Cells were grown in the presence or absence of 100 n*M* DEX and harvested at 10- to 12-h intervals, up to 48 h. In two separate experiments, shown in Fig. 5B,C, Cdc2 levels were markedly reduced by 36 h and then remained lower. These data show that expression of both components of the mitotic kinase complex is inhibited by glucocorticoids at a time just prior to an alteration of the 235-1 cell growth rate.

### *Cell-Cycle Inhibitors: p27Kip1*

Growth inhibition is often accompanied by increased expression of proteins in two families of Cdk inhibitors, p21 and p27 *(24,46–48)*. In addition, glucocorticoid has been shown to induce p27Kip in Hela cells during growth suppression *(49)*. We examined the expression of p27Kip1 in control and DEX-treated 235-1 cells. Quadruplicate cultures were established and collected after 0 and 2 d of DEX treatment. Two sets of replicates were treated with DEX

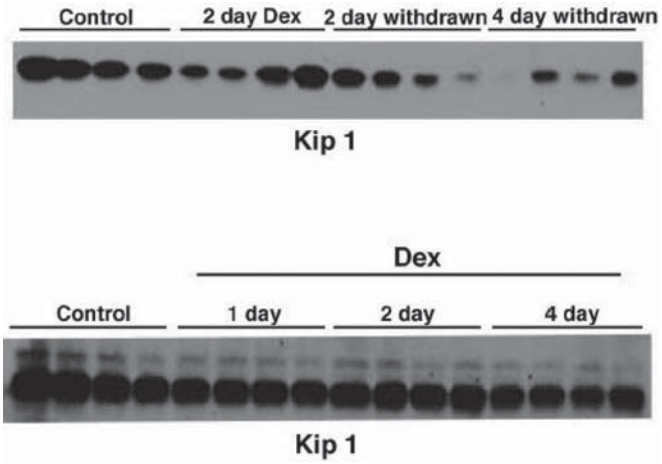


**Fig. 6.** Effect of DEX treatment on p27Kip. Quadruplicate cultures were established and collected after 0–4 d of DEX treatment. Two sets of replicates were also treated for 2 d, then withdrawn from DEX for 2 or 4 d. Equal aliquots of protein were analyzed for p27Kip by Western blotting. (**Top**) Withdrawal experiments; (**bottom**) 0- to 4-d treatment with 100 n*M* DEX.

for 2 d, and then the DEX was removed and the cells were cultured for an additional 2 or 4 d. As shown in Fig. 6, treatment with DEX for 2 d had no consistent effect on levels of this p27Kip1. However, withdrawal of DEX led to a sustained downregulation of p27Kip1 levels. Thus, while growth inhibition by DEX does not appear to induce any change in p27 levels, the cells responded to removal of a growth suppressor by reducing their expression of the p27Kip1 CDK inhibitor. We speculate that this may be part of a general response to the removal of a growth inhibitor, rather than a specific response to glucocorticoid.

## Discussion

Our data support the conclusion that glucocorticoid exposure leads to a gradual decrease in the growth rate of 235-1 cells. This decrease was such that cell growth continued slowly, but cell numbers were 40% of control levels by 6 d of treatment, consistent with our previously published study *(8)*. The cells exhibited no morphologic signs of cell death or loss from the culture. The decrease in growth rate was not accompanied by alterations in the early G1 regulatory proteins c-Myc (Fig. 2) or cyclin D3 (not shown). Thus, the DEX-induced block must act at another point in the cell cycle. There is precedent for growth inhibition by glucocorticoids in the absence of alteration in c-Myc. It has been reported that glucocorticoid inhibits the growth of L929 fibroblasts, with no concomitant inhibition of *c-myc* expression *(50)*.

Expression of the late G1 regulatory protein Rb2 increased gradually with time in culture, and with DEX treatment, while p107 and p116 Rb were unchanged. The retinoblastoma family of proteins functions as regulators of the G1 restriction point, the time at which cells commit to the next round of DNA synthesis *(21)*. An increase in the level of these proteins would be expected to reduce the rate of passage through G1 *(37)* and could explain the reduced growth rate of DEX-treated cells. The levels of p130/Rb2 have also been reported to increase within 8–24 h in growth-inhibited mouse embryo fibroblasts and human melanoma cells *(51,52)*. A higher-mobility band at 120 kDa, which crossreacts with the antibody to the Rb2 protein, appeared in 235-1 cells by 36 h of DEX treatment. This is well before detectable differences in cell numbers were found and, therefore, may represent an early causative factor for growth inhibition. Several published reports also demonstrate a 120-kDa form of Rb2 *(38,39)*. This band appears in rat fibroblasts in the absence of serum and disappears within 6 h of growth stimulation *(39)*. The 120-kDa crossreacting band has been identified as the hypophosphorylated form of the Rb2 protein in these reports, based on the known alterations in mobility and phosphorylation that occur during the cell cycle. Treatment of 235-1 cell extracts with λ-phosphatase revealed that the 120-kDa band that we detected is not the hypophosphorylated form of Rb2. The fact that this band appeared consistently at 36 h of DEX treatment and that it became apparent in control cells as they were becoming confluent after 4 d in culture is suggestive of a regulated, specific response to growth inhibition. Furthermore, the fact that the intensity of the 120-kDa band increased with no concomitant decrease in the amount of p130 supports the idea that the 120-kDa protein is not simply a nonspecific proteolytic fragment.

The published data on proteolytic cleavage of Rb family proteins by caspases *(41,42,53)* indicates production of C-terminal fragments. This is consistent with our data since only an N-terminal-directed antibody detects both the p120 and p130 proteins; however, the sizes of known cleavage products do not correspond to the protein we detected. Some information currently available suggests the production of alternative forms of Rb family proteins. Both p116 Rb and p107 are expressed in alternately spliced forms *(54–56)*, though there are no published data on p130/Rb2. In at least one case, an engineered altered form of Rb was shown to have enhanced growth inhibitory capacity in osteosarcoma cells *(57)*. This suggests the possibility of a role for natural variants in growth inhibition as well. We are currently subjecting isolates of the Rb2 proteins to mass spectrometric analysis by matrix-assisted laser desorption ionization time of flight to determine whether p120 is a fragment of Rb2 or a novel Rb-related protein.

The M-phase proteins cyclin B and Cdc2 were reduced in DEX-treated 235-1 cells between 1 and 2 d of treatment. Again, this is earlier than changes in cell numbers. Glucocorticoid has also been shown to inhibit the levels of Cdc2 (CDK1) in lymphoid cells *(58)*. Recently published data show that glucocorticoids induce the accumulation of Rb2/E2F4 complexes in growth-inhibited osteoblasts *(59)* and that Rb2 inhibits the activity of the S-phase kinase CDK2

*(60)*. Since this kinase activity is required to progress through S-phase into G2/M, it is possible that these two phenomena are part of the same block to cell-cycle progression. The magnitude of the changes in the levels of these proteins was relatively modest, between 30 and 50%. There are two possible explanations for why small changes in the levels of regulatory proteins may be sufficient to cause significant growth inhibition. The 235-1 cell cultures were not synchronized, which may reduce the magnitude of change observed in the levels of proteins associated with particular cell-cycle phases. In addition, because these proteins are necessary to complete passage through the cell cycle, it is possible that small changes in their levels are sufficient to cause the reduction in growth rate noted.

The levels of the CDK inhibitor p27Kip1 can be induced by glucocorticoids during growth inhibition *(49)*; thus, its regulation in 235-1 cells was of interest. Contrary to expectations, the level of p27 was unchanged by exposure of 235-1 cells to DEX and underwent a sustained decrease when DEX treatment was withdrawn. This implies that, rather than being directly inhibited by DEX, p27 is indirectly responsive to growth state, and it is not responsible for the reduction in growth rate in response to DEX. Taken together, our results suggest that glucocorticoid inhibits 235-1 cell growth by one or both of these means: (1) a late G1 block that limits passage through the R point by increasing the levels of hypophosphorylated Rb2; (2) a late cell-cycle block that limits expression of the components of the mitotic kinase complex, Cdc2/cyclin B.

An interesting and still open question is, what is the proximate mechanism for glucocorticoid inhibition of growth in the 235-1 cell line? Glucocorticoids act via nuclear hormone receptors, which are ligand-activated transcription factors. Therefore, a primary effect of these hormones would be expected to occur within 2–6 h of addition *(61,62)*. The earliest detectable change in the cell-cycle proteins that we examined was 24–36 h, well after this initial period. In addition, several genes known to be directly regulated by glucocorticoids in other cell lines (c-myc, cyclin D3, p27) were unaffected in the 235-1 cells. The most economical explanation for this is that inhibition of cell growth is a result of some other direct action of glucocorticoids in these cells. One candidate for this action is the inhibition of PRL gene expression. Glucocorticoids inhibit PRL gene expression by approx 50% in rat pituitary cell lines *(7,8)* via a negative DNA regulatory element *(13)*. The rate of PRL gene transcription is halved in $GH_3$ cells after 20 h of glucocorticoid treatment *(7)*. In 235-1 cells, we have observed this level of inhibition in PRL mRNA levels within 1 d of DEX treatment (Spangler and Delidow, unpublished observation). It has been shown that in these same lines PRL acts as an autocrine growth factor *(9,10)*. The rapid inhibition of PRL gene expression by glucocorticoid fits well within the time course for the earliest changes in cell-cycle regulatory proteins noted here (24–36 h). Since several other glucocorticoid inhibitory mechanisms do not appear to be active (inhibition of c-myc, induction of p27), it is reasonable to suggest that inhibition of PRL gene expression or action may result in the growth suppression observed in our studies.

Furthermore, it has been shown that glucocorticoid administration in rats reduces pituitary levels of the neuropeptide galanin *(63)*. Galanin is a mitogen for 235-1 cells and stimulates their secretion of PRL *(3)*. Thus, it is also possible that glucocorticoid may act by interrupting a positive autoregulatory loop between PRL and galanin. Galanin has been shown to colocalize in the same granules with PRL in normal pituitary *(4)*; however, there is no information yet on whether 235-1 cells produce this peptide.

Another potential explanation is that glucocorticoids may interfere with PRL growth regulatory effects by interrupting its signaling pathway. This mechanism is well supported in the literature. PRL signaling in mammary cells is inhibited by altered levels of SOCS proteins (suppressors of cytokine signaling) *(64)*. SOCS 3, a known target of glucocorticoid action *(65)*, has been shown to act in a negative feedback loop downstream of the PRL receptor *(66)*. In addition, stimulation of mammary cell growth by PRL can be blocked by glucocorticoid acting at a post-PRL receptor signaling step *(67)*. We will investigate these possibilities in the future.

## Materials and Methods

### *Cell Culture*

The 235-1 cells were maintained in tissue culture flasks in DMEM–high glucose (Life Technologies, Gaithersburg, MD) containing 10% defined iron-supplemented newborn calf serum (Hyclone, Logan, UT), 15 m*M* HEPES (pH 7.4), 100 U/mL of penicillin, and 100 µg/mL of streptomycin. The cells were split 1:20 once per week and were plated on 100-mm tissue culture dishes (Falcon) at least 1 d before beginning an experiment. Cells were fed every 2 d. DEX (Sigma, St. Louis, MO) was dissolved in ethanol to make a 1 m*M* stock solution. A 100 µ*M* working solution was made by dilution in sterile PBS. Cells were treated with 100 n*M* DEX, for 6 h to 6 d, as described in the figure legends, and were refed and treated every 2 d. Treatment with vehicle alone (10 µL of 9.5% ethanol in PBS per 100-mm dish) had no effect on 235-1 cells (data not shown). At the end of the experimental period, cells were collected by scraping, washed once in 1 mL of PBS, and analyzed as described subsequently. All experiments were conducted using at least duplicate samples and were repeated two to three times.

### *Western Blot Analyses*

Cell growth was assessed by removing a 20-µL aliquot from the PBS wash for counting using a hemocytometer. Protein extracts were made by pelleting cells from PBS, then lysing them in 2 to 3 pellet vol (up to 200 µL) of HEPES lysis buffer (10 m*M* HEPES, 150 m*M* NaCl, 1.5 m*M* EDTA,

1% NP40, 1 m*M* phenylmethylsulfonyl fluoride, 0.68 mg/mL of pepstatin, 0.5 mg/mL of leupeptin). Protein content was determined by bicinchoninic acid assay (Pierce, Rockford, IL), and 50–100 µg of each sample was resolved by denaturing gel electrophoresis on 7.5, 10, or 12.5% polyacrylamide gels, as appropriate. Samples were transferred to nitrocellulose by electroblotting and Western blotted according to the manufacturer's recommendations with antibodies against cell-cycle-related proteins (c-myc; Oncogene Science, Cambridge, MA; cell-cycle antibody kit; Transduction, Lexington, KY; cyclin B1, Rb, p107, and p130/Rb2 C-terminal; Santa Cruz Biotechnology, Santa Cruz, CA; Rb2/p130 N-terminal; Transduction). The dilutions of primary antibodies used were according to manufacturer's instructions and ranged from 1:1000 to 1:3000. All steps were carried out at room temperature, with gentle agitation. Blots were blocked with 3% nonfat dried milk in TBST (10 m*M* Tris, pH 8.0; 150 m*M* NaCl; 0.05% Tween-20) for 1 h and incubated with primary antibody diluted in the same solution for 1 h. Blots were washed once for 5 min in TBST alone and then incubated with the appropriate peroxidase-conjugated secondary antibody (1:3000 in TBST plus 3% nonfat dried milk) for 1 h. Completed blots were washed three times (15 min each) in TBST and signals were detected using enhanced chemiluminescence (SuperSignal reagent; Pierce). Signal intensities were quantitated by scanning densitometry (Molecular Dynamics model PD densitometer, with ImageQuant software).

### *Northern Blot Hybridization Analysis*

Total cellular RNA was prepared as previously described *(7)*. Twenty-microgram aliquots were separated on 1% agarose-formaldehyde gels and transferred to nylon by capillary blotting. Membranes were probed using a random-primed cDNA probe against murine *c-myc* (kindly provided by Dr. R. M. Niles, Marshall University School of Medicine), and the signal was detected by autoradiography.

### *Phosphatase Treatment of Cellular Extracts*

Cellular lysates were prepared as already described. Aliquots corresponding to 200 µg of cellular protein were incubated in the recommended buffer and 1000 U of λ protein phosphatase (New England Biolabs, Beverly, MA) at 37ºC for 0–30 min. Samples were then mixed with an equal volume of gel electrophoresis sample buffer, denatured, and subjected to electrophoresis on 7.5% SDS-polyacrylamide gels. Samples were transferred to nitrocellulose and analyzed for Rb2-related proteins by Western blotting.

## Acknowledgments

We gratefully acknowledge the expert technical and graphic arts assistance of Margaret McFarland. We also acknowledge the support of our colleagues Melissa Franklin, Phillip Spangler, Joe Remias, Tracey Johnson, Rena Jones, and the members of the Department of Biochemistry and Molecular Biology. We thank Dr. R. M. Niles for his review and comments on the manuscript, and for providing the c-*myc* probe. This work was supported by a Research Experiences for Undergraduates supplement to National Science Foundation grant no. IBN-8507173 and by the Marshall University School of Medicine.

## References

1. Saiardi, A., Bozzi, Y., Baik, J. H., and Borrelli, E. (1997). *Neuron* **19,** 115–126.
2. Spady, T. J., McComb, R. D., and Shull, J. D. (1999). *Endocrine* **11,** 217–233.
3. Hammond, P. J., Smith, D. M., Akinsanya, K. O., Mufti, W. A., Wynick, D., and Bloom, S. R. (1996). *J. Neuroendocrinol.* **8,** 457–464.
4. Wynick, D., Small, C. J., Bacon, A., Holmes, F. E., Norman, M., Ormandy, C. J., Kilic, E., Kerr, N. C., Ghatei, M., Talamantes, F., Bloom, S. R., and Pachnis, V. (1998). *Proc. Natl. Acad. Sci. USA* **95,** 12,671–12,676.
5. Asa, S. L., Ramyar, L., Murphy, P. R., Li, A. W., and Ezzat, S. (2001). *Mol. Endocrinol.* **15,** 589–599.
6. Ramsdell, J. S. (1991). *Endocrinology* **128,** 1981–1990.
7. Delidow, B. C., Billis, W. M., Agarwal, P., and White, B. A. (1991). *Mol. Endocrinol.* **5,** 1716–1722.
8. Spangler, P. R. and Delidow, B. C. (1998). *J. Cell. Physiol.* **174,** 115–124.
9. Krown, K. A., Wang, Y.-F., Ho, T. W. C., Kelly, P. A., and Walker, A. M. (1992). *Endocrinology* **131,** 595–602.
10. Krown, K. A., Wang, Y.-F., and Walker, A. M. (1994). *Endocrinology* **134,** 1546–1552.
11. van Wijk, P. A., van Neck, J. W., Rijnberk, A., Croughs, R. J., and Mol, J. A. (1995). *Mol. Cell. Endocrinol.* **111,** 13–19.
12. Sakai, D. D., Helms, S., Carlstedt-Duke, J., Gustaffson, J.-A., Rottman, F., and Yamamoto, K. R. (1988). *Genes Dev.* **2,** 1144–1154.
13. Somasekhar, M. B. and Gorski, J. (1988). *Gene* **69,** 13–21.
14. Billis, W. M., Delidow, B. C., and White, B. A. (1992). *Mol. Endocrinol.* **6,** 1277–1284.
15. Distelhorst, C. W. (1997). *Adv. Pharmacol.* **41,** 241–270.
16. Johnson, G. L. and Vaillancourt, R. R. (1994). *Curr. Opin. Cell. Biol.* **6,** 230–238.
17. Heikkila, R., Schwab, G., Wickstrom, E., Loke, S., Pluznik, D. H., Watt, R., and Neckers, L. M. (1987). *Nature* **328,** 445–449.
18. Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R., Pittelkow, M. R., Münger, K., Howley, P. M., and Moses, H. L. (1990). *Cell* **61,** 777–785.
19. Pietenpol, J. A., Holt, J. T., Stein, R. W., and Moses, H. L. (1990). *Proc. Natl. Acad. Sci. USA* **87,** 3758–3762.
20. Holt, J. T., Redner, R. L., and Neinhuis, A. W. (1988). *Mol. Cell. Biol.* **8,** 963–973.
21. Bartek, J., Bartkova, J., and Lukas, J. (1996). *Curr. Opin. Cell. Biol.* **8,** 805–814.
22. Lundberg, A. S. and Weinberg, R. A. (1998). *Mol. Cell. Biol.* **18,** 753–761.
23. Johnson, D. G. and Walker, C. L. (1999). *Annu. Rev. Pharmacol. Toxicol.* **39,** 295–312.
24. Elledge, S. J. and Harper, J. W. (1994). *Curr. Opin. Cell. Biol.* **6,** 847–852.
25. Kwon, T. K., Buchholz, M. A., Chrest, F. J., and Nordin, A. A. (1996). *Cell Growth Diff.* **7,** 1305–1313.
26. Macleod, K. F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B., and Jacks, T. (1995). *Genes Dev.* **9,** 935–944.
27. Nagahara, H., Ezhevsky, S. A., Vocero-Akbani, A. M., Kaldis, P., Solomon, M. J., and Dowdy, S. F. (1999). *Proc. Natl. Acad. Sci. USA* **96,** 14,961–14,966.

28. Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. (1995). *Genes Dev.* **9,** 1831–1845.
29. Mulder, K. M., Zhong, Q., Choi, H. G., Humphrey, L. E., and Brattain, M. G. (1990). *Cancer Res.* **50,** 7581–7586.
30. Rhee, K., Breshnan, W., Hirai, A., Hirai, M., and Thompson, E. A. (1995). *Cancer Res.* **55,** 4188–4195.
31. Shindo, H., Tani, E., Matsumuto, T., Hashimoto, T., and Furuyama, J. (1993). *Acta Neuropathol.* **86,** 345–352.
32. Gregory, M. A. and Hann, S. R. (2000). *Mol. Cell. Biol.* **20,** 2423–2435.
33. Forsthoefel, A. M. and Thompson, E. A. (1987). *Mol. Endocrinol.* **1,** 899–907.
34. Rories, C., Lau, C. K., Fink, K., and Spelsberg, T. C. (1989). *Mol. Endocrinol.* **3,** 991–1001.
35. Rogatsky, I., Trowbridge, J. M., and Garabedian, M. J. (1997). *Mol. Cell. Biol.* **17,** 3181–3193.
36. Qian, X., Kulig, E., Jin, L., and Lloyd, R. V. (1998). *Endocrinology* **139,** 2058–2067.
37. Grana, X., Garriga, J., and Mayol, X. (1998). *Oncogene* **17,** 3365–3383.
38. Cinti, C., Claudio, P. P., Howard, C. M., Neri, L. M., Fu, Y., Leoncini, L., Tosi, G. M., Maraldi, N. M., and Giordano, A. (2000). *Cancer Res.* **60,** 383–389.
39. Jinno, S., Hung, S.-C., Yamamoto, H., Lin, J., Nagata, A., and Okayama, H. (1999). *PNAS* **96,** 13,197–13,202.
40. Janicke, R. U., Walker, P. A., Lin, X. Y., and Porter, A. G. (1996). *EMBO J.* **15,** 6969–6978.
41. An, B. and Dou, Q. P. (1996). *Cancer Res.* **56,** 438–442.
42. Chen, W. D., Otterson, G. A., Lipkowitz, S., Khleif, S. N., Coxon, A. B., and Kaye, F. J. (1997). *Oncogene* **14,** 1243–1248.
43. Pines, J. (1992). *Curr. Opin. Cell. Biol.* **4,** 144–148.
44. Damiens, E., El Yazidi, I., Mazurier, J., Duthille, I., Spik, G., and Boilly-Marer, Y. (1999). *J. Cell. Biochem.* **74,** 486–498.
45. Teixeira, C. and Pratt, M. A. (1997). *Mol. Endocrinol.* **11,** 1191–1202.
46. Coats, S., Flanagan, W. M., Nourse, J., and Roberts, J. M. (1996). *Science* **272,** 877–880.
47. Liu, Y., Martindale, J. L., Gorospe, M., and Holbrook, N. J. (1996). *Cancer Res.* **56,** 31–35.
48. Wang, Q. M., Jones, J. B., and Studzinski, G. P. (1996). *Cancer Res.* **56,** 264–267.
49. Samuelsson, M. K. R., Pazirandeh, A., Davani, B., and Okret, S. (1999). *Mol. Endocrinol.* **13,** 1811–1822.
50. Frost, G. H., Rhee, K., Ma, T., and Thompson, E. A. (1994). *J. Steroid Biochem. Mol. Biol.* **50,** 109–119.
51. Collado, M., Medema, R. H., Garcia-Cao, I., Dubuisson, M. L. N., Barradas, M., Glassford, J., Rivas, C., Burgering, B. M. T., Serrano, M., and Lam, E. W.-F. (2000). *J. Biol. Chem.* **275,** 21,960–21,968.
52. Murai, T., Nakagawa, Y., Maeda, H., and Terada, K. (2001). *J. Biol. Chem.* **276,** 6797–6806.
53. Jang, J. S. and Choi, Y. H. (1999). *Int. J. Mol. Med.* **4,** 487–492.
54. Kim, K. K., Soonpaa, M. H., Wang, H., and Field, L. J. (1995). *Genomics* **28,** 520–529.
55. Shew, J. Y., Chen, P. L., Bookstein, R., Lee, E. Y., and Lee, W. H. (1990). *Cell Growth Differ.* **1,** 17–25.
56. Latil, A., Pesche, S., Valeri, A., Cussenot, O., Fournier, G., and Lidereau, R. (1999). *Int. J. Cancer* **80,** 632, 633.
57. Antelman, D., Perry, S., Hollingsworth, R., Gregory, R. J., Driscoll, B., Fung, Y. K., and Bookstein, R. (1997). *Oncogene* **15,** 2855–2866.
58. Rhee, K., Reisman, D., Bresnahan, W., and Thompson, E. A. (1995). *Cell Growth Differ.* **6,** 691–698.
59. Smith, E., Redman, R. A., Logg, C. R., Coetzee, G. A., Kasahara, N., and Frenkel, B. (2000). *J. Biol. Chem.* **275,** 19,992–20,001.
60. De Luca, A., MacLachlan, T. K., Bagella, L., Dean, C., Howard, C. M., Claudio, P. P., Baldi, A., Khalili, K., and Giordano, A. (1997). *J. Biol. Chem.* **272,** 20,971–20,974.
61. O'Malley, B. W. and Tsai, M. J. (1992). *Biol. Reprod.* **46,** 163–167.
62. Meier, C. A. (1997). *J. Recept. Signal Transduct. Res.* **17,** 319–335.
63. Brogan, R. S., Coney, L. K., Wehrenberg, W. B., Beretta, G., and Giustina, A. (1999). *Metabolism* **48,** 792–796.
64. Pezet, A., Favre, H., Kelly, P. A., and Edery, M. (1999). *J. Biol. Chem.* **274,** 24,497–24,502.
65. Paul, C., Seiliez, I., Thissen, J. P., and Le Cam, A. (2000). *Eur. J. Biochem.* **267,** 5849–5857.
66. Tomic, S., Chughtai, N., and Ali, S. (1999). *Mol. Cell. Endocrinol.* **158,** 45–54.
67. Olazabal, I., Munoz, J., Ogueta, S., Obregon, E., and Garcia-Ruiz, J. P. (2000). *Mol. Endocrinol.* **14,** 564–575.