Negative Regulation by Glucocorticoids Through Interference with a cAMP Responsive Enhancer

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Although steroid hormone receptors are known to activate gene expression by binding to specific hormone-dependent enhancers, the mechanisms by which steroids inhibit the transcription of specific genes are unknown. It is shown here by gene transfer studies that the same glucocorticoid receptor that activates gene expression can negatively regulate expression of the human glycoprotein hormone α-subunit gene. Glucocorticoid inhibition was conferred by a 52-nucleotide region that also contains elements crucial both for adenosine 3′,5′-monophosphate (cAMP) responsiveness and for placental-specific expression of this gene and was observed only under conditions in which these elements were functioning as enhancers. Purified glucocorticoid receptor was found to bind to DNA that overlap the cAMP responsive elements sites in this region. It is hypothesized that steroid receptors negatively regulate gene expression by interfering with the activity or binding of other important transcription factors.

STEROID HORMONES EXERT NEGATIVE regulatory functions in humans and other mammals. The inhibitory effects of glucocorticoids on immunological and inflammatory responses and connective tissues form the basis for their frequent therapeutic use for a number of medical conditions. These effects are predominantly caused by the inhibition of expression of specific genes important in these processes (*1*). Glucocorticoids specifically inhibit the transcription of several genes including collagen (types I and IV), stromelysin, and proopiomelanocortin through negative regulation mediated by 5′-flanking sequences (*2*). However, the precise regions on which the steroid acts and the mechanisms by which these inhibitory influences occur are not known.

Reproductive function is adversely affected by elevated levels of glucocorticoids (*1*), possibly involving negative regulation of the gonadotropin hormones (*3*). These glycoprotein hormones, which are composed of a common α subunit and distinct β subunits, include chorionic gonadotropin, luteinizing hormone, and follicle-stimulating hormone. They are crucial for reproduction in humans (*4*) and are negatively regulated by estrogens and androgens. The α subunit is also a component of thyroid-stimulating hormone, which is negatively regulated by thyroid hormones (*5*). Some of the negative effects of steroid and thyroid hormones occur at the level of transcription or mRNA (*6*); however, regulation of the α-subunit gene by glucocorticoids has not been studied at the molecular level.

We have investigated the role of glucocorticoids in modulating α-subunit gene expression using DNA-mediated gene transfer. Hybrid genes [α-cat and α168-cat (7)] consisting of 1.8 kb and 165 bp of 5′-flanking DNA, respectively, linked to the chloramphenicol acetyltransferase gene (CAT) were cotransfected with a human glucocorticoid receptor cDNA expression plasmid [RSVGR (*8*)] into a human placental choriocarcinoma cell line, JEG-3, which produces both the α and β subunits of chorionic gonadotropin (*9*). Addition of the glucocorticoid dexamethasone decreased CAT activity by 90% from either α-cat or α168-cat (Fig. 1A). In contrast, dexamethasone strongly induced CAT activity from a hybrid gene containing the glucocorticoid-response elements from mouse mammary tumor virus (MMTV) long terminal repeat on the herpes simplex virus thymidine kinase (tk) promoter and the CAT gene [MMTV-TKcat (*10*)], a response previously characterized (*8, 11*). Neither response was observed using a receptor gene mutated in the DNA-binding region (*8*), indicating a dependence on DNA binding by the added receptor and a lack of endogenous receptor in this cell line. The tk promoter alone (TKcat) did not respond to glucocorticoids in the presence of functional receptors. Thus, the same receptor mediates both positive and negative effects on transcription in JEG-3 cells. Further, negative regulation by glucocorticoids is conferred to the α-subunit gene by sequences between −168 and +45 and is dependent on steroid and DNA binding by the receptor.

Several elements important for α-subunit gene expression have been defined in the steroid-responsive region (Fig. 1B). Two tandem cAMP (adenosine 3′,5′-monophosphate)-responsive elements (CREs) are located between −146 and −111 (*7, 12*). These CREs also contribute to placental-specific expression of the α-subunit promoter, but they are insufficient to confer placental specificity to a heterologous promoter. Full placental expression of the α-subunit gene, or generation of placental specificity on a heterologous promoter, requires cooperation of the CREs with a tissue-specific element [TSE (*7*)]. This TSE is not an enhancer alone but is dependent on the CREs to confer tissue specificity (*7*). A TSE-binding protein specific to placental cells and a CRE-binding protein common to many cell types have been identified in nuclear extracts (*7*). The CRE-binding protein may be similar to the protein that binds to the CRE of somatostatin and is phosphorylated in vitro by cAMP-dependent protein kinase (*13*). Since CRE-binding activity is not increased in nuclear extracts of cells with elevated levels of cAMP (*7, 13*), the CRE-binding protein may respond to cAMP by increasing its activity rather than its concentration or affinity for DNA.

Though positive glucocorticoid regulatory elements (GREs) are known to act as enhancers (*11*), negative regulation by steroids has not been characterized in this manner. We tested several fragments from the α-subunit 5′-flanking region linked to the tk promoter in JEG-3 cells (Fig. 2) in the presence or absence of forskolin, which activates the CREs by increasing intracellular cAMP levels. Fragments −224 to +4 or −224 to −100, containing both the TSE and the CREs that act together as a placental-specific enhancer, produced the expected 10-fold tissue-specific enhancement and 15-fold forskolin induction (*7*). Glucocorticoids caused a fourfold inhibition of tk transcription from either the enhanced level (tissue specific) or the higher cAMP-induced level without interfering with the magnitude of the cAMP response (15-fold). A fragment containing only the TSE (−224 to −136), which confers little tissue-specific or cAMP-responsive enhancement to the tk promoter alone (*7*), does not confer glucocorticoid inhibition. In addition, a fragment containing just the CREs (−152 to −100), which has a minimal effect on the tk promoter in the absence of forskolin, did not confer significant negative regulation. However, when induced by cAMP to enhance

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TKcat expression, the fourfold inhibition by the glucocorticoid was revealed. The inhibitory glucocorticoid actions are therefore exerted through the 52-bp CRE-containing region. As in the case of glucocorticoid-mediated enhancement (*11*), the negative responsiveness can occur through a DNA sequence that is separable from, and upstream of, the promoter and can be conferred onto a heterologous promoter. However, the negative glucocorticoid effect occurs only when the CRE-containing region is active as an enhancer as a result of either elevated cAMP levels or the actions of the TSE. In addition, the steroid-responsive region does not contain intrinsic glucocorticoid-dependent silencing activity, since it did not inhibit basal expression of TKcat in the presence of the glucocorticoid receptor (GR) and glucocorticoids unless it was induced to act as an enhancer.

Since GR induces gene expression by binding to specific DNA sequences, we performed deoxyribonuclease (DNase) I and methylation protection analyses (*14*), using the α-subunit 5′-flanking DNA and purified rat liver glucocorticoid receptor, to determine whether GR binding sites were present in the region conferring inhibition of expression. Three GR-dependent protected regions (Fig. 3A) were identified and carefully quantitated by densitometry. The most prominent was located from −93 to −122, overlapping the downstream CRE (−128 to −111); a second site from −140 to −153 overlapped the upstream CRE (−146 to −129). The third protected region, −84 to −62, overlaps a binding site (CAAT) for another previously identified nuclear factor (*7*). However, fragments from which this region had been deleted (−224 to −100 or −152 to −100) conferred glucocorticoid inhibition to the tk promoter (Fig. 2), suggesting that this site [CAAT (*15*)] is not the primary one involved in this regulation. Previously identified GREs commonly vary in their degree of homology to the consensus sequence and in the number of protected guanine residues (*16*). The three GR binding sites we identified show modest homology to the consensus sequence (Fig. 3B) and protection of the primary conserved methylated guanine at position 11 among others (*17*). Thus, the glucocorticoid receptor complex may interfere with placental-specific enhancement mediated by the CREs, by binding directly to the α-subunit 5′-flanking DNA at these GR binding sites that overlap the CREs, such that negative regulation occurs through interference with the binding or function of the CRE-binding proteins.

The in vitro binding and methylation protection data suggest that negative regulation by glucocorticoids is the result of GR binding to the α-subunit gene; however, it remains possible that the glucocorticoid receptor could be influencing the expression or activity of the CRE-binding protein more indirectly. Since inhibition of the fold cAMP-dependent enhancement was not observed, glucocorticoids are unlikely to be interfering with the process of cAMP induction itself. It is also unlikely that steroid-mediated stimulation of the production of a protein that affects CRE function is operative, since we have found that negative regulation by glucocorticoids and induction by cAMP of endogenous α-subunit mRNA levels can occur in mouse pituitary tumor cells when protein synthesis is blocked by cycloheximide (*18*). Additional evidence for the role of the GR binding sites, which overlap the outer edge of each of the duplicated CREs, was obtained by the use of plasmids from which the central 18 bp of the CREs was deleted, forming a single CRE with GR binding sites overlapping both outer edges. This mutation, which decreases basal expression in JEG-3 cells sixfold (*7*), is negatively regulated to a significantly greater degree by glucocorticoids (*18*).

Transcriptional repressors have only recently been described for eukaryotic genes such as mammalian insulin, fos, and α- and β-interferon genes (*19*). Though some systems have autonomous silencer elements

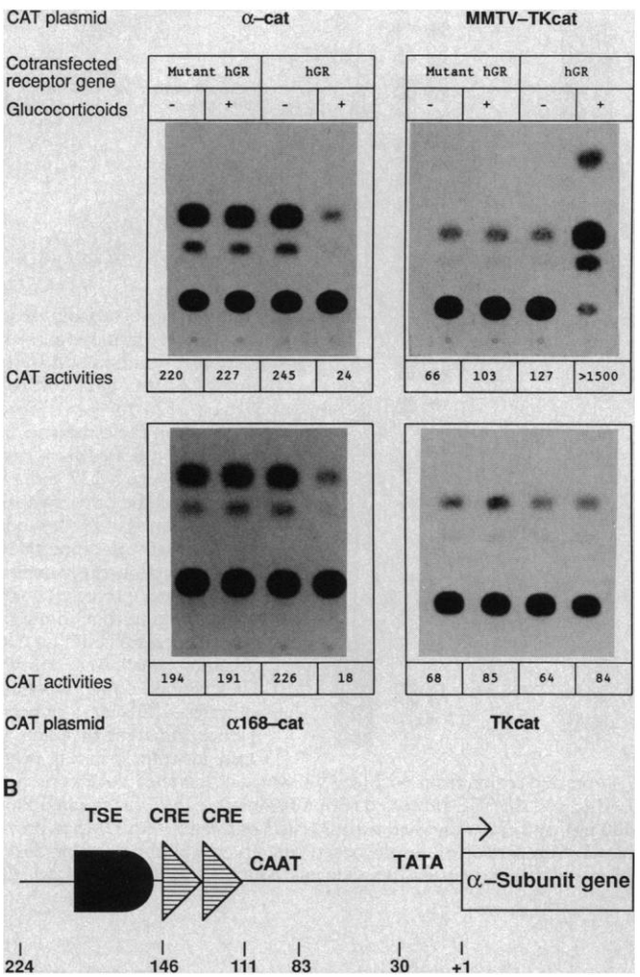

Fig. 1. Negative regulation of the α-subunit gene in placental cells. (**A**) Expression of the α-subunit gene using cotransfection with human glucocorticoid receptor expression vectors. CAT analysis of JEG-3 cell transfections containing a CAT reporter plasmid {α-cat, α168-cat, MMTV-TKcat [originally p5A (*10*)], or TKcat} cotransfected with vectors (*8*) expressing either the intact receptor (RSVGR) or a mutant receptor (I422, which has an insertion of three amino acids in the DNA-binding domain) with or without the addition of the glucocorticoid dexamethasone ($5 \times 10^{-7} M$ was used routinely, though $10^{-9} M$ was fully effective). CAT activities are expressed as acetylated [^{14}C]chloramphenicol counts per microgram of cell protein per hour and are the average of three experiments. Human placental JEG-3 cells (*9*) were plated in medium containing 5% charcoal-stripped fetal calf serum for 24 hours, then transfected as described (*7*) with 2.5 μg of the cat reporter plasmid indicated, 7.5 μg of carrier plasmid (pUC8), 5 μg of either RSVGR or I422 (*8*), and 2.5 μg of a control plasmid, RSVβgal (*22*). Extracts were prepared after 48 hours, and protein concentrations, β-galactosidase activities, and CAT assays were performed as previously described (*7*). Negative regulation by glucocorticoids was equally effective with 0.5 μg of RSV-GR plasmid, indicating that GR is in excess in these conditions. (**B**) Functional map of the 5′-flanking region of the α-subunit gene. Enhancer and promoter elements (*7*) are shown diagrammatically: TSE, a tissue-specific element, which participates in placental-specific expression and serves as the binding site for a placental-specific DNA-binding protein; CRE, two 18-bp cAMP-responsive elements, which are involved in both tissue-specific expression and cAMP regulation, each of which binds a protein found in many cell types [CRE-binding protein (*7, 13*)]; CAAT and TATA, the positions of the consensus sequences common to many eukaryotic promoters (*15*). The arrow indicates the transcriptional initiation site that was previously confirmed by S1 analysis (*7*). Effects of glucocorticoids on the α168 promoter in cotransfections with GR or mutant GR into JEG-3 cells were also confirmed by S_1 nuclease analysis of RNA.

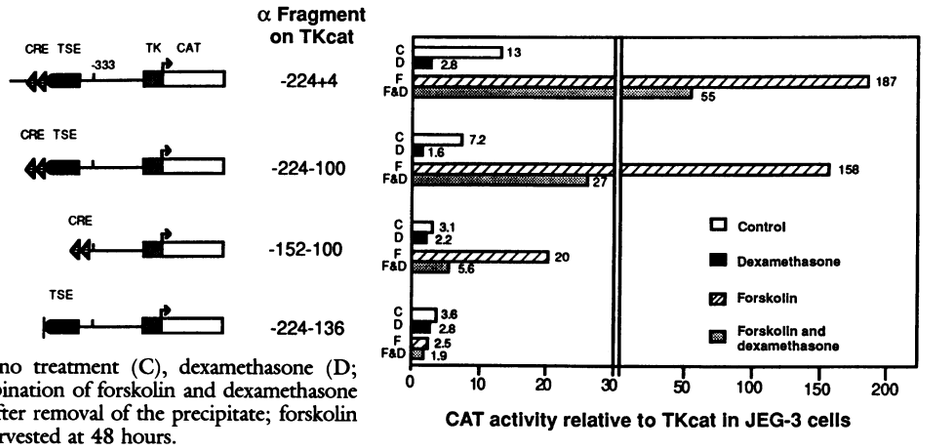

Fig. 2. Effect of fragments of the α-subunit 5′-flanking region on expression from the tk promoter in response to glucocorticoids and cAMP in placental cells. Diagrams on the left represent the plasmids used in these experiments, including the location and orientation of TSE and CRE. The α-subunit DNA fragments were inserted in opposite orientation, 333 bp 5′ to the tk promoter (−109 to +55) in TKcat (*7*), and cotransfected into JEG-3 cells with RSVGR as described for Fig. 1. CAT activities of the α-TKcat constructions are represented relative to the activity of TKcat (the value of which was set to one) and are the average of three experiments with SEM values from 3 to 30% of the reported values. The bars represent the response of each construction to no treatment (C), dexamethasone (D; $5 \times 10^{-7} M$), forskolin (F; 10 μ*M*), and the combination of forskolin and dexamethasone (F&D). Dexamethasone was added immediately after removal of the precipitate; forskolin was added 12 to 15 hours before the cells were harvested at 48 hours.

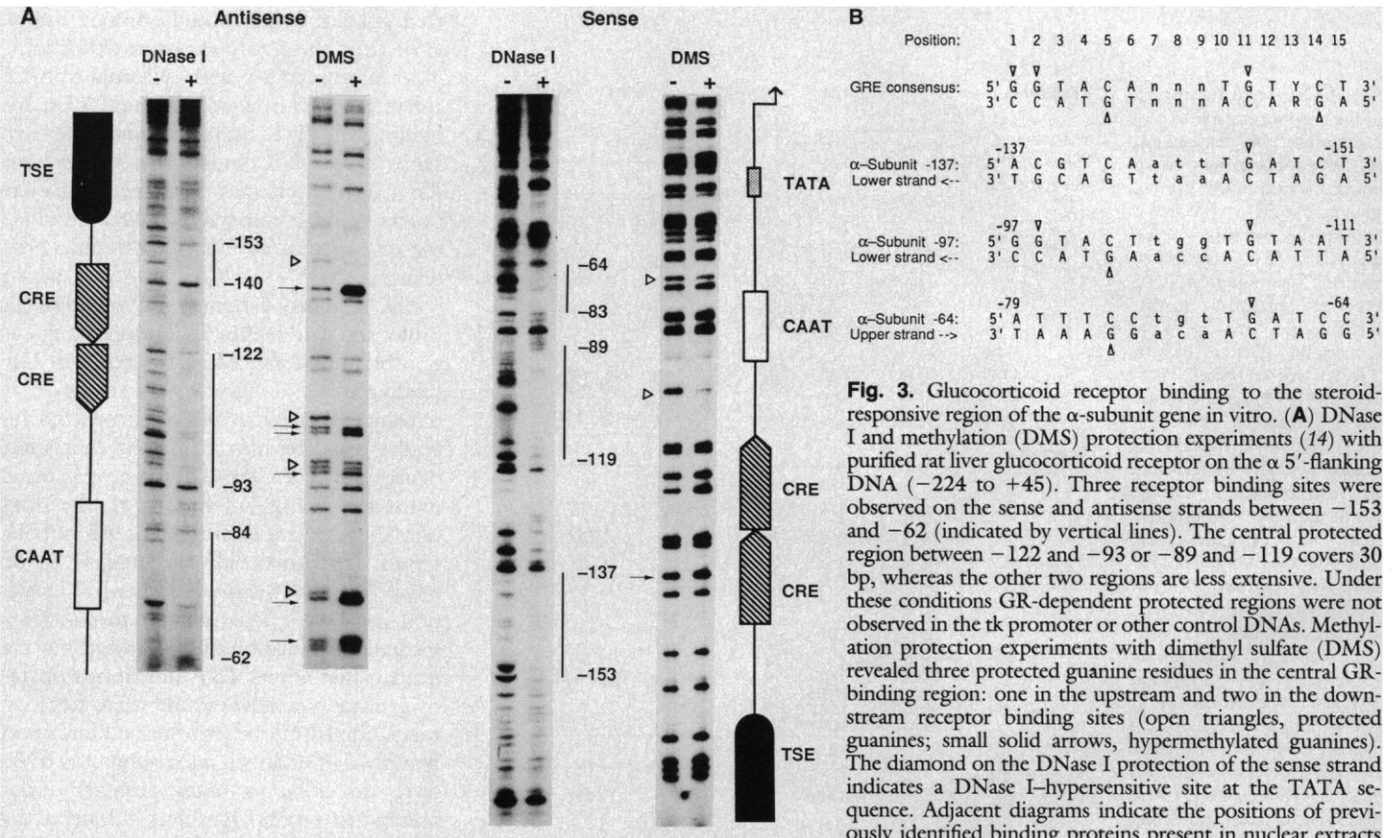

Fig. 3. Glucocorticoid receptor binding to the steroid-responsive region of the α-subunit gene in vitro. (**A**) DNase I and methylation (DMS) protection experiments (*14*) with purified rat liver glucocorticoid receptor on the α 5′-flanking DNA (−224 to +45). Three receptor binding sites were observed on the sense and antisense strands between −153 and −62 (indicated by vertical lines). The central protected region between −122 and −93 or −89 and −119 covers 30 bp, whereas the other two regions are less extensive. Under these conditions GR-dependent protected regions were not observed in the tk promoter or other control DNAs. Methylation protection experiments with dimethyl sulfate (DMS) revealed three protected guanine residues in the central GR-binding region: one in the upstream and two in the downstream receptor binding sites (open triangles, protected guanines; small solid arrows, hypermethylated guanines). The diamond on the DNase I protection of the sense strand indicates a DNase I–hypersensitive site at the TATA sequence. Adjacent diagrams indicate the positions of previously identified binding proteins present in nuclear extracts of placental cells (*7*). The open box indicates a DNase I–protected region from −72 to −92, which covers the CAAT consensus sequence (−82 to −88). The CREs are indicated from −111 to −128 and −129 to −146 and the TSE-protected region begins at −159. The isolated Bam HI to Xba I 269-bp fragment (30 ng) was incubated with (+) or without (−) GR (500 ng), and either digested with DNase I or treated with DMS as previously described (*14*), but in the presence of 115 m*M* NaCl and 11% polyethylene glycol. (**B**) Comparison of the glucocorticoid receptor binding sites on the α-subunit gene with a consensus GRE. Sequences protected from DNase I by GR are compared to a consensus GRE sequence compiled by Beato *et al.* (*16*). Guanine residues protected from methylation by GR binding are indicated by arrows.

(*20*), others may be repressed by the displacement of transactivator proteins from enhancer or promoter elements. In the case of the sea urchin histone H2B-1 gene, a displacement protein present in non-expressing cell types appears to sterically prevent interaction of the CAAT-binding protein with its binding site (*21*).

Our experiments suggest a solution to the paradox of how a positive transactivating protein could simultaneously mediate negative regulation of gene expression. In this model, the receptor-hormone complex binds the same sequence recognized in positive regulation and mediates negative regulation through interference with the binding or action of other transactivating proteins crucial for gene expression. It is clear from our experiments that receptor encoded by a single gene mediates both negative and positive regulation, since GR is introduced by transfection of a cloned expression vector. The GRE- and CRE-containing α-subunit DNA fragment does not have intrinsic glucocorticoid-dependent silencing activity, since it cannot confer negative regulation to the tk promoter unless the CREs are induced to act as enhancers. These CREs can become enhancers either through induction by cAMP or cooperation with the placental

TSE. In either case, the addition of glucocorticoids and GR then results in decreased transcription, demonstrating interference with the activity of the CRE and CRE-binding protein. The presence of GR binding sites that overlap both CREs supports a model in which negative control is exerted through direct GR binding to DNA sequences that overlap the CREs, with consequent blockage of CRE activity. This mechanism of interference between transcription factors may apply not only to negative regulation by steroid receptors but also to negative regulation in other systems.

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Abnormalities in Structure and Expression of the Human Retinoblastoma Gene in SCLC

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Small cell lung cancer (SCLC) has been associated with loss of heterozygosity at several distinct genetic loci including chromosomes 3p, 13q, and 17p. To determine whether the retinoblastoma gene (*Rb*) localized at 13q14, might be the target of recessive mutations in lung cancer, eight primary SCLC tumors and 50 cell lines representing all major histologic types of lung cancer were examined with the *Rb* complementary DNA probe. Structural abnormalities within the *Rb* gene were observed in 1/8 (13%) primary SCLC tumors, 4/22 (18%) SCLC lines, and 1/4 (25%) pulmonary carcinoid lines (comparable to the 20 to 40% observed in retinoblastoma), but were not detected in other major types of lung cancer. *Rb* messenger RNA expression was absent in 60% of the SCLC lines and 75% of pulmonary carcinoid lines, including all samples with DNA abnormalities. In contrast, *Rb* transcripts were found in 90% of non-SCLC lung cancer lines and in normal human lung. The finding of abnormalities of the *Rb* gene in SCLC and pulmonary carcinoids (both neuroendocrine tumors) suggests that this gene may be involved in the pathogenesis of a common adult malignancy.

IN SEVERAL CHILDHOOD TUMORS, including retinoblastoma and Wilm's tumor, there is growing evidence to indicate that the inactivation of both alleles of certain genes triggers tumorigenesis (*1–3*). The genomic locus determining susceptibility to retinoblastoma has been mapped to chromosome 13q14 (*4*), and several groups have obtained complementary DNA (cDNA) clones derived from this region that detect a DNA segment with properties of the putative retinoblastoma (*Rb*) gene (*5–7*). Evidence for this gene being the site of recessive mutations leading to tumor formation in retinoblastoma is based on the finding of structural changes within the gene, including internal homozygous deletions in a number of retinoblastomas, and the presence of altered or absent messenger RNA (mRNA) expression in the majority of both sporadic and familial forms of the tumor.

Specific chromosomal deletions have now been reported in various adult tumors (*8*), suggesting that "recessive oncogenes" may be important in the pathogenesis of these malignancies. In the case of small cell lung cancer (SCLC), a consensus deletion of DNA in the region 3p14–3p21 has been identified in virtually all cases (*9–11*). Several studies have also identified nonrandom changes involving other chromosomes, including chromosome 13. For example, one cytogenetic study of SCLC reported that chromosome 13 was the most frequently underrepresented chromosome, with 17 out of 21 SCLC lines having absent or hypodiploid numbers of chromosome 13 (*12*). In two recent studies, polymorphic probes from the long arm of chromosome 13 that spanned the region 13q12–13q33 demonstrated a reduction to homozygosity at one or more informative loci in primary tumor tissue from 18 of 23 patients with SCLC (*11, 13*). SCLC is an aggressive adult tumor which phenotypically resembles retinoblastoma in that both display properties of neural or neuroendocrine differentiation (*14, 15*) and both can have deregulated N-*myc* expression (*16, 17*). These observations, coupled with the recent report of structural abnormalities of the *Rb* gene in ~20% of osteosarcomas and other mesenchymal tumors (*18*), prompted us to examine the

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