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# Expression of Insulinlike Growth Factor (IGF) and IGF-Binding Protein Genes in Human Lung Tumor Cell Lines


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***Background:*** **The presence of multiple, low-molecular-weight, insulinlike growth factor (IGF)-binding proteins in lung tumor cell-conditioned medium and lung cancer patient serum has been recently reported.** ***Purpose:*** **To begin to elucidate the genetic basis for these observations, the present study examines the expression by lung tumor cell lines of three IGF-binding protein genes, namely, IGFBP-1, IGFBP-2, and IGFBP-3. Since IGF-binding proteins are thought to modulate the biologic action of the IGFs, the relationship between the expression of IGF-binding protein genes and the genes encoding IGF-I and IGF-II also has been investigated.** ***Methods:*** **Gene expression was studied in four small-cell lung cancer (SCLC) and three non–small-cell lung cancer (NSCLC) cell lines using Northern blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR) for IGFBP-1.** ***Results:*** **IGFBP-1 gene expression was detected by Northern blot analysis in one NSCLC cell line only. However, RT-PCR revealed that the IGFBP-1 gene was expressed in all four SCLC cell lines and in two of the three NSCLC lines. Northern blot analysis of IGFBP-2 gene expression demonstrated that all lung tumor cell lines expressed this gene. A low level of IGFBP-3 gene expression was detected in one SCLC cell line and in all three NSCLC cell lines. All lung tumor cell lines expressed the IGF-II gene as determined by Northern blot analysis. In marked contrast, none of the lines showed evidence of IGF-I gene expression using this method. However, RT-PCR revealed a low level of IGF-I gene expression in one SCLC and one NSCLC cell line only.** ***Conclusions:*** **These observations indicate 1) that IGF-binding proteins secreted by lung tumors are encoded by at least three different genes; 2) that there may be a close association between IGF-II and IGFBP-2 gene expression, such that, where there is production of IGF-II, IGFBP-2 is the principal BP; and 3) that the IGF-II gene is more widely expressed than the IGF-I gene in human lung tumor cell lines. [J Natl Cancer Institute 84:628–634, 1992]**


An increasing number of proteins with insulinlike growth factor (IGF)-binding characteristics have been isolated from various body fluids, tissue extracts, and


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cell lines (*1*). On the basis of extensive protein and complementary DNA (cDNA) sequencing studies, these proteins have been classified (*2*) into six distinct groups. IGFBP-1, also named placental protein 12 (pp12) (*3*), IBP-1 (*4*), BP-25 (*5*), BP-28 (*6*), and alpha pregnancy-associated endometrial globulin (*7*), has been purified from amniotic fluid and several other sources. Its cloned cDNA sequence predicts a molecular mass of 25 kd, and expression of the cDNA encoding IGFBP-1 in COS cells results in the synthesis of protein with a relative molecular mass ($M_r$) of 30 kd on nonreduced sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (*4*). IGFBP-2 (*8*) is the human homologue of a protein isolated from rat BRL-3a cells (*9*) and has been isolated from human serum (*10*). The cDNA sequence of the human protein predicts a molecular mass of 31 kd; under nonreducing conditions, the expressed protein has an $M_r$ of 36 kd (*8*). IGFBP-3, also known as BP-53 (*11*), is a growth hormone-dependent protein, originally purified from human plasma. On nonreduced SDS-PAGE, the protein appears as a glycoprotein doublet consisting of a major 53-kd and a minor 47-kd component (*11*). The cDNA for this protein predicts a molecular mass of 28 kd for the nonglycosylated protein (*12*). IGFBP-4, isolated from human osteoblastoma- (*13*) and prostatic carcinoma- (*14*) conditioned media and human serum (*10*), has a predicted molecular mass of 22 kd and migrates as a 28- to 30-kd IGF-binding protein on nonreduced gels. A further IGF-binding protein, IGFBP-5, isolated from cerebrospinal fluid (*15*) and human serum (*10*), has a predicted molecular mass of 26 kd for the mature protein and an $M_r$ of 24 kd for the nonglycosylated protein. Finally, a sixth IGF-binding protein, IGFBP-6, has been purified recently from pig ovarian follicular fluid, and cDNA clones encoding rat and human IGFBP-6 have also been isolated and characterized (*16*). All six IGF-binding proteins are distinct from the type I and type II receptors for IGFs; importantly, stimulatory (*1,17-19*) and/or inhibitory (*1,20,21*) effects on cell growth have been demonstrated for certain IGF-binding proteins.

Recently, multiple IGF-binding proteins have been shown to be secreted by human lung tumor cells both in vitro (*22,23*) and in vivo (*23*). These proteins, under nonreducing conditions, range in size from 12 kd to 30 kd and may be encoded by one or more of the aforementioned genes. To determine whether lung tumor cells secrete multiple different IGF-binding proteins, the present study examines the expression of the IGFBP-1, IGFBP-2, and IGFBP-3 genes in a panel of small-cell lung cancer (SCLC) and non–small-cell lung cancer (NSCLC) cell lines and examines the relationship between IGF-binding protein gene expression and expression of the genes encoding IGF-I and IGF-II.

## Materials and Methods

### Cell Lines

Full details of the derivation and characterization of SCLC cell lines COR-L47, COR-L51, and COR-L88 and large-cell lung cancer cell line COR-L23 have been described (*24*). The classic SCLC cell line NCI-H69 was donated by Drs. D. Carney and A. Gazdar (National Cancer Institute Navy Medical Oncology Branch, Bethesda, Md.). The squamous cell lung carcinoma cell line BEN and the lung adenocarcinoma cell line MOR were from Dr. M. Ellison (Ludwig Institute, Sutton, Surrey, England). All cell lines were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (both medium and serum from GIBCO BRL, Paisley, Scotland).

### RNA Preparation

Cells in logarithmic phase of growth were collected by centrifugation at 300*g* for 10 minutes and suspended in 100 μL of medium. A solution containing 6.0 *M* guanidine hydrochloride and 0.2 *M* sodium acetate (pH 5.5) was added to the cells (20 mL per $5 \times 10^7$ cells), and the DNA was sheared by vigorous homogenization in a Virtis homogenizer (Virtis Co., Gardiner, N.Y.). RNA was precipitated by the addition of a half volume of 95% ethanol followed by incubation at –20 °C overnight. The pelleted precipitate was dissolved in a solution containing 7.0 *M* urea, 0.35 *M* NaCl, 50 m*M* Tris (pH 7.5), 1 m*M* EDTA, and 0.2% SDS and then was extracted once with phenol-chloroform. RNA was precipitated from the aqueous phase using two volumes of ethanol, washed with 70% ethanol, air dried, and dissolved in sterile, double-distilled water.

Poly(A)$^+$ RNA was prepared from total RNA using a messenger RNA (mRNA) purification kit (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.).

### Northern Blot Analysis

Five micrograms of poly(A)$^+$ RNA in 10 m*M* sodium phosphate buffer (pH 7.0) was denatured in 1.0 *M* glyoxal for 1 hour at 50 °C. The RNA was electrophoresed in a 1.4% agarose gel in 10 m*M* sodium phosphate buffer and was transferred by Northern blotting to nylon filters. After treatment for 2 minutes with UV light, the nylon filters were baked at 80 °C for 2 hours before hybridization.

The IGFBP-3 (*12*) and the IGF-I and IGF-II cDNA probes (*25*) were supplied by Genentech Inc., San Francisco, Calif., and by Dr. G. Bell, Howard Hughes Medical Institute, Chicago, Ill., respectively. The IGFBP-1 (*4*) and the IGFBP-2 (*8*) cDNAs, both cloned into the vector PTZ19 (Pharmacia LKB Biotechnology Inc.), the IGFBP-3 cDNA, cloned into the pUC119 vector (*12*), and the IGF-I and IGF-II cDNAs, cloned into the pKT218 vector (Pharmacia LKB Biotechnology Inc.), were separated from their vectors by treatment with *Eco*RI followed by agarose gel electrophoresis. The *Eco*RI fragments, still in the gel slice, were radiolabeled by transcribing the fragments using mixed oligonucleotides to initiate transcription. The radiolabeled probes were separated from unincorporated nucleotide triphosphates using Sephadex G50 (Pharmacia LKB Biotechnology, Inc.) and boiled for 3 minutes before use. A mouse β actin probe, PRT3 (donated by Dr. John Rogers, Laboratory of Molecular Biology, Medical Research Council), was similarly labeled to confirm equal loading of RNA.

The labeled probe, at a concentration of $10^6$ counts per minute per milliliter, was hybridized to the filter in 1 *M* NaCl and 0.1 *M* trisodium citrate (6× SSC), 5% dextran sulfate, 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinyl pyrrolidone, 0.1% SDS, and 150 μg/mL sonicated salmon sperm DNA at 65 °C for 18 hours. The filter was washed with 6× SSC and 0.1% SDS at 65 °C to

remove unhybridized probe prior to autoradiography.

### Reverse Transcriptase Polymerase Chain Reaction Analysis of IGFBP-1 and IGF-I Gene Expression

Synthetic oligonucleotides, designed on the basis of the nucleotide sequence of the mRNA encoding IGFBP-1 and IGF-I, were synthesized using an Applied Biosystems 380 DNA Synthesizer (Applied Biosystems, Warrington, England). The sequences of the IGFBP-1 specific primers were the following:

(B1) 5'GCTCCCCATGCTGCAGAGG-CAGGG3' corresponding to nucleotides 386-409.
(B2) 5'TACATTAAAATACATCTGG-CAGTT3' complementary to nucleotides 823-800.

The 437-base-pair (bp) amplification product is unique to IGFBP-1.

The sequences of the primers used for reverse transcriptase polymerase chain reaction (RT-PCR) analysis of IGF-I gene expression were the following:

(IG-1) 5'TCTTGAAGGTGAAGAT-GCACACCA3' corresponding to nucleotides 238-261.
(IG-2) 5'AGCGAGCTGACTTGGCA-GGCTTGA3' complementary to nucleotides 540-517.

The 302-bp amplification product is common to IGF-IA and IGF-IB.

Ten micrograms of total RNA was reversed transcribed into first-strand cDNA by addition of 5 µL 0.1 *M* dithiothreitol, 2.5 µL 5 m*M* deoxyribonucleoside triphosphate (dNTP) (Pharmacia LKB, Milton Keynes, England), 20 pmol of either oligonucleotide B2 or IG-2, and 5 µL 5× reverse transcriptase buffer (500 m*M* Tris [pH 8.3], 60 m*M* $MgCl_2$, and 400 m*M* KCl). After heating for 10 minutes at 70 °C, the reaction mixture was cooled to 25 °C, and 2 units of avian myeloblastosis virus reverse transcriptase (Anglian Biotec Ltd., Colchester, England) were added. Following incubation at 42 °C for 1 hour, a 5-µL aliquot of first-strand cDNA was added to 20 pmol of either oligonucleotide primers B1 and B2 or IG-1 and IG-2, 5 µL 5 m*M* dNTP, and 5 µL 10× Thermus aquaticus (Taq) polymerase buffer—0.67 m*M* Tris (pH 8.8), 9.17 *M* $(NH_4)_2SO_4$, 0.1 *M* $MgCl_2$, 0.1 *M* bromomercaptoethanol, and 2 mg/mL gelatin—in a total volume of 50 µL. Two units of Taq polymerase (ILS Ltd., London, England) were added, and amplification (35 cycles) was performed using a PHC-1 automated cycler (Techne Ltd., Duxford, England). Annealing was for 2 minutes at 55 °C, polymerization was at 72 °C for 3 minutes, and denaturation was at 95 °C for 1 minute. Forty microliters of the reaction mixture was then electrophoresed on 1.4% agarose gels in the presence of ethidium bromide. Amplified products were detected by UV light transillumination and by autoradiography following Southern blotting and hybridization with either IGFBP-1 cDNA or IGF-I cDNA.

## Results

### IGFBP-1 Gene Expression

Using a $^{32}P$-labeled *Eco*RI fragment derived from cDNA clone W85 as a probe, a single transcript of approximately 1.5 kb was detected in the squamous cell lung carcinoma cell line BEN only (data not shown). However, Southern blot analysis of amplification products produced by RT-PCR (Fig. 1) revealed that the IGFBP-1 gene was expressed in all SCLC cell lines, the BEN cell line, and the large-cell lung carcinoma cell line COR-L23, as evidenced for each cell line by the hybridization of an amplification product having the expected molecular mass of 437 bp to the radiolabeled IGFBP-1 cDNA probe. Only the lung adenocarcinoma cell line MOR failed to show IGFBP-1 gene expression.

### IGFBP-2 Gene Expression

Fig. 2 (panel a) shows that IGFBP-2 gene expression was detected by Northern blot analysis in all SCLC and NSCLC cell lines. Two transcripts were present in all cell lines: a 4-kb and a 1.4-kb species. The 4-kb species could not be removed by washing filters in 0.1× SSC and 0.1% SDS. Fig. 2 (panel b) shows hybridization of mRNAs with the mouse β actin cDNA probe.

### IGFBP-3 Gene Expression

A single 2.5-kb IGFBP-3 gene transcript was detected by Northern blot analysis in one of the four SCLC cell lines and in all NSCLC cell lines studied (Fig. 3, panel a). In addition, a smaller mRNA species was detected in the MOR cell line. Detection of IGFBP-3 gene expression required long exposure times of up to 10 days. Hybridization of lung tumor mRNAs to the mouse β actin probe is shown in Fig. 3 (panel b). The apparent absence of IGFBP-3 transcripts in SCLC cell lines NCI-H69, COR-L47, and COR-L88 was investigated further using RT-PCR. No evidence of IGFBP-3 gene expression in these cell lines was observed using this method (data not shown).

### IGF-I and IGF-II Gene Expression

The IGF-I cDNA probe used in this study hybridized to 1.1-kb and 6.3-kb transcripts in human liver. However, no evidence of IGF-I gene expression was obtained by Northern blot analysis in any of the lung tumor cell lines examined (data not shown). The expression of the IGF-I gene was investigated further using RT-PCR. Fig. 4 shows that, with this method, amplification products of the expected size were detected in one of the four SCLC and in one of the three NSCLC cell lines.

Fig. 5 shows Northern blot analysis of IGF-II gene expression and demonstrates the presence of IGF-II gene transcripts in all lung tumor cell lines examined. A 5.3-kb transcript was detected in liver poly(A)$^+$ RNA. Three transcripts, 3.5 kb, 4.8 kb, and 6 kb, were detected in SCLC cell lines NCI-H69 and COR-L47 and in the lung adenocarcinoma cell line MOR. Only the 6-kb and 4.8-kb transcripts were present in COR-L51. A weak but detectable 4.8-kb transcript only was observed in NSCLC cell lines COR-L23 and BEN. SCLC cell line COR-L88 failed to show IGF-II gene expression.

## Discussion

We have shown previously that SCLC and NSCLC cell lines produce multiple low-molecular-weight IGFBPs and that sera from lung cancer patients contain elevated levels of these proteins (*23*). Affinity cross-linking studies indicated that lung tumor cells may secrete up to four different molecular-weight species, including 30-kd, 28-kd, 25-kd, and 12-kd proteins. The present study has inves-

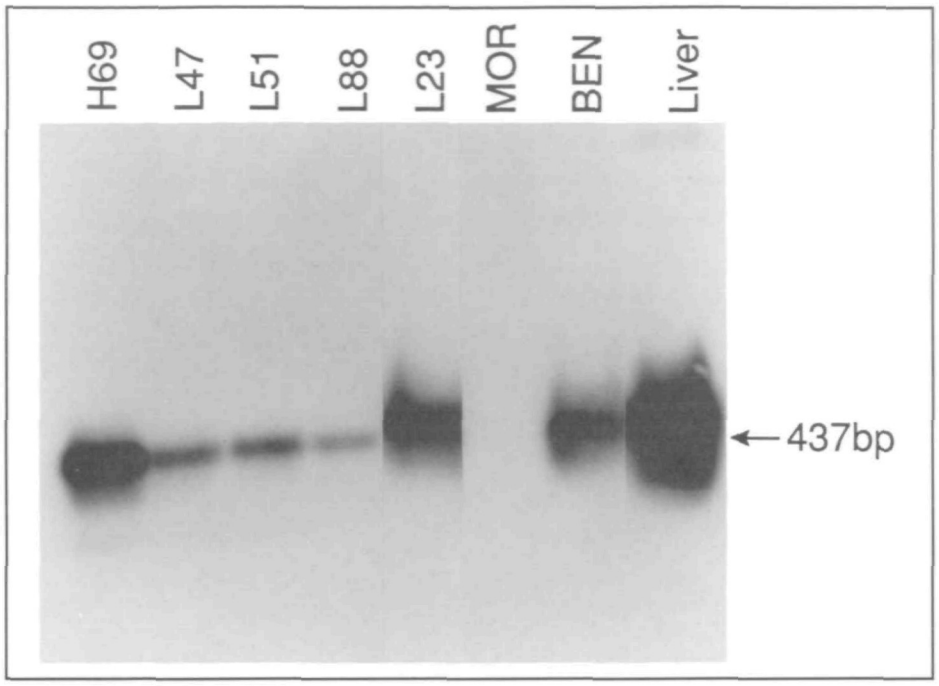


**Fig. 1.** *Detection of IGFBP-1 gene expression in SCLC and NSCLC cell lines by RT-PCR and Southern* blotting, followed by hybridization with a 1.2-kb radiolabeled IGFBP-1 cDNA probe (clone w85).

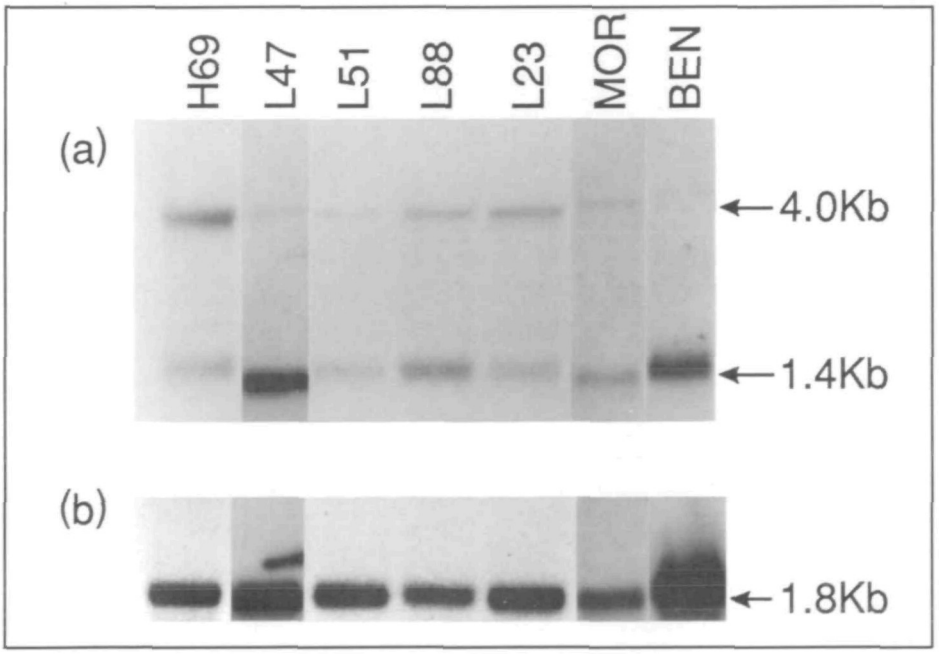


**Fig. 2.** Northern blot analysis of IGFBP-2 gene expression in SCLC and NSCLC cell lines (panel a). Blots were probed with a 1.5-kb fragment containing approximately 60 bp of 5′ untranslated and approximately 470 bp of 3′ untranslated regions. In all cell lines, 4.0-kb and 1.4-kb IGFBP-2 transcripts are present. The actin signal for each cell line is shown in panel b.

tigated the expression of three genes encoding IGFBPs, namely, IGFBP-1, IGFBP-2, and IGFBP-3, in these cells and demonstrates the concomitant expression of two of these genes in four of seven cell lines and of all three genes in the remaining three cell lines studied. IGFBP-1 gene expression, though undetectable in most cell lines by Northern blot analysis, does occur in most SCLC and NSCLC lung tumor cells. This expression is evidenced by hybridization of low-molecular-weight amplification products generated from enzymatic amplification from IGFBP-1 mRNA via RT-PCR, with the IGFBP-1 cDNA probe. Expression of this gene correlates with the secretion of a 25-kd IGFBP by lung tumor cell lines, as indicated by the observation that this protein is secreted by all cell lines except MOR—the only line showing no IGFBP-1 gene expression. IGFBP-2 gene expression was readily detected by Northern blot analysis in all cell lines examined, and pilot studies using an IGFBP-2-specific radioimmunoassay confirm the secretion of this protein by the lung tumor cell lines studied here. Finally, all NSCLC cell lines and one of the four SCLC lines showed IGFBP-3 gene expression. The findings of the present study are in marked contrast to those of a recent report in which IGFBP-2 gene expression only was detected in lung tumor cell lines (*26*). Detection of IGFBP-1 gene expression in the present study is attributable to the use of a sensitive RT-PCR method, and detection of IGFBP-3 transcripts is attributable to the use of greater amounts of poly(A)$^+$ RNA than those used in the Northern blot analyses of the earlier study.

The findings of the present study indicate that, of the three IGF-binding proteins studied, IGFBP-2 is the principal one produced by lung tumor cell lines. The marked differences in the relative levels of IGFBP-1, IGFBP-2, and IGFBP-3 gene expression detected in the various lines examined may reflect differences in IGF-binding protein gene activation, variation in mRNA stabilities, or expression of the IGFBP-1 and IGFBP-3 genes in a subset of cells only. A number of factors have been shown to influence the expression of IGF-binding protein genes, including insulin which decreases IGFBP-2 gene expression (*27*), growth hormone which decreases transcription of the IGFBP-1 gene (*28*), and dexamethasone which increases both IGFBP-1 (*29*) and IGFBP-3 (*30*) mRNAs in hepatocytes. Studies are in progress to identify factors involved in the hormonal regulation of tumor-derived IGF-binding protein production.

The sizes of the IGFBP-1 and IGFBP-3 gene transcripts reported here for lung tumor cells are consistent with those detected in human liver (*4,12*). In contrast, normal adult liver contains only a 1.4-kb IGFBP-2 mRNA, whereas most lung tumor cell lines contained this transcript and a 4-kb mRNA. We have recently detected this transcript in fetal lung fibroblasts (Reeve JG: unpublished

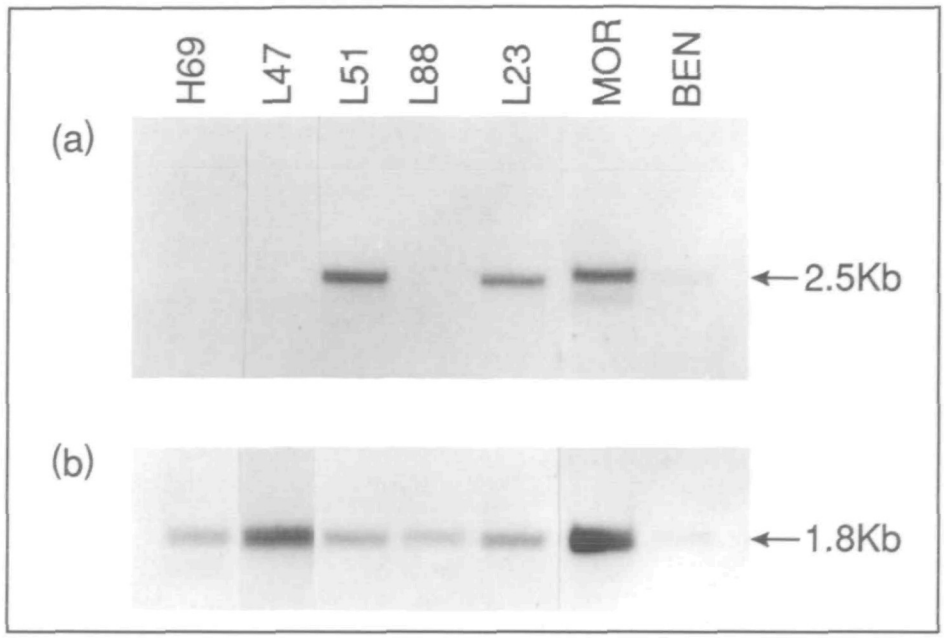


**Fig. 3.** Northern blot analysis of IGFBP-3 gene expression in SCLC and NSCLC cell lines (panel a). Blots were probed with a 2.5-kb fragment including the full coding region of human IGFBP-3. A 2.5-kb transcript is present in only one SCLC cell line. In contrast, all three NSCLC cell lines express the IGFBP-3 gene. A 2.0-kb transcript is also detected in the lung adenocarcinoma cell line MOR. The actin signal for each cell line is given in panel b.

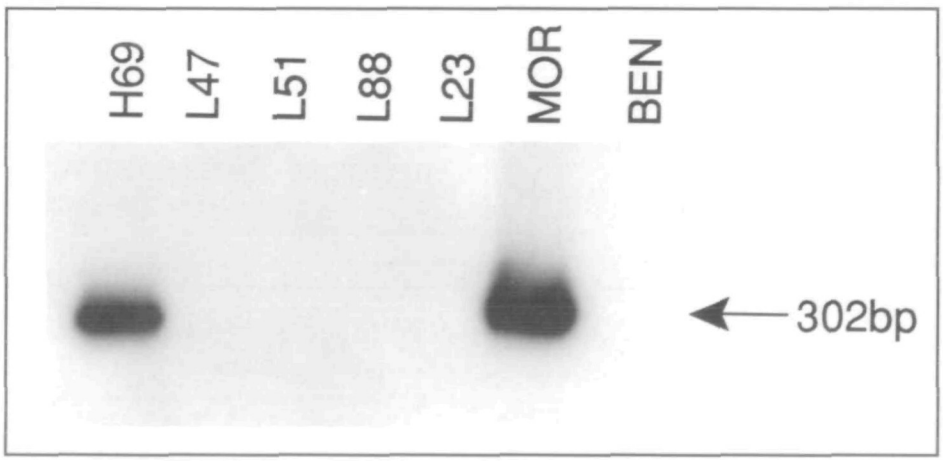


**Fig. 4.** Detection of IGF-I gene expression in lung tumor cell lines by RT-PCR followed by Southern blotting and hybridization to the 0.66-kb phigf-I cDNA probe. Amplification products having the expected size of 302 bp were detected in the SCLC cell line NCI-H69 and the lung adenocarcinoma cell line MOR only.

data), and it is also present in fetal liver and in the HepG2 and the WRL-68 embryonic liver lines (*31*). In the liver, the presence of the 4-kb transcript appears to be inversely related to the degree of differentiation, in that adult liver contains only the mature 1.4-kb mRNA, fetal liver contains both the 1.4-kb and the 4-kb transcripts, but cultured WRL-68 embryonic liver cells contain only the 4-kb mRNA. The presence of the 4-kb transcript in human lung tumor cells and fetal lung fibroblasts may indicate that the presence of this transcript is also differentiation related in the lung. Studies are in progress to investigate the relative amounts of these two transcripts in a variety of normal fetal and adult tissues.

Although the biological significance of IGF-binding protein production by lung tumor cells is not known at this time, a number of studies have shown that these proteins can modulate cellular responses to IGF stimulation. IGF-binding proteins have been shown to inhibit the effects of IGF-I and IGF-II on fibroblast DNA synthesis (*20,21*), to increase the binding of IGF-I to its receptor (*32*), to potentiate markedly the replication of human, mouse, and chicken fibroblasts in response to IGF-I stimulation (*17*), and to enhance the cellular DNA synthesis response of cultured porcine aortic smooth muscle cells to IGF-I (*19*). Given that both IGF-I and IGF-II have been shown to promote the proliferation of SCLC cells in vitro (*33,34*), IGF-binding proteins may regulate the mitogenic action of the IGFs in these cells.

A number of studies have reported the secretion of immunoreactive IGF-I (*23,33*) and the presence of IGF-I receptors (*22, 34*) in SCLC cell lines. Such observations, together with the mitogenic responsiveness of these cells to IGF-I stimulation and the growth-inhibitory effects of antibodies to the IGF-I receptor, have led to the conclusion that IGF-I is an important autocrine growth factor for SCLC (33-36). However, the present study is the first to examine the expression of the IGF-I gene in SCLC cell lines and shows that the majority of SCLC cell lines studied failed to express the IGF-I gene as determined by RT-PCR, perhaps challenging the importance of this peptide in the autocrine growth of SCLC. This finding, as well as the low level of IGF-I gene expression found in the only positive SCLC cell line, is surprising, given that several of the cell lines investigated have been shown to secrete low levels of immunoreactive IGF-I (*23*). However, the antiserum used to determine IGF-I secretion showed 3% cross-reactivity with IGF-II. Given the observed expression of the IGF-II gene in all the lines studied, secretion of IGF-II, and not IGF-I, seems more likely. Together with the observation that exogenous IGF-II promotes SCLC cell proliferation (*33*) and stimulates DNA synthesis in NSCLC cell lines (Reeve JG, Schwander J, Bleehen NM: manuscript submitted for publication), the findings of the present study support the contention that IGF-II may be more widely involved in the autocrine growth of lung tumors than IGF-I.

IGF-II gene expression in lung tumor cell lines is particularly interesting, given that IGF-II is an embryonal mitogen (*37-40*) and is thought to play an important role in lung differentiation and maturation (*41*). Transcription of the IGF-II gene in the fetus is driven by three distinct promoters (*42-44*) and yields three major transcripts of 6.0 kb, 4.8 kb, and 1.9 kb (*45*). In adult tissues, including the lung,

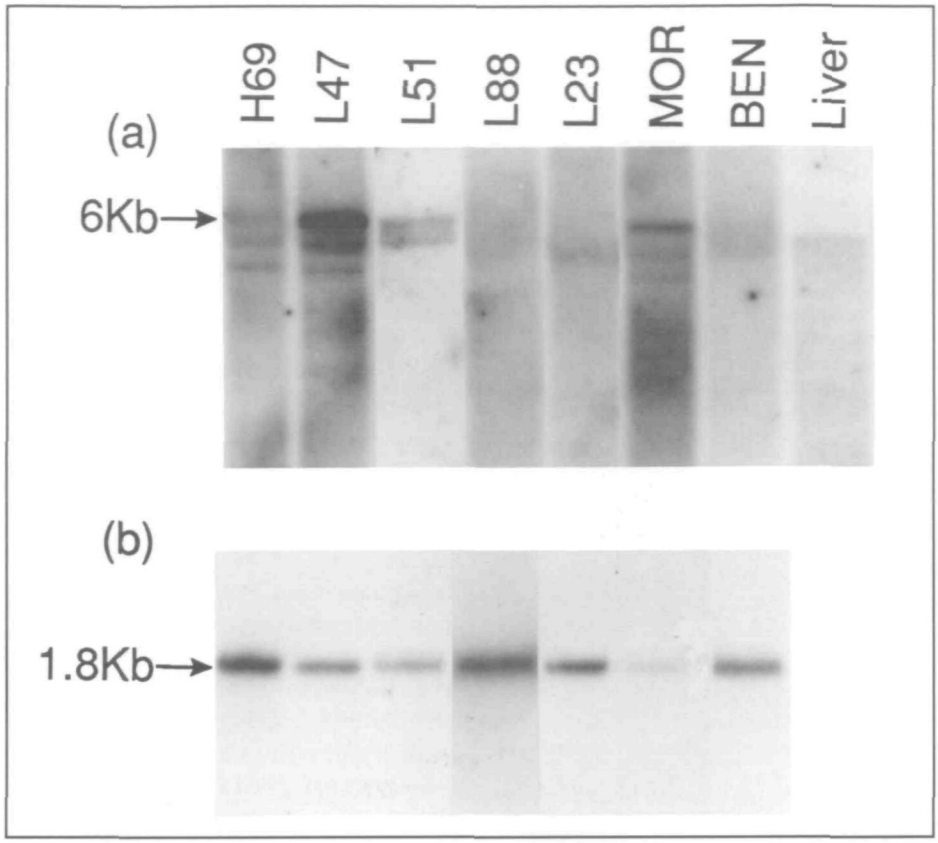


**Fig. 5.** IGF-II gene expression in SCLC and NSCLC cell lines detected using the 1.1-kb phigf-II cDNA probe and Northern blot analysis (panel a). Each track contains approximately 5 μg mRNA, and filters were exposed for 10 days. Panel b shows the actin signal for each track.

all three promoters are markedly suppressed (*42,43,45*). Hence, in the present study, the detection of abundant 6.0-kb and 4.8-kb mRNA species in lung tumor cells may represent re-activation of IGF-II fetal promoters during lung carcinogenesis. Recent circumstantial evidence has also implicated IGF-II in the genesis of developmental tumors, such as hepatoblastoma, Wilms' tumor, and rhabdomyosarcoma (*45*), but elevated expression is also seen in hepatocellular carcinoma (*46*), colon carcinoma (*47*), liposarcoma (*47*), and fibrosarcoma (*48*).

The IGF-II gene has been assigned to 11p14.1 (*49*) and is in the immediate vicinity of the Wilms' tumor susceptibility gene locus. In the Wilms' tumor, increased expression of the IGF-II gene is thought to arise either through juxtaposition of the Wilms' tumor locus and the IGF-II gene via chromosomal deletion or through re-activation of a set of embryonic genes, which includes the IGF-II gene, as a consequence of the recessive mutation in the Wilms' tumor locus (*45*). Since chromosome 11p is one of several sites of frequent cytogenetic deletion and loss of heterozyosity in lung cancers (*50,51*), it is tempting to speculate that similar mechanisms may be responsible for re-expression of the IGF-II gene in lung tumors. Studies are in progress to investigate this possibility.

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# Induction by Estrogen Metabolite 16α-Hydroxyestrone of Genotoxic Damage and Aberrant Proliferation in Mouse Mammary Epithelial Cells

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***Background:*** **Estrogens are potent mammary tumor promoters influencing post-initiational events via epigenetic mechanisms. The upregulation (i.e., induction) of the C16α-hydroxylation pathway during 17β-estradiol ($E_2$) biotransformation has been associated with mammary cell transformation. The action of $E_2$ metabolites on tumorigenic transformation, however, is poorly understood.** ***Purpose:*** **The newly established mammary epithelial cell line C57/MG, derived from the C57BL mouse strain, was used to examine whether $E_2$ or its metabolites, 16-hydroxyestrone (16α-$OHE_1$) and estriol ($E_3$), function as initiators of mammary cell transformation.** ***Methods:*** **DNA repair (hydroxyurea-insensitive thymidine uptake), estrogen metabolism ($^3H$ exchange to form $^3H_20$), hyperproliferation (increased cell number), and acquisition of anchorage-independent**

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