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The *CCL6* Chemokine Is Differentially Regulated by c-Myc and L-Myc, and Promotes Tumorigenesis and Metastasis[1]

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ABSTRACT

The CCL6 chemokine gene was identified as a direct positive target of the L-Myc oncoprotein in interleukin 3-dependent 32D myeloid cells. A mutant form of c-Myc, lacking a region of the NH_2-terminal domain necessary for transcriptional repression (c-MycΔMBII), also up-regulated CCL6. Chromatin immunoprecipitation showed that L-Myc, c-MycΔMBII, and full-length c-Myc all bound the *CCL6* promoter, although the latter was inactive in transcriptional up-regulation. Exogenously added *CCL6* induced marked apoptosis in some cell types. However, in 32D cells, the coexpression of c-Myc and *CCL6* abrogated interleukin 3 dependence and produced a highly leukemogenic phenotype. In two solid tumor models, *CCL6* overexpression also accelerated tumor growth, and/or enhanced local and metastatic spread in association with marked apoptosis of the tumor capsule and adjacent normal tissues. Our results show that *CCL6* can be either a positive or negative target for Myc oncoproteins. The chemokine may alter tumor behavior by relieving its growth factor dependency and by promoting invasiveness as a result of local tissue apoptosis.

INTRODUCTION

Three members of the *MYC* oncogene family (*C-MYC*, *N-MYC*, and *L-MYC*) are dysregulated frequently in human cancers. Some of the most common examples are seen in Burkitt's and AIDS-related lymphomas, where a large fraction of tumors are associated with translocations between *C-MYC* and one of the immunoglobulin loci; in neuroblastoma, which is often associated with *N-MYC* amplification; and in SCLC,[3] where *L-MYC* amplification may occur concurrently with that of *C-MYC* and/or *N-MYC* (1).

Myc oncoproteins belong to the helix-loop-helix leucine zipper family of transcription factors (2). After their heterodimerization with another helix-loop-helix leucine zipper protein, Max, Myc proteins bind to specific DNA elements (E-boxes) of the sequence CAC/TGTG (3). Transcriptional activation of the bound gene is then mediated by an NH_2-terminal segment of the Myc protein termed the TAD (4). Two segments of the TAD, termed MBI and MBII, are highly conserved among the three Myc proteins, and play important roles in target gene regulation (5).

Myc proteins can also participate in transcriptional repression, although this appears to be mediated primarily through different target gene sequences, most notably initiator or NFY-B sites (6). Several studies have demonstrated that down-regulation of certain genes is particularly dependent on MBII (5, 6). Until now, repression mediated subsequent to E-box binding has not been described.

A major goal in the study of Myc proteins has been to identify their target genes. A number of these have now been characterized, mostly as a result of the application of DNA microarray technology (6–12). Many of the target genes encode proteins involved in cell cycle control, DNA synthesis, metabolism, apoptosis, and extracellular matrix formation. Some are also transforming when overexpressed (5, 6).

A related goal that has received little attention concerns the degree of overlap among the transcriptional targets for the three *MYC* genes. Indirect evidence suggesting that some, if not all, of these targets are the same, derives from studies demonstrating that the embryonic lethality of c-Myc−/− mice can be rescued by N-Myc (13), and that each of the *MYC* genes can rescue the growth deficit of c-Myc−/− fibroblasts and promote apoptosis (14). On the other hand, indications that the target genes may differ is provided by observations that the transforming ability of L-Myc is 10–100 times less potent than c-Myc or N-Myc, and that L-Myc−/− animals are phenotypically normal (15, 16).

In the current work, we provide evidence that *CCL6*, a member of the CC or β chemokine family (also known as *C10/SCYA6)*, is directly up-regulated by L-Myc but is repressed by c-Myc in a MBII-dependent manner. Although the exogenous addition of *CCL6* is well tolerated by some cell types, it is markedly proapoptotic for others. *CCL6* overexpression also dramatically alters the *in vitro* and *in vivo* behavior of several cell types, either by relieving them of their growth factor dependence and/or by markedly enhancing their tumorigenic and metastatic potential.

MATERIALS AND METHODS

Cells and Antibodies. 32D cells transfected with wild-type murine c-Myc (32D-c-Myc cells) and the control, parental vector (32D-neo cells), have been described previously (17) as have 32D cells expressing c-Myc with deletions in the TAD (10). These latter included 32D-c-MycΔ2–42, 32D-c-MycΔMBI (deletion of amino acids 42–66), 32D-c-MycΔ70–130, 32D-c-MycΔMBII (deletion of amino acids 133–147), and 32D-c-MycΔTAD (deletion of amino acids 2–147). All of the latter lines produced their respective mutant proteins at levels comparable with that of the wild-type protein in 32D-c-Myc cells (10). All of the 32D-derived cell lines were grown in RPMI 1640 plus 10% FBS (Life Technologies, Inc., Grand Island, NY). Unless otherwise stated, cell lines also contained 10% conditioned medium from IL-3-producing WEHI-3B cells and 400 μg/ml G-418 (Life Technologies, Inc.). Most other cell lines were grown either in RPMI 1640 or DMEM containing 10% FBS or supplemented calf serum (Life Technologies, Inc.). Human SCLC lines were kindly provided by Dr. Michael Birrer (National Cancer Institute).

All of the antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). These included the 9E10 anti-Myc epitope tag monoclonal antibody (sc-40), rabbit polyclonal anti-c-Myc (sc-764), rabbit polyclonal anti-L-Myc (sc-790), and murine monoclonal antiactin antibody (sc-8432). A polyclonal antibody raised against bacterially expressed, full-length max, has been described previously and used for immunoprecipitations (18). Horseradish peroxidase-conjugated secondary antibodies were also purchased from Santa Cruz and were used according to the directions of the supplier.

Microarray Analysis and Northern Blotting. Polyadenylated mRNA was isolated from 32D-c-Myc and 32D-L-Myc cells as described previously (10). Reverse transcription, labeling with Cy3 and Cy5 chromophores, and hybridization to murine cDNA microarrays (GEM Microarrays; Incyte Pharmaceutical, St. Louis, MO), were performed as described previously (10). Northern

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[3] The abbreviations used are: SCLC, small cell lung cancer; TAD, transactivation domain; MBI, Myc Box I; MBII, Myc Box II; FBS, fetal bovine serum; IL, interleukin; ChIP, chromatin immunoprecipitation; nt, nucleotide; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling; CLL, chronic lymphocytic leukemia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

blots were performed with 5 μg of total RNA/lane, blotted to Nytran membranes (Schleicher & Schuell, Keene, NH), and hybridized in UltraHyb solution (Ambion, Austin, TX).

Plasmids, Recombinant DNA, and Transfections. A cDNA clone encoding the entire *CCL6* coding region was obtained from Incyte Genomics, Inc. The coding region was amplified with the following primers: forward: 5′-*CGC GTC GAC* ACC ATG AGA AAC TCC AAG ACT-3′ and reverse: 5′-*CGC CTC GAG* AGC AAT GAC CTT GTT CCC AGA TC-3′. In these sequences, italicized nts indicate engineered "GC clamps," and *Sal*I and *Xho*I restriction enzyme sites, respectively, and underlined triplets indicate the initiator methionine codon and the final triplet in the coding region. After digestion of the amplified product with *Sal*I and *Xho*I, it was isolated by agarose gel electrophoresis and cloned into the *Xho*I site of the pSVL-3′MT-puro cloning vector (18). The resultant clone contained the *CCL6* cDNA cloned in-frame at its 3′ end to a c-Myc epitope tag. The vector was linearized in the plasmid backbone for electroporation into 32D cells as described previously (17). Alternatively, supercoiled plasmid DNA was transfected into A549 or HeLa cells using Lipofectamine (Life Technologies, Inc.). Stable transfectants were selected in 1 μg/ml of puromycin (Sigma, St. Louis, MO).

The murine *CCL6* promoter region between nts −1423 and +49 relative to the start of mRNA transcription was amplified from bacterial artificial chromosome DNA that was kindly supplied by Dr. Hisayuki Nomiyama (Kinki University School of Medicine, Osaka, Japan). PCR primers consisted of the following sequences: forward, 5′-*CGC CTC GAG* TAA GTT GGG CAT GAA CTG GGC A-3′ and reverse, 5′-*CGC AAG CTT* CCT CCT GGA GGA CAG GCC C-3′, where italicized bases denote engineered "GC clamps," and *Xho*I and *Hind*III sites, respectively, to facilitate unidirectional cloning. After amplification, the fragments were digested with *Xho*I and *Hind*III, isolated by agarose gel electrophoresis, and cloned into the *Xho*I + *Hind*III-digested luciferase reporter vector pGL2-puro. This vector was constructed by ligating a ~1 kb blunt-ended *Eco*RI-*Nhe*I DNA fragment containing a puromycin resistance cassette from the pAPuro vector (19) into the blunted-ended unique *Sal*I site of the pGL2 luciferase reporter vector (Promega, Madison, WI).

Electrophoresis and Western Analyses. SDS-PAGE, Western blotting, and enhanced chemiluminescence detection were performed as described previously (17, 18). In most cases, 25 μg of whole cell lysate/lane was used. To detect secreted, epitope-tagged *CCL6*, 32D cells were maintained for 24 h in tissue culture medium lacking serum. The supernatant was then concentrated 10-fold using PAGE-Perfect (Geno Technology, Inc., St. Louis, MO) before SDS-PAGE.

ChIP Experiments. These were performed essentially as described (20) except that Staph A cells were replaced by Protein A-Agarose (Affigel; Bio-Rad, Hercules, CA). Isolated chromatin from ~6×10^6 cell equivalents was used for each point. To precipitate sheared, formaldehyde cross-linked chromatin, antibodies were added to a final dilution of 1:500. One-tenth of the final precipitated DNA (2 μl) was used in each PCR reaction. PCR primers were synthesized to allow amplification of a 353-bp region from the murine *CCL6* promoter (nt −1416 to −1063) that contained all six of the E-box elements depicted in Fig. 2*A*. The PCR primer sequences were: forward, 5′-GGG CAT GAA CTG GGC ACT GCT CCA-3′ and reverse, 5′-AAC TGC CAG GAA CTC CAG CTG CAG-3′. Other PCR primers were designed to amplify a 301-bp fragment from the 3′-untranslated region of the *CCL6* gene (GenBank accession no. AB051897), located ~6 kb downstream of the promoter (forward 5′-GGC AGG CAT TGT CAC CCA CTT TCT-3′ and reverse 5′-CAT CTC AGC TAG ATA TTA CCC GGC-3′); a 295-bp segment flanking both E-box elements located in the first intron of the murine *ODC* gene (GenBank accession no. JO3733.1: forward 5′-CAG CCA GCA GCT CGG CGC CAC CT-3′ and reverse CGA GGT CCA GGA GCA GCT GCC TTC-3′); and a 308-bp segment spanning nt 5252–5560 of the murine *junB* promoter (GenBank accession no. U20735: forward 5′-GTC GCC AGG CCA GCG TAG GAG G-3′ and reverse 5′-CTG TTC CAT TTT CGT GCA CAT CCG-3′). In addition to the above input PCR primer pairs, PCR reactions (100 μl) contained 1× PCR buffer (Promega), variable concentrations of $MgCl_2$, 100 μM of each deoxynucleoside triphosphate, and 2.5 units of Taq DNA polymerase (Promega). A total of 40 cycles were performed in a DNA Thermal Cycler 480 (Perkin-Elmer, Inc., Foster City, CA). The resultant amplified fragments were then resolved by 2% agarose gel electrophoresis. Confirmation that they corresponded to the regions of interest was obtained by hybridization of the blotted fragments with an end-labeled oligonucleotide synthesized to an internal sequence or by restriction digestion with enzymes recognizing internal sites.

TUNEL and Luciferase Assays. These were performed as described previously (14).

RESULTS

Differential Expression of *CCL6*. To identify transcripts that were differentially regulated by c-Myc and L-Myc, we compared the DNA microarray profiles of 32D-c-Myc and 32D-L-Myc cells. These cell lines were derived from the IL-3-dependent 32D murine myeloid cell line after stable transfection with c-Myc or L-Myc expression vectors. Pooled transfectants expressed high levels of the respective oncoprotein and showed similar rates of accelerated apoptosis in response to IL-3 withdrawal (17).

Most transcripts that are differentially expressed between 32D-c-Myc and control cells (10) did not register in the current comparison between 32D-c-Myc and 32D-L-Myc cells, thus indicating similar transcriptional regulation by the two oncoproteins (data not shown). One exception was that of the CC chemokine *CCL6*, which showed a 7.5-fold greater relative up-regulation in 32D-L-Myc cells.

To confirm the differential expression of *CCL6* detected by microarray analysis, we performed Northern analysis on RNAs extracted from 32D-c-Myc or 32D-L-Myc cells, either during logarithmic growth, or after a 12-h period of IL-3 deprivation. RNAs from control 32D cells, transfected with the empty parental vector (32D-neo cells), were also included in the analysis. As seen in Fig. 1*A*, *CCL6* transcripts were detected only in 32D-L-Myc cells, thus confirming the results of the DNA microarray analysis. Two distinct transcripts were noted, which may reflect the utilization of alternate consensus polyadenylation signals at positions 998 and 1308 of the murine cDNA sequence in the GenBank database (accession no. NH009139). The withdrawal of IL-3 had no discernible effect on the abundance of *CCL6* transcripts.

The failure to detect *CCL6* transcripts in 32D-neo and 32D-c-Myc cells could indicate that c-Myc either lacked the ability to up-regulate the *CCL6* gene or that that the gene was actively suppressed by c-Myc. To distinguish between these possibilities, we performed Northern analyses on RNAs extracted from 32D cells stably expressing c-Myc proteins with defined deletions in the TAD (10). These included MBI (amino acids 43–69), MBII (amino acids 131–147),

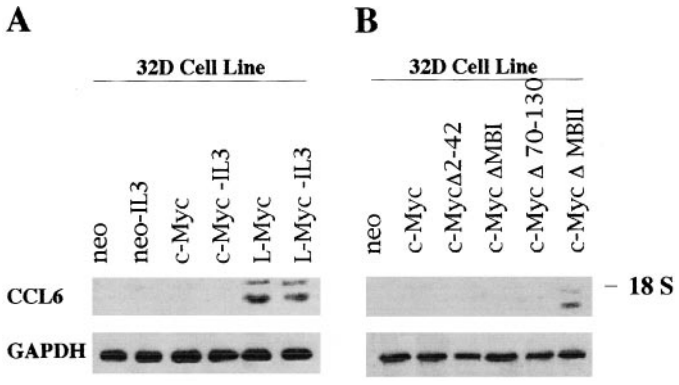

Fig. 1. Northern analysis of *CCL6* expression in 32D cell lines. *A*, each of the indicated cell lines was harvested during logarithmic growth in the presence of IL-3 or after 12 h in the absence of the cytokine (−IL-3). Total RNAs were then isolated, and 5 μg of each was used for Northern blotting as described previously (10). After hybridization to a murine *CCL6* cDNA probe (*top panel*), the blot was stripped and rehybridized with a *GAPDH* probe to control for RNA loading (*bottom panel*). *B*, Northern analysis of RNAs extracted from 32D cell lines expressing various TAD deletion mutants of c-Myc (10). Total RNAs were extracted from logarithmically growing cells and hybridized with the *CCL6* probe described above (*top panel*). The blot was then stripped and rehybridized with the *GAPDH* probe (*bottom panel*).

and the less conserved inter-Myc Box regions comprising amino acids 2–42 and 70–130. We have shown previously that these mutant proteins are expressed at levels equivalent to that of full-length, wild-type c-Myc, and that each of these regions is responsible for controlling the expression of distinct subsets of c-Myc-responsive genes in 32D cells (10). As seen in Fig. 1*B*, cells expressing the c-MycΔMBII mutant showed a marked up-regulation of CCL-6. Taken together with the data presented in Fig. 1*A*, these results indicate that the expression of *CCL6* is markedly up-regulated by L-Myc in 32D cells, that the chemokine in actively suppressed by wild-type c-Myc, and that the MBII domain plays an important role in this suppression.

The *CCL6* Gene Is a Direct Transcriptional Target for L-Myc. To determine whether the *CCL6* gene was a direct transcriptional target for L-Myc and c-Myc, we first sequenced ~2.0 kb of the murine *CCL6* promoter. Examination of this region showed that it contained six variant E-box elements (CATGTG; Ref. 3) located between −1130 and −1380 relative to the presumed start of mRNA transcription, with five of the sites residing within a 110 nt span between −1130 and −1220 (Fig. 2*A*). To determine the responsiveness of the promoter to various c-Myc and L-Myc proteins, the region between −1425 and +50 nt was amplified and cloned into the pGL2-Puro luciferase reporter plasmid. The vector was then linearized in its backbone and cotransfected along with pCMV-β-gal into each of the 32D cell lines described above. After selection in puromycin, the stably transfected pooled clones were assayed for expression of luciferase and β-galactosidase, with the latter being used to control for differences in transfection efficiencies (<two-fold in all of the cases). As seen in Fig. 2*B*, significant increases in luciferase activity relative to those of control 32D-neo cells were seen only in 32D-L-Myc and 32D-c-MycΔMBII cells.

To determine whether the effects of L-Myc and c-MycΔMBII on the *CCL6* promoter were direct, we performed ChIP studies on several of the above cell lines (20). In these experiments, *in vivo* cross-linking of DNA-bound protein was achieved by the direct addition of formaldehyde to cells suspended in tissue culture medium. Whole chromatin was then isolated from the cells, sheared to an average size of 300–500 bp, and immunoprecipitated by the addition of anti-c-Myc, anti-L-Myc, or control antibodies. After the reversal of cross-links, the precipitated DNAs were initially used as PCR templates with primers that spanned the E-box-containing *CCL6* promoter region. Analysis of the PCR products (Fig. 2*C, top panel*) revealed several features. First, the *CCL6* promoter region was not amplified from any of the cell lines after control chromatin precipitation performed in the absence of antibody or with a nonspecific antibody. Second, the promoter region was amplified from 32D-neo, 32D-c-Myc, and 32D-c-MycΔMBII-derived chromatin precipitated with anti-c-Myc antibody but not from the same cells precipitated with anti-L-myc antibody. In several independent experiments, the intensity of these latter two bands ranged between 1.6 and 3 times that of the former. This was in good agreement with the Western blots shown in Fig. 1*C* and our previous reported results (10) indicating that 32D-c-Myc and 32D-c-MycMBII cells contained about three times as much c-Myc protein and control 32D-neo cells. Third, after ChIP with anti-L-Myc antibody, the promoter region was amplified only from 32D-L-Myc cells. Finally, the promoter region was not amplified from 32D-L-Myc-derived chromatin after immunoprecipitation with anti-c-Myc antibody. Taken together with the findings presented in Fig. 1 and Fig. 2*A*, these findings suggest that the *CCL6* promoter, while capable of binding either endogenous or overexpressed c-Myc, remains unresponsive to the oncoprotein unless its repression is eliminated by the removal of MBII. The inability to detect bound c-Myc protein in 32D-L-Myc cells additionally suggests that c-Myc and L-Myc proteins compete for the same binding site(s) in the *CCL6* promoter, but that the latter, when expressed at high levels, can displace the former. This is consistent with the finding that endogenous c-Myc protein can still be detected in 32D-L-Myc cells.[4] Therefore, the absence of *CCL6* promoter-bound c-Myc is not because of a silencing of the endogenous c-Myc gene and the subsequent decay of c-Myc.

Various control ChIP experiments were also performed (Fig. 2, *C* and *D*). In these experiments, and using the same chromatin precipitates, we were unable to amplify a region from the 3′-untranslated region of the *CCL6* gene, which resides ~6 kb downstream of the promoter region and contains no known E-box elements. In contrast, The precipitation pattern for the first intron of the ornithine decarboxylase gene, which contains two c-Myc-responsive E-box elements (21), mimicked that of the *CCL6* promoter. In other experiments, none of the anti-Myc antibodies was able to precipitate chromatin containing the promoter region of the *junB* gene, which is not known to be a Myc target gene. Finally (Fig. 2*D*), an anti-*Max* antibody (22) was able to precipitate the *CCL6* promoter region from all of the cell lines, consistent with the notion that all of the Myc proteins bind to their cognate sites as Myc-Max heterodimers.

Finally, to demonstrate that c-Myc and L-Myc transcriptionally compete for the *CCL6* promoter, we performed reciprocal competitive transient transfection experiments. In the first, 32D-L-Myc cells were transfected with a constant amount of the *CCL6* promoter-luciferase reporter vector described above and increasing amounts of a c-Myc expression vector. As seen in Fig. 2*E* (*right panel*), luciferase activity decreased as a function of c-Myc input. In the second study, 32D-c-Myc cells were transiently transfected with a constant amount of *CCL6*-luciferase reporter and increasing amounts of an L-Myc expression vector. As seen in the *left* panel this resulted in a dose-dependent enhancement of luciferase activity. Both sets of results are consistent with the idea that each of the transiently expressed Myc proteins competes with its stably expressed relative for regulation of the *CCL6* promoter.

***CCL6* Cooperates with c-Myc to Promote the Survival of 32D Cells.** To gain a better understanding for the function of *CCL6*, it was stably expressed as an epitope-tagged fusion protein in several of the above 32D cell lines. Pooled clones were then examined for both cell-associated and secreted *CCL6*. As seen in Fig. 3, *A* and *B*, all of the cell lines transfected with the *CCL6* expression vector expressed and secreted high levels of the chemokine. *CCL6* was not detected in cells or supernatants derived from empty vector control transfectants. Each of the c-Myc proteins was also present in these cells at levels comparable to those as described previously by us (Fig. 3*C;* Ref. 10).

Because some chemokines have been reported to affect the survival of certain cell types (23), we assessed the ability of each of the above 32D cell lines to survive and proliferate in the absence of IL-3. As seen in Fig. 3*C*, neither the enforced expression of *CCL6* alone, nor the combination of L-Myc+*CCL6* nor of c-MycΔMBII+*CCL6* was sufficient to abrogate IL-3 dependence. Indeed, as in the case of control 32D cell, all of these lines succumbed to apoptotic death within 72 h of removal of the cytokine. However, the combination of c-Myc+*CCL6* not only prevented apoptotic death, but allowed for continued cell proliferation after IL-3 withdrawal. The 32D-c-Myc+*CCL6* cells doubled approximately five times in the course of the first 4 days of the study, thus indicating that the assumption of IL-3-independent growth was immediate and thus a property of all of the cells in the culture. However, in the presence of IL-3, these cells did not proliferate any more quickly than did control cells (data not shown). Thus, we conclude that *CCL6* not only overcomes the potent

[4] F. Y. and E. V. P., unpublished observations.

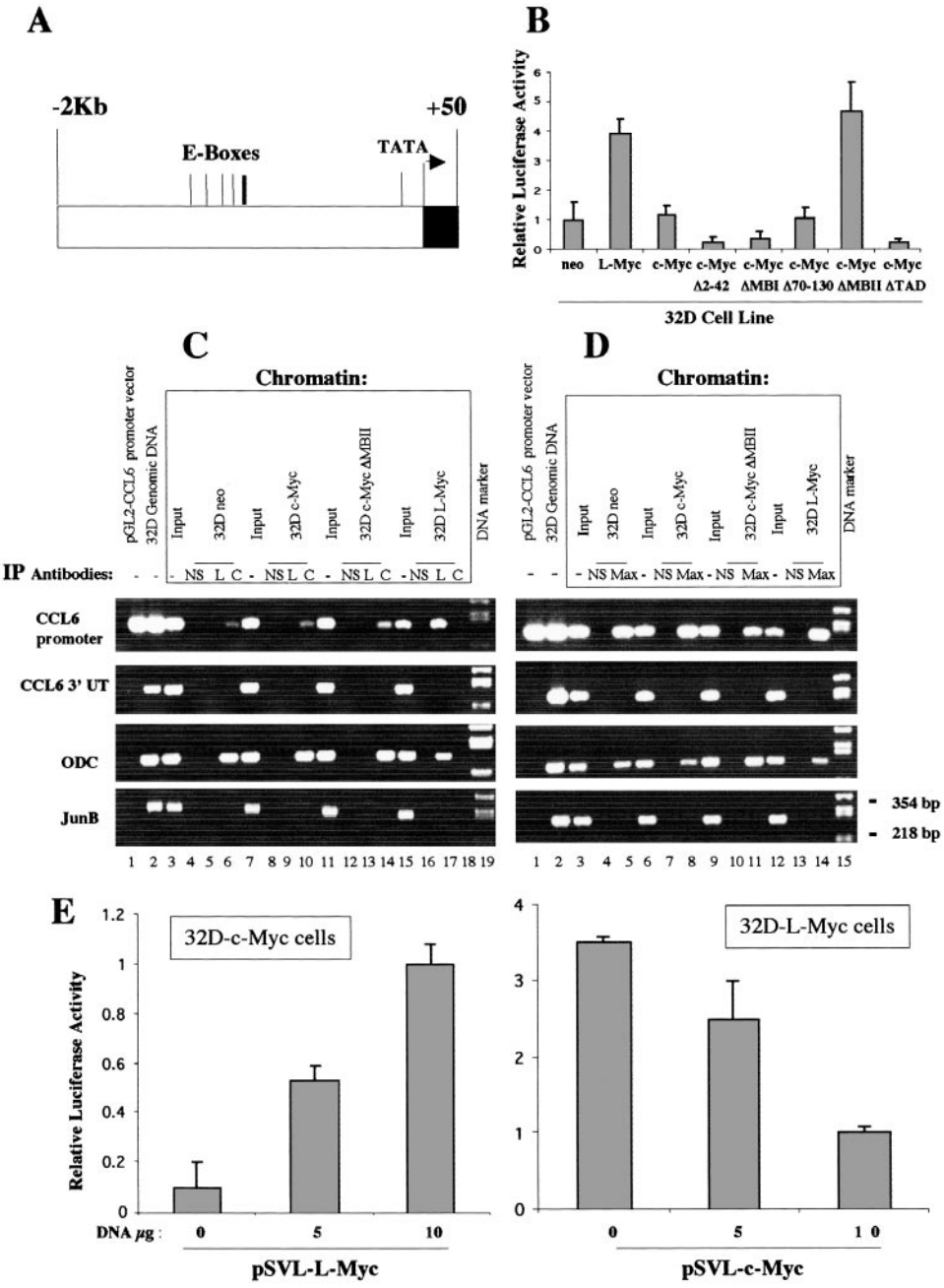

Fig. 2. Characterization of the murine *CCL6* promoter. *A*, diagrammatic representation of the promoter between −2.0 kb and +50 bp relative to the presumed start of transcription initiation. □, the 5′-flanking region; ■, the 5′-untranslated region. A consensus TATA element, located at position −31 nt relative to the start of transcription (*arrow*) is indicated. The positions of six consensus E-box elements, all of the sequence CATGTG, are indicated by *hash marks*. *B*, results of luciferase assays. The region between −1425 and +50 of the *CCL6* promoter was amplified by PCR and cloned into the pGL2-puro luciferase reporter vector. After stable cotransfection of this and a pCMVβgal control vector, luciferase assays were performed on pooled clones as described previously (14). Assay results were adjusted for differences in β-galactosidase activities among the various cell lines (<two-fold). The results shown represent the averages of triplicate assays; *bars*, ±1 SE. *C*, chromatin immunoprecipitation assays. Purified chromatin from each of the indicated sources was cross-linked with formaldehyde, sheared, and then immunoprecipitated with the indicated antibodies [*NS*, nonspecific (*Lanes 4, 8, 12,* and *16*); L, anti-L-Myc (*Lanes 5, 9, 13,* and *17*); and *C*, anti-c-Myc (*Lanes 6, 10, 14,* and *18*)]. After reversal of the cross-links, the *CCL6* promoter between nt −1163 and −1416 was amplified by PCR (40 cycles). The PCR products were resolved on a 2% agarose gel. Control PCR reactions in the *top panel* included DNAs derived from the pGL2-*CCL6* promoter-luciferase plasmid described in *B* (*Lane 1*), 32D-neo total genomic DNA (*Lane 2*), and total input chromatin, before immunoprecipitation, derived from each of the indicated cell lines (*Lanes 3, 7, 11,* and *15*). In the *second panel*, each of the above samples was amplified with primers designed against the 3′-untranslated region of the *CCL6* gene as a control for shear size. In the *third panel*, samples were amplified with primers spanning the two E-boxes present in the first intron of the *ODC* gene as a positive control for Myc protein binding, and in the *last panel*, samples were amplified with primers to the *junB* promoter, which was used as a control for a sequence that does not bind c-Myc. Confirmation that each amplified segment actually corresponded to the stated region was obtained by showing that it hybridized with an end-labeled oligonucleotide homologous to an internal region of the fragment or contained predicted restriction enzyme sites (data not shown). *D*, immunoprecipitation using either a nonspecific antibody (*NS: Lanes 3, 6, 9,* and *12*) or anti-Max antibody (*Max: Lanes 4, 7, 10,* and *13*). Input chromatin samples before immunoprecipitation (*Lanes 2, 5, 8,* and *11*) were also included. PCR primers are as in Fig. 2*C*. *E*, competitive transient transfections experiments. In the *left panel*, 32D-c-Myc cells were transiently transfected by electroporation with 2 μg of the above-described *CCL6* promoter-luciferase, the indicated amount of the pSVL-L-Myc expression vector, and sufficient pSVLneo vector DNA to provide a final total DNA amount of 13 μg. Two days later, equivalent numbers of cells were harvested, normalized for β-galactosidase, and assayed for luciferase activity. The results shown represent the average of triplicate determinations; *bars*, ±1 SE, and are expressed relative to the amount of luciferase in the absence of cotransfected pSVL-c-Myc (0 μg DNA). Note that luciferase activity increased as input pSVL-L-Myc DNA increased. In the *right panel*, the reciprocal experiment was performed in 32D-L-Myc cells. In contrast to the previous experiment, increasing the amount of input pSVL-c-Myc DNA resulted in decreased activity of the *CCL6* promoter vector.

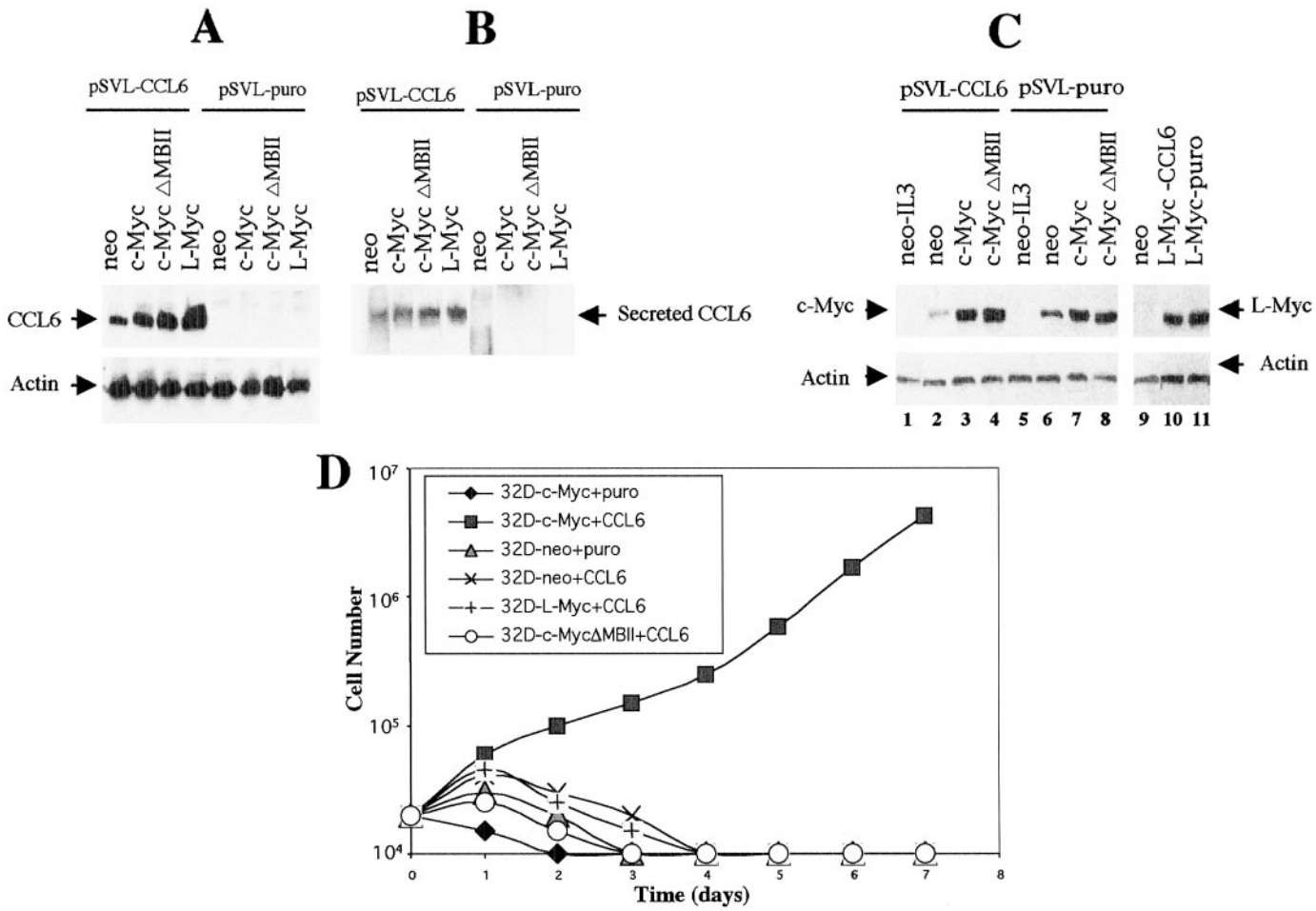

Fig. 3. Expression of *CCL6*. Each of the indicated 32D cell lines (10, 17) was transfected either with an epitope-tagged *CCL6* expression vector or with the empty pSVL-puro vector alone. *A*, after selection of stable transfectants, pooled clones were washed several times in PBS, lysed, and examined by SDS-PAGE, and Western blotted for the expression of cell-associated *CCL6* protein (*top panel*). The *bottom panel* shows the same blot probed with an antiactin antibody as a control for protein loading. *B*, secretion of epitope-tagged *CCL6* into the tissue culture medium. The above cell lines were plated into fresh medium at 5×10^5 cells/ml and then maintained for the next 48 h. Filtered medium was concentrated and subjected to Western blotting to detect *CCL6* protein. *C*, *CCL-6* transfected cells express Myc proteins at levels comparable with those of control cells. *Top panel*, lysates from each of the cell lines shown in *B* were resolved by SDS-PAGE, blotted, and then incubated with anti c-Myc (*Lanes 1–8*) or anti L-Myc (*Lanes 9–11*) antibodies. Lysates in *Lanes 1* and *5* were prepared from 32D-neo cells that had been deprived of IL-3 for 16 h so as to deplete endogenous c-Myc protein. *Lanes 2* and *6* show the levels of endogenous c-Myc protein in lysates from proliferating 32D-neo cells grown in IL-3-containing medium. All other *lane*s contained lysates obtained from IL-3-deprived cells to allow the detection of ectopically expressed c-Myc or L-Myc proteins in the absence of endogenous c-Myc protein. *Bottom panel*, the same blots probed with an antiactin antibody as a control for protein loading. *D*, coexpression of c-Myc and *CCL6* promotes IL-3-independent growth of 32D cells. The indicated cell lines were maintained at >90% viability in IL-3-containing medium. At the start of the experiment, the cells were washed three times in IL-3-free culture medium and then resuspended at 2×10^4 cells/ml in IL-3-free RPMI 1640 containing 10% FBS. The total number of viable cells was determined manually on a daily basis. This experiment was repeated twice more with independently transfected populations of cells and produced very similar results (data not shown).

c-Myc-mediated proapoptotic phenotype associated with growth factor removal (24), but actually cooperates with c-Myc to impart a new behavior to the cells, namely that of growth factor (IL-3) -independent proliferation.

***CCL6* Promotes Apoptosis in Some Cell Types.** Despite the ability of *CCL6* to impart IL-3-independent growth of 32D-c-Myc cells, we encountered considerable difficulty in establishing stable *CCL6*-expressing clones of certain other cell types. This suggested that *CCL6* might exert pleiotropic effects and that in some cells it might actually induce apoptosis. To test this, we either cocultivated various known tumorigenic and nontumorigenic cell lines with *CCL6*-overexpressing 32D cells or grew them in *CCL6*-conditioned medium. As seen in Fig. 4, *A* and *B*, several of the cell lines tested showed enhanced apoptosis in the presence of *CCL6* as confirmed by trypan blue staining and TUNEL assay. Although our survey was not exhaustive, it suggested that nontransformed cells are more sensitive to the proapoptotic effects of *CCL6* than are transformed cells. However, exceptions were seen in both cases.

Tumorigenic Capacity of *CCL6*-overexpressing Cell Lines. The IL-3 independence of 32D-c-Myc+*CCL6* cells (Fig. 3) prompted us to investigate their *in vivo* survival. At the same time, we wished to study the consequences of enforced *CCL6* overexpression on the behavior of known tumorigenic cell lines. Therefore, based on the apoptotic profiles of the cell lines depicted in Fig. 4*A*, demonstrating that A549 lung cancer cells and HeLa cells were resistant to the proapoptotic effects of *CCL6*, we established several *CCL6*-overexpressing clones from each line. Control clones were also established from cells transfected with the empty parental vector. As seen in Fig. 5*A*, several of the clones examined from each of the former transfections expressed and secreted high levels of *CCL6*. Under standard culture conditions, all of the *CCL6*-overexpressing clones grew at rates that were indistinguishable from those of their vector control counterparts (data not shown).

One clone of each of the above cell types, as well as 32D cells described previously, were next inoculated s.c. into nude mice, which were then monitored weekly for tumor development. Of the various

32D cell lines, only 32D-c-Myc+*CCL6* cells were tumorigenic (Fig. 5*B*). These formed readily detectable tumors within 2–3 weeks and grew rapidly over the ensuing 3–4 weeks. Similarly, A549-*CCL6* cells grew at a much faster rate than their control A549-puro counterparts (Fig. 5*C*). In the former case, tumors first became detectable within 4–5 weeks of inoculation, compared with 6–7 weeks for A549-puro tumors. In contrast to our findings with 32D and A549 cells, the growth rates of HeLa-*CCL6* tumors were virtually indistinguishable from those of control HeLa-puro tumors (Fig. 5*D*). We have subsequently obtained similar results in smaller groups of animals with two additional A549-*CCL6* clones and one additional HeLa-*CCL6* clone (data not shown).

Histological examination of tumors derived from 32D-c-Myc+*CCL6* cells (Fig. 6, *A* and *B*), showed them to be comprised almost exclusively of immature myeloid elements that could readily be distinguished from neighboring cells by their strong myeloperoxidase positivity (Fig. 6*C*). These tumors readily breached the surrounding capsule and invaded adjacent normal structures such as s.c. fat and muscle. Distant metastases were also identified, and involved lung, liver, heart, and kidney. In some animals, complete replacement of the bone marrow was observed. Cells derived from primary tumors retained their IL-3 independence when recultured *in vitro* and expressed *CCL6* protein at levels comparable with those observed before *in vivo* proagation (data not shown).

Given the proapoptotic effects of *CCL6* on certain cell types observed previously, we also performed TUNEL assays on some of the tissue sections taken from the above animals. Considerable numbers of apoptotic cells were seen adjacent to the invading tumor cells in most samples (Fig. 6*D*).

Histological examination of tumors derived from A549-*CCL6* cells also showed dramatic differences from A549-puro cell-derived tumors of comparable size. Whereas the latter nearly always remained confined to their capsules or pseudocapsules, the former aggressively invaded surrounding tissues (Fig. 6*E*). Indeed, in many cases, it was difficult or impossible to discern a capsule. Metastases to liver, lung, and spleen were also observed frequently (Fig. 6, *F* and *G*). TUNEL assays revealed the presence of robust apoptosis in tissues near the periphery of the infiltrating tumors and within the tumor capsules (Fig. 6*H*). In contrast, TUNEL assays performed on A549-puro tumor-bearing animals showed a notable lack of apoptosis in the tumor capsule or adjacent tissues (Fig. 6*N*).

Although the expression of *CCL6* did not influence the actual growth rates of HeLa cell-derived tumors (Fig. 5*D*), microscopic examination nonetheless provided evidence for their more aggressive behavior. Whereas neoplasms originating from HeLa-puro clones did infiltrate adjacent tissue, they seldom showed evidence of distant metastatic spread, and little evidence for apoptosis of adjacent normal tissues was observed (Fig. 6, *O* and *P*). In contrast, HeLa-*CCL6*-derived tumors showed a much more aggressive pattern of local tissue invasion and distal metastases (Fig. 6, *I–K*). In virtually all of the cases, this was accompanied by a marked apoptotic response of normal tissues adjacent to the tumor edges (Fig. 6*L*).

Taken together, our results indicate that *CCL6* can cause tumorigenic conversion of otherwise growth factor-dependent and nontumorigenic cells, and can greatly augment the *in vivo* growth rates and/or invasive/metastatic capabilities of other tumor cell lines established previously. In each of these cases, this is accompanied by a marked apoptotic reaction of normal cells in the path of the advancing tumor.

Expression of *CCL6* by SCLC Cell Lines. Human SCLC is the only known human neoplasm associated consistently with amplification of the L-MYC locus, which occurs in 10–15% of such cases (1). In some instances, this is associated with a coamplification of *C-MYC* and/or *N-MYC* (1). We examined the expression of each of these genes, as well as *CCL6* in 8 SCLC cell lines, some of which had been determined previously to overexpress L-Myc.[5] As seen in Fig. 7, 4 of the lines (N691, H510, H1836, and H889) expressed high levels of L-Myc transcripts, 2 (H378B and H1694) expressed considerably lower levels, and 2 (H298 and H345) expressed an undetectable amount. High levels of c-Myc expression were seen in 3 cases (H510, H298, and H378B), lower levels in a fourth (H345), and barely detectable, basal levels in the remainder. Finally, a single cell line (H345) expressed fairly high levels of N-Myc.

Rehybridization of these blots with the murine *CCL-6* probe showed high level expression of the chemokine in one cell line (N691). Interestingly, this was one of the two lines that did not also express c-Myc or N-Myc, and of these two, it expressed higher levels of L-Myc. These results indicate that *CCL6* expression may be de-

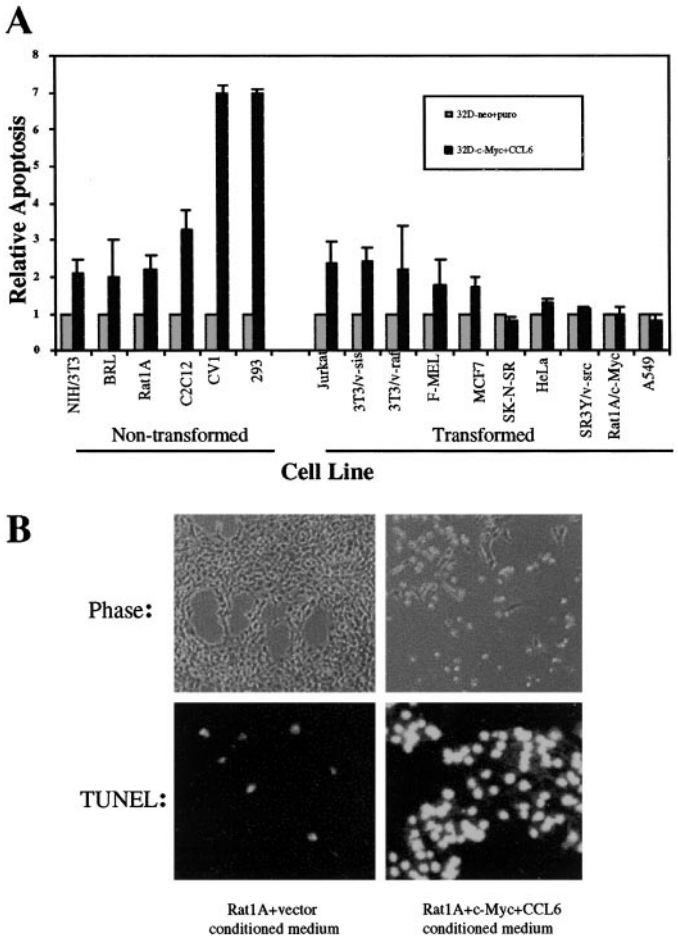

Fig. 4. *A*, *CCL6* promotes apoptosis in some cell lines. The indicated target cell lines were maintained in log phase growth at the bottom of a *trans*-well plate. In the upper chamber of the plate, 32D-c-Myc+*CCL6* or 32D-neo+puro cells were seeded at a density of 10^6 cells/ml. All cells were then maintained in RPMI 1640 containing 1% FBS for the next 48 h at which time cell viability in the lower chambers was determined using trypan blue exclusion and confirmed by TUNEL assay. Relative apoptosis was expressed as a ratio of the percentage of apoptotic cells after cocultivation with 32D-c-Myc+*CCL6* cells *versus* 32D-neo+puro cells. In several repeat experiments, the range of apoptosis in the target populations cocultivated with 32D-neo+puro cells ranged from 3 to 10%; *bars*, ±SD. *B*, *CCL6*-containing conditioned medium promotes apoptosis in Rat1a fibroblasts. Rat1a cells were maintained in log-phase growth in the presence of 10% FBS. At the initiation of the experiment all cells were >90% viable. Serum concentrations were then reduced to 1%. One-tenth volume of conditioned medium from control 32D-neo+puro cells or from 32D-c-Myc+*CCL6* cells was then added. The lines were maintained for an additional 3 days at which time phase contrast microscopy (*top panels*) and TUNEL assays (*bottom panels*) were performed (magnification ×200).

[5] M. Birrer, personal communication.

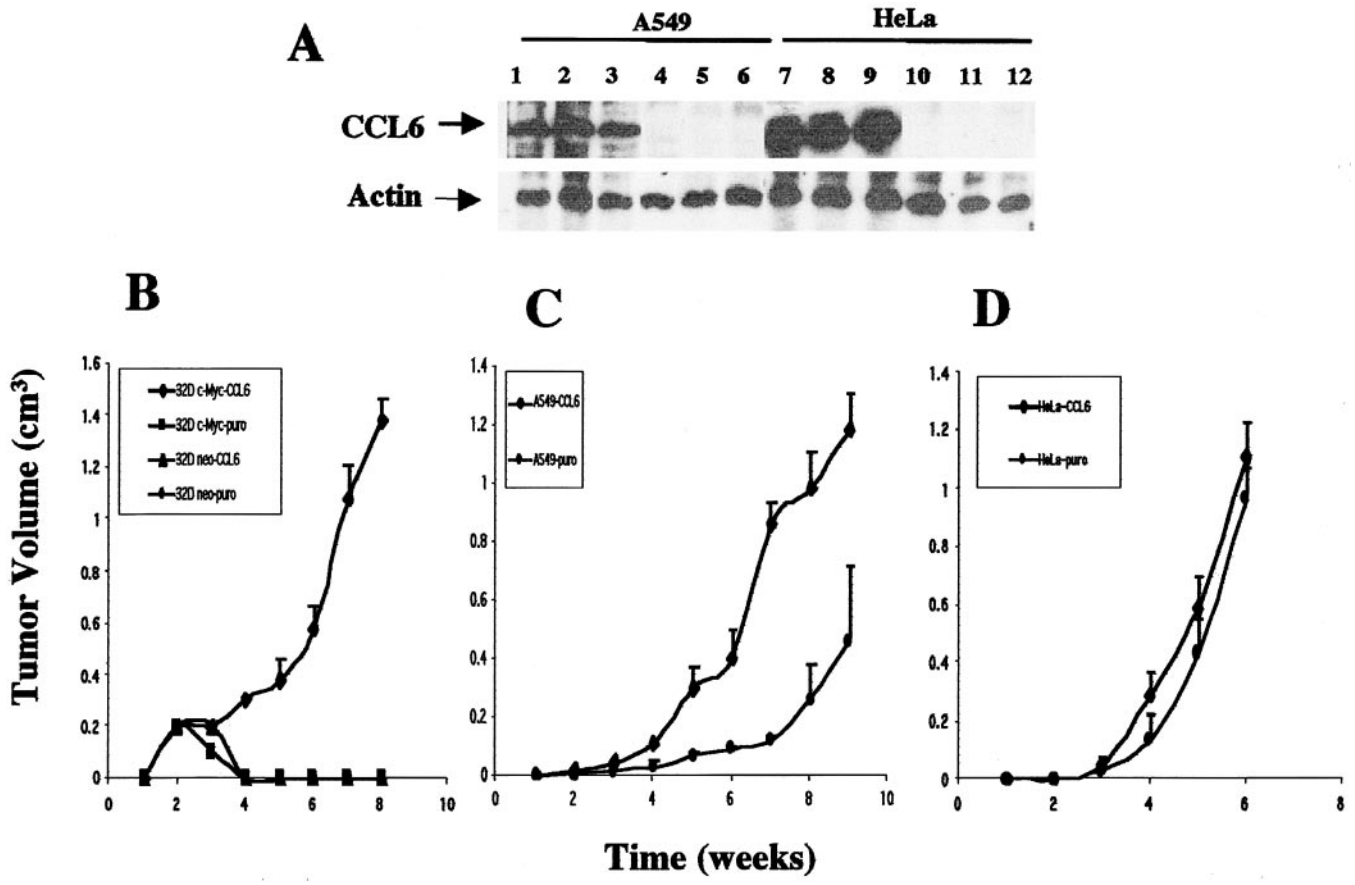

Fig. 5. *A*, expression of *CCL6* by human A549 lung cancer cells or HeLa cells. Single clones of each cell type were selected in puromycin-containing medium after stable transfection with either the pSVL-*CCL6* expression vector *(Lanes 1–3* and *7–9*) or with the empty parental vector *(Lanes 4–6* and *10–12*). Each clone was then examined by Western blotting for the expression of *CCL6* (*top panel*) or actin (*bottom panel*) as described in the Legend to Fig. 3*A*. *B*, c-Myc and *CCL6* cooperate to promoter tumorigenic conversion of 32D cells. Equal numbers of 32D-neo+puro, 32D-c-Myc+puro, 32D-neo+*CCL6*, and 32D-c-Myc+*CCL6* cells (Fig. 3) were inoculated s.c. into individual groups of five mice. Animals were then monitored weekly for the appearance of tumors. *C*, A549-puro or A549-*CCL6* cells were inoculated s.c. into five nude mice each. Animals were monitored weekly for the appearance of tumors. *D*, HeLa-puro and HeLa-*CCL6* were each inoculated into nude mice as described for *B*. All tumor volumes were calculated weekly. Each of the A549-*CCL6* and HeLa-*CCL6* clones showed similar rates of tumorigenesis; *bars*, ±SD.

regulated in SCLC cell lines in association with L-MYC gene amplification and overexpression, but that this is not always the case.

DISCUSSION

One of the questions concerning the *MYC* oncogene family is why its members have been evolutionarily conserved. Many studies have supported the general idea that the expression of c-Myc, N-Myc, and L-Myc is differentially regulated, both at the tissue level and during ontogeny (1, 25). Their association with specific tumor types (1) additionally suggests highly specialized roles for each, as does the finding that homozygous deletion of either c-Myc or N-Myc results in embryonic lethality (26, 27), whereas deletion of L-Myc does not (28). Despite the evidence suggesting differential functions of these genes, little information has emerged regarding the molecular basis for this.

Chemokines are a large group of low molecular weight chemotactic cytokines that were originally identified as mediators of leukocyte trafficking (23, 29–31). More recently, they have been shown to exert a variety of effects on cellular proliferation, apoptosis, and neoplastic cell migration. With regard to the latter, it has been shown that breast cancer cells frequently express the chemokine receptors CXCR4 and CCR7 on their surfaces, and that their predilection to metastasize to liver, lung, and bone reflects the expression of specific chemokines by these organs (32). Similarly, infiltration of lymph nodes by CLL cells, a prognostically significant feature of the disease, may be associated with expression of CCR7 by the CLL cells. This allows their migration through high endothelial venules of the lymph nodes, which express the CCR7 ligands CCL19 and CCL21 (33). In several experimental model systems, enforced expression of individual chemokines has been reported to either enhance or inhibit the metastatic and/or invasive properties of tumors (reviewed in Refs. 29, 30).

In the current work, we have identified *CCL6*, a member of the CC or β chemokine family, as a specific and direct positive target for L-Myc. Thus, *CCL6* represents the first reported target for a Myc oncoprotein that is not similarly regulated by other members of the family. Indeed, our data are compatible with a model in which c-Myc, rather than exerting no effect on the *CCL6* gene, actually represses it. The basis for this conclusion rests on several findings. First, overexpression of c-MycΔMBII is associated with an up-regulation of *CCL6* comparable with that seen with L-Myc. This suggests that c-MycΔMBII behaves in a dominant-negative fashion. Second, ChIP experiments indicate that endogenous c-Myc, as well as ectopically expressed c-Myc and L-Myc occupy the same region(s) of the *CCL6* promoter. Third, overexpression of L-Myc results in the displacement of endogenous c-Myc from the *CCL6* promoter. The observation that the *CCL6* gene remains repressed after a prolonged period of IL-3 starvation, during which time endogenous c-Myc levels should be markedly reduced (10), suggests that other alterations of *CCL6*-

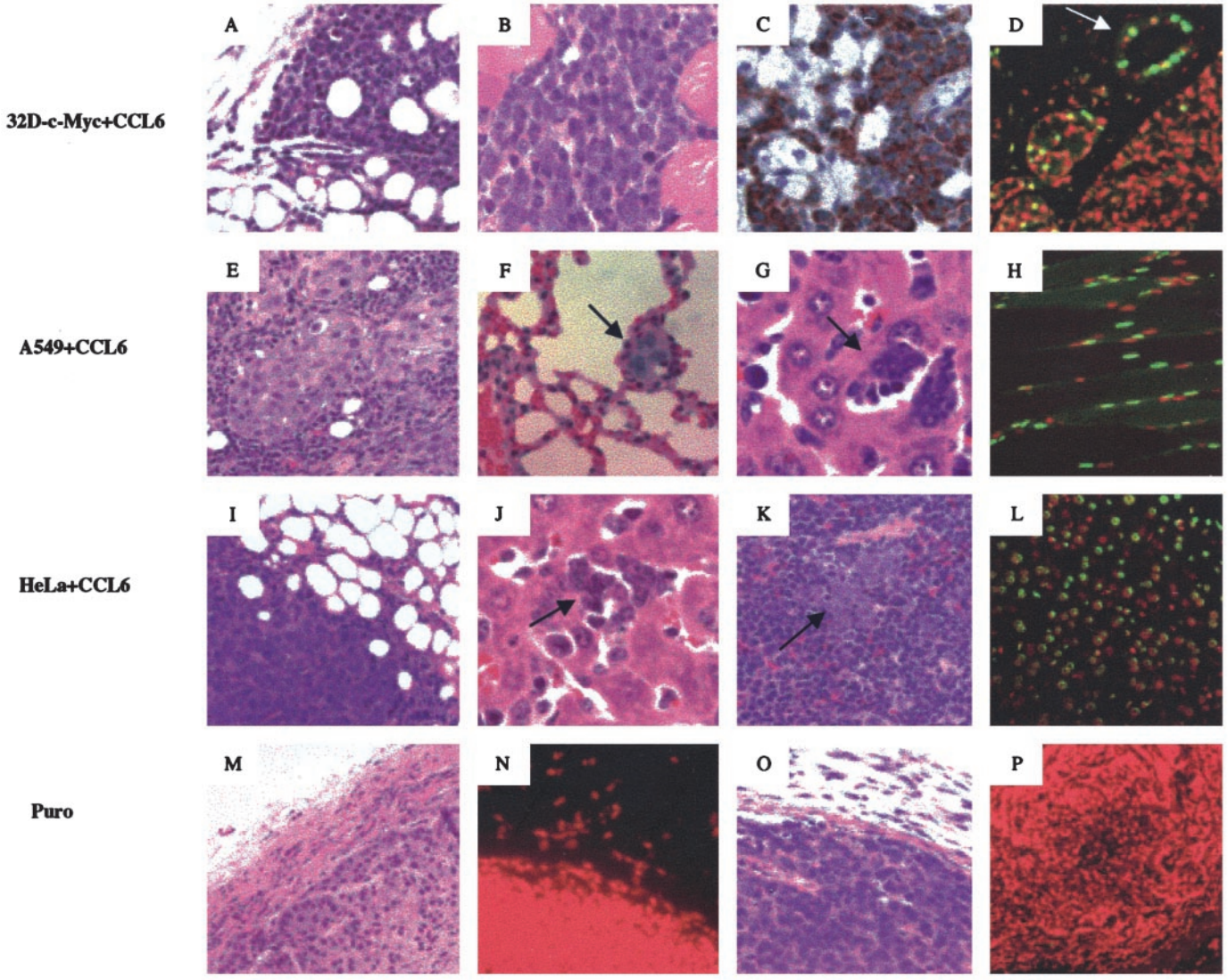

Fig. 6. Histological appearances of various *CCL6*-overexpressing tumors and relevant normal tissues. *First row,* tissue sections from animals bearing tumors derived from 32D-c-Myc+*CCL6* cells. *A,* H&E-stained tissue section demonstrating destruction of the tumor capsule/pseudocapsule and invasion of the surrounding s.c. tissue; *B,* H&E stain demonstrating invasion of muscle tissue by tumor cells; *C,* myeloperoxidase stain confirming the myeloid nature of tumor cells; *D,* TUNEL assay showing apoptosis (*green fluorescence*) of a glandular structure adjacent to infiltrating tumor tissue (*arrow*). *Second row,* tissue sections from animals bearing A549+*CCL6* tumors. *E,* H&E stain of tumor cells showing invasion of the capsule; *F* and *G,* H&E stains of lung and liver sections, respectively, showing invasion by tumor cells (*arrows*); *H,* TUNEL assay showing apoptosis in muscle tissue adjacent to infiltrating tumor. *Third row,* tissue sections from animals bearing HeLa+*CCL6* tumors. *I,* H&E stain of tumor cells showing invasion into the surrounding tissue with loss of the capsule/pseudocapsule; *J,* H&E stain of liver showing infiltration by tumor cells (*arrow*); *K,* spleen section showing widespread replacement of the normal architecture by tumor cells (*arrow*); *L,* TUNEL assay showing apoptosis in liver adjacent to infiltrating tumor cells. *Fourth row: M* and *N,* H&E stain of a tumor section from an animal bearing a tumor derived from A549+puro cells. *M,* note the minimal destruction of the tumor capsule; *N,* TUNEL assay performed on a section similar to that in *M* showing absence of apoptosis in tissue adjacent to the tumor; *O,* H&E stain of a tumor section derived from HeLa+puro cells. Although some invasion of the capsule is seen, no evidence for adjacent tissue apoptosis was noted (*P*).

associated chromatin are likely to be necessary for the activation of the gene.

The binding of c-Myc to its positively regulated target genes typically occurs at consensus E-box elements located within the promoter or first intron (6, 7). Negative regulation by c-Myc is much less well understood, although it appears more complex and until now has not been identified as being mediated by E-box binding (6). Rather, in several cases thus far described, it involves the interaction of c-Myc with non-E-box elements of the promoter, particularly initiator or NF-YB binding sites (6, 34, 35). Indeed, it is not completely clear that this regulation actually involves the direct binding of c-Myc to the gene (36–38). The work reported here suggests that E-boxes can, at least in the case of the *CCL6* gene, serve as either positive and negative elements, and that the ultimate mode of regulation depends on the nature and disposition of the bound Myc oncoprotein. However, we cannot rule out the possibility that c-Myc may be also be repressing the *CCL6* gene through other, non-E-box-dependent mechanisms.

The means by which c-Myc activates the transcription of its target genes include the recruitment of histone acetylase complexes, ATP-dependent chromatin remodeling, and promoter clearance (reviewed in Ref. 6). In the first case, considerable evidence indicates that MBII plays a crucial role in the interaction of c-Myc with TRRAP, a large protein that serves as a nucleation center for histone acetylases and their associated proteins (5, 39). On the other hand, proteins such as Bin1, MM-1, and pag have been shown to interact with the MBII region of c-Myc, and, in some cases, to serve as negative regulators of certain c-Myc target genes and biological functions (6, 40). Taken together, these observations suggest that the MBII region of c-Myc may be involved in both transcriptional activation and repression as

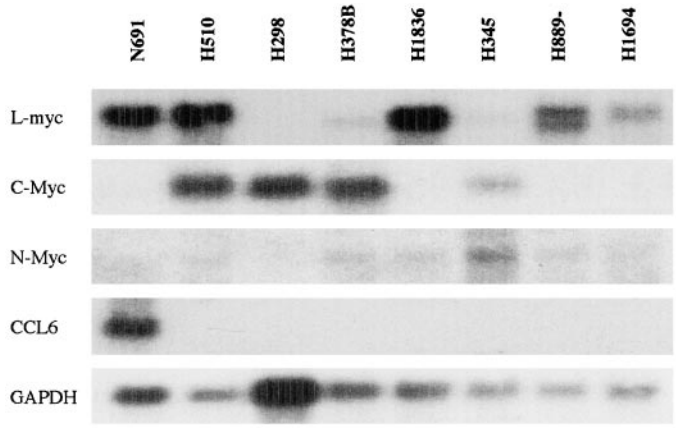

Fig. 7. Expression of Myc family members and *CCL6* in human SCLC cell lines. The indicated cell lines were harvested during late log-phase growth, and total RNAs were extracted. Northern blots were hybridized with coding region probes for each of the indicated human Myc family members or with the coding region for murine *CCL6*. The *bottom blot* was hybridized with GAPDH as a control for total RNA loading.

we have suggested previously (10). Which of these activities predominates may depend on the relative abundance of these various MBII-interacting proteins, tissue-specific factors, and non-MBII regions of the TAD. Despite the high degree of similarity between the MBII regions of c-Myc and L-Myc, they nonetheless manifest distinctly different functions and divergent sequences outside of MBI and MBII. Therefore, the differences between c-Myc and L-Myc on the *CCL6* gene may well involve regions of the TAD encompassing more than their MBII regions.

A surprising feature of *CCL6* is its ability to confer IL-3-independent growth and aggressive tumorigenic behavior to 32D cells. This appears to be a novel property of *CCL6*, which has been suggested previously to play roles in wound healing and cardiac allograft rejection (41, 42). However, whereas *CCL6* expression was necessary for these properties, it was not sufficient, being seen only when cells concurrently overexpressed c-Myc. Indeed, neither L-Myc nor c-MycΔMBII was able to substitute for c-Myc. Given that L-Myc is a much weaker transforming agent than c-Myc (15) and that the MBII domain is required for the transforming abilities of c-Myc (5, 39, 43), it is tempting to speculate that the ability of *CCL6* to cooperate with c-Myc to impart growth factor independence and alter tumor behavior is related in some way to its activity as a potent transforming oncoprotein.

We were unable to compare the tumorigenic behavior of 32D-c-Myc+*CCL6* cells to that of any other 32D cell line because only the former demonstrated IL-3 independence and autonomous tumor growth. However, the observation that *CCL6* expression altered the growth and/or invasive behaviors of two other already tumorigenic cell lines (Fig. 5 and Fig. 6) argues that the chemokine functions in ways other than to promote cell survival. Indeed, the ability of *CCL6* to induce apoptosis of certain cell types both *in vitro* and *in vivo* suggests that one of its major effects is to enhance tumor invasion by disrupting local tissue barriers. Overexpression of *CCL6* may also allow for cellular migration into organs that express the *CCL6* receptor, precisely the inverse effect that has been described for chemokine receptor-expressing mammary carcinoma and *CLL* cells (32, 33).

One of the best known attributes of c-Myc is its ability to induce apoptosis (24, 44–46). It has been argued that this may represent a cellular response that recognizes the impending neoplastic catastrophe arising as a consequence of c-Myc deregulation, and that eliminates such incipient transformed cells in favor of host survival (41). Numerous *in vitro* and *in vivo* studies have supported this model by demonstrating the dire consequences of suppressing these apoptotic pathways (46–50). However, additional cellular safeguards, which suppress the emergence of other properties essential to the transformed state, such as tissue invasion, have not been as intensively investigated. Given the strong synergy that exists between c-Myc and *CCL6* with regard to tumorigenesis and metastatic spread, it is tempting to speculate that the active suppression of *CCL6* in the face of c-Myc overexpression may represent an additional cellular mechanism to ensure against additional tumorigenic evolution, even after proapoptotic responses have been circumvented.

In conclusion, the studies reported here have identified the *CCL6* chemokine gene as a direct positive transcriptional target for L-Myc and a direct negative target for c-Myc. The opposite actions of these two oncoproteins appear to be the result of their binding to identical or closely adjacent E-box elements in the *CCL6* promoter. In turn, the level of positive or negative regulation appears to be determined by a balance between these two related Myc oncoproteins. An implication of these findings is that either of these proteins might by itself be capable of regulating *CCL6* expression provided that MBII interactions with various cofactors was permitted. The consequences of combined *CCL6* and c-Myc deregulation are to transform non-neoplastic cells to a tumorigenic and highly invasive phenotype, and to markedly enhance the tumorigenic behavior of cells transformed previously. These studies characterize for the first time differences in the transcriptional repertoire between c-Myc and L-Myc. In addition, they identify novel chemokine properties that bear investigating in other contexts.

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