The human *myc* gene family: structure and activity of L-*myc* and an L-*myc* pseudogene

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We have determined the nucleotide sequence and transforming activity of the human L-*myc* gene and a processed L-*myc* pseudogene (L-*myc* ψ). We demonstrate by cotransformation assays that a 10.6-kb *EcoRI* fragment derived from a human placental library contains a complete and functional L-*myc* gene including transcriptional regulatory sequences sufficient for expression in rat embryo fibroblasts. Organization of the L-*myc* gene was determined by comparing its sequence to those of the L-*myc* ψ gene and an L-*myc* cDNA clone derived from a human small cell lung carcinoma. Our results show that L-*myc* has a three-exon organization similar to that of the c-*myc* and N-*myc* genes. The putative L-*myc* gene product consists of 364 amino acids and contains five of the seven homology regions highly conserved between c-*myc* and N-*myc*. These conserved regions are located along the entire length of the putative L-*myc* protein and are interspersed among nonconserved regions. While the putative L-*myc* gene product is of a smaller size when compared to the c- and N-*myc* proteins, the relative positions of certain conserved residues occur in corresponding locations along the peptide backbone of the three proteins. In addition, comparison of the human and murine L-*myc* gene sequences indicate that the relatively large 5' and 3' untranslated regions are evolutionarily conserved, but that these sequences are totally divergent between the L-, c-, and N-*myc* genes. Finally, we demonstrate that, like the N- and c-*myc* genes, the L-*myc* gene can cooperate with a mutant Ha-*ras* gene to cause malignant transformation of rat embryo fibroblasts in culture. Our analyses clearly prove that L-*myc* represents a functional member of the *myc* oncogene family and further delineate structural features that may be important for the common and divergent functions of the members of this gene family.

[Key Words: Nucleotide sequences; cDNA; cotransformation assay; processed pseudogene; *myc* gene family]

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The *myc* oncogene family consists of three known members: c-, N-, and L-*myc* (for review, see Alt et al. 1986; DePinho et al. 1987). The c-*myc* gene was first identified as the cellular homolog of an avian retroviral transforming gene, v-*myc* (Sheiness and Bishop 1979). Amplified N-*myc* genes were identified in human neuroblastomas on the basis of homology to the c-*myc* gene (Kohl et al. 1983; Schwab et al. 1983). The c-*myc* and N-*myc* genes have a three-exon organization with a major coding domain located within exons 2 and 3 (Battey et al. 1982; DePinho et al. 1986; Kohl et al. 1986; Stanton et al. 1986). They encode similarly sized nuclear phosphoproteins (Abrams et al. 1982; Donner et al. 1982; Alitalo et al. 1983; Hann et al. 1983; Ikegaki et al. 1986; Persson et al. 1986; Ramsay et al. 1986; Sullivan et al. 1986; Slamon et al. 1986) which contain highly conserved clusters of amino acids (DePinho et al. 1986; Kohl et al. 1986; Stanton et al. 1986) that are likely to be important for nuclear targeting (Stone et al. 1986), nucleic acid binding (Donner et al. 1982; Persson and Leder 1984; Watt et al. 1985; Spector et al. 1987), and in vitro transforming activities (Land et al. 1983; Schwab et al.

1985; Yancopoulos et al. 1985; Stone et al. 1987). L-*myc* was identified first as a *myc*-related sequence that was amplified in a subset of human small cell lung carcinomas (SCLC) (Nau et al. 1985) and independently isolated from normal murine and human genomes on the basis of homology to N-*myc* (Zimmerman et al. 1986; see below). Limited nucleotide sequence analysis of a portion of the human L-*myc* gene revealed two short stretches of nucleotide sequence that were highly homologous to sequences conserved between the N- and c-*myc* genes (Nau et al. 1985), suggesting that L-*myc* might be an additional member of the *myc* gene family.

The physiological role of *myc*-family genes is not known; however, it is generally believed that these genes play a fundamental role in cellular proliferation and perhaps differentiation (for review, see Alt et al. 1986; Kelly and Siebenlist 1986). That they serve essential but distinct physiological function(s) is strongly supported by conservation of c-, N-, and L-*myc* as independent sequences over a large phylogenetic distance (King et al. 1986; Van Beneden et al. 1986; Collum and Alt, unpubl.). A potential role for c-*myc* expression in

development was suggested by the observation that deregulated expression of this gene affects B-cell differentiation in transgenic mice (Adams et al. 1985) as well as the differentiation of myeloid erythroleukemias in vitro (Coppola and Cole 1986; Dmitrovsky et al. 1986; Lachman et al. 1986). Analysis of *myc*-family gene expression during murine (Jakobovits et al. 1985; Zimmerman et al. 1986) and human (DePinho and Alt, in prep.) development demonstrated that the *c-myc* gene is expressed at substantial levels in most developing tissues; in contrast, high-level N- and L-*myc* expression is very restricted with respect to tissue and developmental stage. A striking example of differential *myc*-family gene expression occurs in the B-cell differentiation pathway. Precursor B-lymphoid cells express both N- and *c-myc*, but only *c-myc* is expressed in later stages of B-lymphoid cell development (Zimmerman et al. 1986). These differential *myc*-family gene expression patterns led to the suggestion that differential or perhaps, combinatorial expression of these genes could be related to the progression of certain cell lineages through differentiation (Zimmerman et al. 1986). Recent evidence suggests that there may be additional members of the *myc*-family (Alt et al. 1986).

To help elucidate the potential content and function of the *myc*-gene family, we have isolated and determined the structure of the complete human L-*myc* gene and a related human genomic sequence. In addition, we have demonstrated for the first time that the human L-*myc* gene has oncogenic potential; the cloned gene can cooperate with an activated Ha-*ras* gene to cause malignant transformation of rat embryo fibroblasts in vitro. In parallel, we have also characterized the nucleotide sequence of the murine L-*myc* gene (Legouy et al. 1987). Our analyses define structural elements that may be important for overlapping and distinct *myc* protein properties and point to sequences that could potentially serve a regulatory role in *myc* gene expression. Together, our findings demonstrate the L-*myc* represents a third functional member of the *myc*-family of cellular oncogenes.

Results

Isolation and structural characterization of the L-myc gene

To determine if *myc*-related sequences, in addition to N- and *c-myc*, existed in the human genome, we assayed *Eco*RI-digested human genomic DNA by Southern blotting procedures for hybridization with second and third exon probes derived from N-, c-, and v-*myc* genes. Under low stringency conditions, these probes hybridized to numerous fragments that did not correspond to either the N- or *c-myc* genes (Alt et al. 1986). The novel *myc*-homologous fragments that hybridized to an N-*myc* exon 2 probe were isolated from a human genomic library that was prepared by cloning human placental DNA digested to completion with *Eco*RI into the λ vector Charon 30A. Two recombinant phage clones, R1.3 and R11.1, that hybridized strongly to probes derived from exons 2 and 3 of both the c-, and N-*myc* genes

were characterized in detail. Restriction mapping and hybridization experiments demonstrated that regions cross-hybridizing with exons 2 and 3 were closely positioned in the 8.8-kb R1.3 insert and widely separated in the 10.6-kb R11.1 insert. Preliminary sequence analyses revealed that phage clone R11.1 contained the short stretch of sequences previously identified within a partial human L-*myc* clone (Nau et al. 1985) and that phage clone R1.3 harbored stretches of sequence highly related to those of R11.1. To examine the structure and organization of these L-*myc*-related human sequences, we determined the nucleotide sequence of the appropriate regions of both clones; clone R11.1 (referred to hereafter as L-*myc*) was found to contain the entire L-*myc* genomic sequence and the clone R1.3 (referred to hereafter as L-*myc* ψ) was found to contain a processed L-*myc* pseudogene (Figs. 1 and 2; and see below). Comparison of the L-*myc* genomic nucleotide sequence with the processed L-*myc* ψ sequence indicates that L-*myc* contains three regions of significant homology with L-*myc* ψ separated by large stretches of unrelated sequence (Fig. 1). At each of the boundaries between conserved (exons) and divergent sequence (introns), there exists a consensus donor and acceptor splice recognition sequence (Mount 1982) (Fig. 1C).

To confirm L-*myc* intron/exon structure suggested by the L-*myc* ψ gene, we prepared a λ phage Charon 16A cDNA library from cytoplasmic poly(A)⁺ mRNA of H510 SCLC cell line and from that of an L-*myc*-transformed rat embryo fibroblast (REF) cell line. A 5'-L-*myc*-exon 3 synthetic oligonucleotide primer (Fig. 1B) or oligo(dT) primer was hybridized to cytoplasmic RNA and extended as described previously (Kohl et al. 1986; Legouy et al. 1987). Recombinant phage were screened for hybridization to various L-*myc* probes (Fig. 2, L-*myc* probes 1–4). Several cDNA clones were identified that hybridized to probes containing putative L-*myc* exon 1 and exon 2 sequences. Characterization of multiple cDNA clones by restriction mapping, nucleotide sequence determination, and S1 nuclease protection experiments indicates that several distinct L-*myc* transcripts result from the differential processing of the first intron (with some species retaining the first intron) and from the alternative utilization of polyadenylation signals located in the second intron (DePinho and Alt, in prep.). Direct nucleotide sequence analysis of one such primer-extended cDNA clone, pPEIII.5, confirmed the intron/exon organization suggested by analysis of the L-*myc* ψ gene (see above). Primer extension analyses (not shown) indicate that the pPEIII.5 cDNA clone extends to within several basepairs of a major transcription initiation site (Fig. 1A, position 138); just upstream from this major cap site is a potential TATAA element (Fig. 1A, position 88) and a region homologous to sequences occurring near the N-*myc* promoter (DePinho et al. 1986). On the basis of the data presented here, however, the existence of additional 5' exons cannot be excluded.

The location of the polyadenylation signal downstream to the third exon of the L-*myc* gene (Fig. 1, position 5452) was determined by comparing the genomic

B.

Figure 1. (See facing page for legend.)

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Figure 2. Structure and organization of the *L-myc* cDNA fragment pPEIII.5; the 10.6-kb *L-myc* genomic fragment R11.1 and the 8.8-kb *L-myc* pseudogene genomic fragment R1.3. A partial restriction map indicates restriction endonuclease sites used in the sequence analysis. An asterisk (*) indicates a restriction site created as a result of linker addition during cloning of the cDNA. Horizontal arrows represent sequencing strategy. The boxed areas indicate the position of exons; shaded areas represent untranslated regions, whereas open areas represent putative translated regions. The dashed border of exon 3 in the *L-myc* pseudogene represents a 2.4-kb region not subjected to nucleotide sequence analysis. Below the restriction map of the *L-myc* genomic fragment are shown four DNA probes used in screening the *L-myc* cDNA library. Probe 1 is a 400-bp *Sma*I fragment encompassing exon 1 and a portion of intron 1. Probe 2 is a 450-bp *Nae*I fragment that contains exon 2. Probe 3 is a 1200-bp *Bam*HI–*Hind*III fragment containing exon 3 translated sequences. Probe 4 is a 1500-bp *Xba*I–*Eco*RI fragment containing only 3' untranslated sequences.

L-myc sequence with the 3' termini of the mouse *L-myc* cDNA (Legouy et al. 1987) and the *L-myc* ψ gene. A consensus polyadenylation sequence motif AATAAA (Proudfoot and Brownlee 1976) occurs at corresponding locations in all the *L-myc* genes and is followed by a poly(A) stretch in the mouse *L-myc* cDNA and the *L-myc* ψ (Fig. 1A,B). Thus, at least one form of the human *L-myc* gene consists of a three-exon organization similar to that of the *c-* and *N-myc* genes (Fig. 2): exon 1 is about 170 bp in length; exon 2 is 505 bp; and exon 3 is 2529 bp. The combined size of these sequences, including a poly(A) stretch of variable length, is approximately 3.5 kb; this length could account for the size of the largest noted human *L-myc* mRNA sequence (~3.5–3.8 kb), which appears to represent a major form of the *L-myc* mRNA (Nau et al. 1986; Fig. 5).

A long open reading frame (ORF) in the *L-myc* gene begins with an ATG codon at position 684 (9 bp downstream from the 5' boundary of exon 2) and extends 1092 nucleotides to an in-phase terminator at position 3522 in exon 3. Multiple termination codons are present in the other two reading frames. If we assign the first ATG of this ORF as coding for the amino terminal residue of the protein, *L-myc* would consist of 364 amino acids with a predicted molecular weight of approximately 38 kD. Recent evidence has indicated the possibility that translation initiation of the *c-myc* mRNA can occur at nonmethionine residues within exon 1 (King et al.

1987); such upstream initiation could theoretically extend the amino terminus of the *L-myc* coding domain an additional 38 or 160 amino acids (in the presence or absence of first intron splicing, respectively) before an in-phase termination codon would be reached at position 202 in exon 1.

The increased size of the *L-myc* mRNA relative to *N-* and *c-myc* appears to result primarily from its larger 3' untranslated region—1900 bp compared with 900 bp for *N-myc* (Kohl et al. 1986; DePinho et al. 1986; Stanton et al. 1986) and 350 bp for *c-myc* (Battey et al. 1982; Bernard et al. 1983). Similar to *c-myc* (Bernard et al. 1983) and *N-myc* (DePinho et al. 1986), the 5' and 3' untranslated regions of the *L-myc* mRNA exhibit about 80% nucleotide sequence conservation between the mouse and human genes (Fig. 3). This degree of homology is comparable to that seen in the translated regions (data not shown). While the untranslated regions of the individual *c-*, *N-*, and *L-myc* genes are conserved across species, the untranslated regions of each type of *myc* gene diverge extensively from one another (data not shown).

myc family gene products are highly related

The putative human and mouse *L-myc* proteins contain 364 and 368 amino acids, respectively (Fig. 4B). Allowing for the four-amino-acid insertion near the 5' border of

[illegible][illegible]

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Figure 4. (See facing page for legend.)

exon 3 in the mouse sequence, the overall homology between mouse and human L-myc proteins is 90% with half of the changes representing conservative amino acid substitutions (Fig. 4B). The predicted size of the L-myc protein is significantly smaller than that of the observed size of human c-myc (Battey et al. 1983; Bernard et al. 1983) or N-myc (Kohl et al. 1986; Stanton et al. 1986) proteins which measure 439 and 464 amino acids, respectively.

Several observations indicate that the proposed L-myc coding region is correct. Detailed comparisons reveal clusters of amino acids that are highly conserved among the three myc proteins. Dot matrix comparison of the human N-myc and c-myc proteins identifies seven clusters of highly conserved amino acids (Fig. 4A, top, first panel). The L-myc protein possesses five of these homology regions which are interspersed between divergent regions and which span the entire length of the proteins (Fig. 4A, top, second panel). One of these five common homology regions (region 3) is encoded by sequences that span the exon 2–exon 3 splice junctions of all three proteins (Fig. 4); the shorter size of the L-myc protein appears to result from the absence of approximately 80 (mostly nonconserved) amino acid residues found within exon 2 between homology regions 2 and 3 of c- and N-myc (Fig. 4). The latter alteration occurs in a region previously noted to be subject to structural variations between myc-related proteins (Ralston and Bishop 1983; Kohl et al. 1986). Hydropathy plots also demonstrate considerable relatedness among c-myc, N-myc, and L-myc. N-myc and c-myc plots are virtually superimposable (data not shown); and N-myc and L-myc have a highly related pattern (Fig. 4A, middle). However, the truncated length of L-myc exon results in closer positioning of homology regions 3 and 4 relative to regions 1 and 2. In this regard, a portion of L-myc homology region 3 appears to be missing a major internal stretch of acidic polyglutamic/aspartic acid residues (termed homology region 3'; Fig. 4A); of potential significance, such an acidic stretch is encoded by sequence further downstream within exon 3 of the L-myc gene. This potential transposition of coding sequences results in the conservation of the spatial relationship within the L-myc protein of this highly acidic amino acid cluster and L-myc

Figure 4. (A) (Top) Dot matrix computer analysis of protein sequence homology between human N-myc and human c-myc (left panel) and human N-myc and L-myc (right panel) was performed with a window of 8 and a stringency of 60%. Regions of homology (CN1, CN2, 1-5) are indicated. An arrow denotes the position of the exon 2–3 exon splice junction. (Middle) Hydrophobic (–) and hydrophilic (+) residues are plotted across the length of the peptide and assigned a hydropathy value, ranging from –3 to +3, as determined by the method of Hopp and Woods (1981). (Bottom) The amino acid sequence of homology regions between c-, N-, and L-myc proteins is shown. A solid vertical line indicates identity between residues; a double dot indicates a conservative amino acid substitution. (B) Dot matrix analysis of protein sequence homology between the mouse and human L-myc protein was performed using a window of 20 and stringency of 90%. Solid vertical lines indicate identity; a double dot indicates a conservative substitution.

homology regions 1 and 2, thereby maintaining the position of these residues along the *L-myc* peptide backbone relative to their position in the *N-* and *c-myc* proteins (Fig. 4A, top and middle).

In recent studies, *in vitro* mutagenesis analyses of the human *c-myc* gene uncovered a number of regions that appear essential for nuclear targeting, nuclear localization, and transformation activity (Stone et al. 1987). Comparison of these regions among the *c-*, *N-*, and *L-myc* proteins demonstrates areas of strong conservation (for summary, see Alt et al. 1987). Gene fusion studies have demonstrated that *c-myc* residues 351–381 are capable of directing the movement of cytoplasmic pyruvate kinase to the nucleus (Stone et al. 1987). There is 70% conservation within this region among all *myc* proteins. Other studies demonstrate that *c-myc* residues 106–143 and 371–412 appear necessary for retention within the nucleus, as opposed to targeting (Stone et al. 1987). Both of these domains are also highly conserved among all *myc* proteins. It is notable that within these coordinates there are residues that are identical in the three proteins (e.g., *c-myc* residues 132–140 are the same as *L-myc* residues 93–101 and *N-myc* residues 113–120).

L-myc can cooperate with ras to transform normal cells in culture

L-myc gene amplification occurs in a subset of human SCLC (Nau et al. 1985). By analogy to *N-myc* gene amplification in human neuroblastomas (Kohl et al. 1983; Schwab et al. 1983), the increased expression of *L-myc* as a result of gene amplification may confer an increased growth potential to malignant cells. The *c-myc* (Land et al. 1983) and *N-myc* (Schwab et al. 1985; Yancopoulos et al. 1985) genes possess similar transforming activities in the rat embryo fibroblast (REF) cotransformation assay. Therefore, to assay the oncogenic potential of the *L-myc* gene and the *L-myc* ψ gene, we tested their ability to cooperate with an activated *Ha-ras* gene to transform early-passage REFs in culture. The 10.6-kb *EcoRI* fragment containing the *L-myc* gene and the 8.8-kb *EcoRI* fragment containing the *L-myc* ψ gene were subcloned into a retroviral expression construct, pVcos7 (Table 1, bottom); the *L-myc* fragment was inserted in both orientations relative to the transcriptional orientation of two Moloney murine leukemia virus long terminal repeats (LTRs). The *L-myc* ψ gene was subcloned only in the same orientation as the Moloney promoter. These constructs were cotransfected with and without the mutant *Ha-ras* gene into early-passage rat embryo fibroblasts.

L-myc constructs that contained the *L-myc* gene in the same transcriptional orientation as the flanking LTRs (pL-*myc*-S) were not able to transform REFs alone but did cooperate with a mutant *Ha-ras* gene. In the *c-*, *N-*, and *L-myc/ras* cotransfections, dense foci became noticeable 7–15 days after the transfection. Foci consisted of individual cells with clearly transformed morphology, rapidly overgrowing and displacing the normal monolayer. The frequency of focus formation in the

various *L-myc/ras* cotransformations was significantly greater than the focus formation observed in the pVcos7/*ras* or *ras* alone controls but was significantly less than the positive cotransfection controls, *c-myc/ras* and *N-myc/ras* (Table 1). The *L-myc* gene in the same transcriptional orientation as the LTRs cotransfected with *ras* exhibited the greatest efficiency of transformation (Table 1; pVL-*myc/ras*; $p < 0.001$). The *L-myc* gene in the opposite transcriptional orientation (pVL-*myc*-AS) and the *L-myc* gene without neighboring LTRs (pL-*myc*) also cooperate with *ras* to induce transformation at a significant efficiency above background controls (Table 1; pVL-*myc*-AS, $p \leq 0.01$; pL-*myc*, $p \leq 0.05$), but the efficiency was somewhat less than the pVL-*myc*-S/*ras* combination. *L-myc* also conferred upon the transformants a high frequency of anchorage-independent growth and tumorigenic potential in young syngeneic hosts (Table 1).

Analysis of DNA prepared from the transformed REF lines demonstrated the presence of multiple copies of the introduced *ras* and *myc* genes (data not shown). Total RNAs prepared from REF transformants and controls were screened for expression of the introduced oncogenes. All of the REF transformants expressed high levels of the introduced *ras* gene (Fig. 5, middle panel). *L-myc* expression could be detected only in permanent REF lines transformed with the various *L-myc/ras* combinations (Fig. 5, bottom panel). The range of *L-myc* expression varied in each of the *L-myc/ras* transformants but greatly exceeded the level of *L-myc* expression seen in normal developing tissues and approached the levels seen in *L-myc*-expressing SCLC. pL-*myc/ras* transformants expressed an approximately 10- to 20-fold lower amount of normal *L-myc* message than the pVL-*myc*-S/*ras* cell lines (Fig. 5, bottom panel). *L-myc* expression levels in tumors derived by injecting transformed REF lines into syngeneic hosts were similar to those observed in the corresponding cultured cell lines (data not shown). Total cellular RNA derived from pVL-*myc*-AS/*ras* transformants was also assayed with SP65-generated *L-myc* antisense and sense probes and found to express normal-sized *L-myc* message (data not shown).

Total RNAs derived from the *L-myc/ras* transformants contain *L-myc*-hybridizing sequences of 3.8, 2.2, and 1.8 kb; sequences of the same size are found in the total RNA of the H510 SCLC (Fig. 5, lower panel) and in human *L-myc*-expressing embryonic tissues (R. DePinho and F. Alt, unpubl.). *L-myc/ras* transformants also exhibited a 5.0-kb transcript that initiates from the viral promoter (Fig. 5, lower panel). The presence of normal-sized *L-myc* message in each of the *L-myc/ras* transformants, including the antisense construct, indicates that *L-myc* promoter elements sufficient for expression in REF lines are contained in the 10.6-kb *L-myc* clone. *ras* alone transformants did not express *L-myc* (Fig. 5, panel C).

L-myc ψ is a processed *L-myc* pseudogene

The genomic organization of the *L-myc* ψ gene corre-

Table 1. Transformation of REFs with *myc*-family and *ras* gene combinations

Transfected DNA	Plates with transformed foci/total plates			Growth in soft agar, lines positive/lines tested	Tumorigenicity, rats with tumors/injected
	Exp. 1	Exp. 2	Exp. 3		
pkomyc + T24-ras	9/12	9/9	9/9	2/2	2/2
pV-Nmyc + T24-ras	—	12/16	8/12	—	—
pV-Lmyc-S + T24-ras	6/11	16/36	17/36	3/4	9/9
pV-Lmyc-AS + T24-ras	6/11	—	7/18	4/4	2/5
pL-myc + T24-ras	3/11	—	4/15	2/2	4/4
pL-myc ψ -S + T24-ras	0/12	—	1/12	—	—
pVcos-7 + T24-ras	—	0/12	2/12	1/2	1/2
pV-Lmyc-S	0/12	—	0/12	—	—
T24-ras	0/12	0/9	2/12	0/2	0/2
None	—	—	—	0/2	0/3

The data for the first three columns are presented as the number of plates with positive foci compared to the total number of plates into which each particular transfection was finally divided; due to the way the cells were passaged after transfection, each plate in the final passage has independent transformants. At 2 weeks after transfection each positive plate had 1–5 foci, whereas at 4 weeks each positive plate had 5–20 foci; presumably some of these were due to metastases. Cell lines derived from *c-myc/ras* foci (two lines examined) or *L-myc/ras* foci (four lines examined) were assayed for both colony formation in soft agar and the ability to cause tumors in young syngeneic rats; each line assayed for tumorigenicity was injected in two rats. As a nontransfected control for these assays (bottom line) we used REFs propagated in culture. Both the colonies in soft agar and the tumors in the rats appeared and grew at approximately the same rate for either the *L-myc/ras* or *c-myc/ras* transformants. These tumors were noted in all cases within 1 week of cell injection, grew rapidly, and resulted in the death of the host animal within 2–3 weeks.

The *L-myc* pVcos7 expression constructs are shown below the table. *L-myc* and *L-myc* ψ inserts were subcloned between two directly repeated Moloney leukemia virus LTR sequences in the transcriptional orientation shown. pVL-*myc*-S and pVL-*myc*-AS constructs contained the *L-myc* gene in the same or opposite transcriptional orientation as the flanking LTRs, respectively. pL-*myc* construct contained the *L-myc* subcloned into the pUC18 plasmid vector and did not contain neighboring LTR sequences. pVL-*myc* ψ -S contained the *L-myc* ψ gene in the same transcriptional orientation as the flanking LTRs. pT24-*ras* construct contains the 6.2-kb *Bam*HI insert harboring the activated (Val-12) human *H-ras* oncogene.

sponds to the structure of the *L-myc* cDNA (Figs. 1B and 2). The *L-myc* ψ gene contains initiation and termination codons in the same relative positions as the *L-myc* gene; however, the *L-myc* ψ gene contains numerous in-phase termination codons due to insertions and deletions that result in frameshift mutations. Nucleotide sequence homology between *L-myc* transcribed regions and the corresponding *L-myc* ψ regions is approximately 90%. However, restriction map comparisons between the *L-myc* ψ and *L-myc* 3' untranslated regions reveal that the *L-myc* ψ gene contains an additional 850 bp within the 2.4-kb region not subjected to direct nucleotide sequence analysis (Fig. 1B, 2.4-kb region is indicated as a series of Ns) and suggests that the *L-myc* ψ gene has undergone extensive alterations in this region (Fig. 2, dashed portion of exon 3). A consensus polyadenylation

signal is followed by a small poly(A) stretch starting at position 1991 which does not exist in the *L-myc* 3'-flanking sequence. Immediately flanking the poly(A) tract, there exists a 9-bp sequence that is directly repeated precisely 5' to where the 5' homology between the *L-myc* and *L-myc* ψ ceases. The repeat sequences, AGAAATAAA and AGAAGTAAA, occur at positions 482 and 1996, respectively. Direct repeats are a sequence motif common to many processed pseudogenes and may be related to the mechanism(s) responsible for pseudogene reintegration in the genome (for review, see Vanin 1984). The *L-myc* ψ gene does not exist in the mouse genome (data not shown) and does not function in the cotransformation assay (see above). Thus, the *L-myc* ψ gene appears to possess all of the structural features of a processed pseudogene: It is a processed, nonfunctional

Figure 5. Expression of introduced *ras* and *myc* genes in the transfected REF cell lines. Ten micrograms of total RNA, prepared from indicated lines, was used in each lane. REF refers to RNA isolated from normal rat embryo fibroblasts. H510 refers to RNA isolated from a human SCLC. All of the *L-myc/ras* transformants listed in Table 1 expressed the introduced *L-myc* construct (not shown); the highest and lowest *L-myc*-expressing transformants are shown. Duplicate filters were probed with a 32 P-labeled 500-bp *Pst*I fragment containing the human *c-myc* exon 2 (A), a 1.6-kb *Hind*III fragment containing the *Ha-ras* gene (B), or a 450-bp *Nae*I fragment containing human *L-myc* exon 2. The introduced *c-myc* gene was driven from the simian virus 40 promoter linked upstream to the second exon, therefore *c-myc* RNA expressed from the construct is smaller than the normal endogenous *c-myc* mRNA.

gene containing a small poly(A) stretch that is flanked by directly repeated sequences. Our preliminary mapping data indicate that *L-myc* ψ is located on human chromosome X (R. DePinho et al., in prep.); previous studies have assigned the *L-myc* gene to human chromosome 1 (Nau et al. 1985).

Discussion

L-myc is the third member of the *myc* oncogene family

Our results clearly demonstrate that the human *L-myc* gene represents an additional functional member of the *myc*-family of cellular oncogenes. This conclusion is based on the observation that the *L-myc* gene and gene product share oncogenic activities and structural features with the other well-characterized *myc*-family members. The common and divergent structural elements described in this report could provide direction in the preliminary dissection of potential functional domains in the *L-myc* and other *myc* proteins. For example, *c-myc* encodes a nuclear protein that has DNA-binding capacity in vitro (Donner et al. 1982; Persson and Leder 1984; Watt et al. 1985), a property attributed to the abundance of basic amino acids at its carboxyl terminus (Persson and Leder 1984). This structural feature is also present in the putative *L-myc* gene product; thus, the *L-myc* protein may also be a nuclear protein with capacity to bind nucleic acids. In general, we have also found that most *c-myc* protein domains necessary for cotransformation activity, nuclear targeting, and retention in the nucleus are well conserved in the *L-myc* protein. Moreover, extremely well-conserved residues that we have identified within these potential functional domains may further delineate critical residues involved in these properties. However, not all of the *c-myc* domains are conserved in *L-myc*; one region that appears

important for nuclear targeting in *c-myc* is not conserved in the putative *L-myc* gene product. Despite the striking degree of conservation of various sequences and aspects of function (e.g., activity in the REF cotransformation assay), the conservation of the *c*-, *N*-, and *L-myc* genes throughout vertebrate evolution as distinct sequences suggests unique physiological activities (see below for further discussion of this point). In this context, the potential roles of the extremely divergent regions that appear at common positions within the various *myc* proteins or regions conserved only between *N*- and *c-myc* need to be assessed.

The mouse and human *L-myc* untranslated regions are well conserved. This remarkable degree of sequence conservation in regions that are not under any selective pressure at the protein level is consistent with a role in the regulation of gene expression. In contrast to the remarkable evolutionary conservation within a given *myc*-family gene, untranslated sequences among the different *myc* family members are totally divergent from each other. The different *myc*-family genes have very distinct expression patterns (Alt et al. 1986; Zimmerman et al. 1986); in this context, it is conceivable that evolutionarily conserved flanking sequences unique to each family member somehow could be involved in mediating differential expression.

L-myc transforming activity

A large body of evidence has suggested that progression to malignancy is a multistep process (for review, see Weinberg 1985). Thus, the *c-myc* gene has been linked with immortalization activity (Mougeon et al. 1984; Ruley et al. 1984) while the action of *Ha-ras* has been correlated with transformation activity (Land et al. 1983; Ruley et al. 1983). Additional studies, however, indicate that *c-myc* expression constructs can induce

transformation in immortalized cell lines (Keath et al. 1984; Kelekar and Cole 1986) and that Ha-*ras* can immortalize primary cells (Spandidos and Wilkie 1984). Currently, it is not clear how the activities of these genes result in the observed spectrum of deregulated growth. We have demonstrated that the L-*myc* gene can cooperate with a mutant Ha-*ras* gene to malignantly transform rat embryo fibroblasts. Although we could not detect a difference in the malignant phenotype between the L-*myc/ras* transformants and the N- or c-*myc/ras* transformants, the L-*myc/ras* transformants did arise at a lower frequency. If the three *myc* genes encode proteins with identical transforming activities, the different efficiencies in the REF transformation assay might result from constructs with different capacities to produce sufficient levels of the respective *myc* proteins. However, in this regard, it is surprising that the L-*myc* gene was able to cooperate with *ras* in the absence of a retroviral enhancer because the N-*myc* gene seemed to require the presence of associated transcriptional enhancer elements. Alternatively, it is possible that the L-*myc* gene product may not possess a transforming activity equivalent to that of the c- or N-*myc* proteins and/or may require additional genetic events to achieve the malignant phenotype; both these possibilities could result in diminished activity of L-*myc* in this assay.

We have demonstrated that the transforming activity of L-*myc* is not limited to the very restricted set of tumors in which the gene has been found to be amplified and overexpressed (to date, only in a subset of SCLC). The apparently limited involvement of L-*myc* in the genesis of naturally occurring tumors is also observed for N-*myc* (clearly implicated only in neuroblastomas and some retinoblastomas and SCLC), but contrasts with that of c-*myc*, which appears to participate in the neoplastic transformation of a much wider variety of cells (for review, see Alt et al. 1986). We have recently demonstrated that the restricted set of tumors in which the N- and L-*myc* genes are amplified and/or overexpressed correlates in part with the tissue- and stage-specific expression patterns of these genes during normal development (Nisen et al. 1986; Zimmerman et al. 1986). Given our current observation that L-*myc*, like N-*myc*, can participate in the in vitro transformation of other cell types (fibroblasts), which do not even express detectable levels of either gene, it seems possible that deregulated expression of these genes contributory to the development of naturally occurring tumors may occur preferentially in cell types that actively express the genes. Another, not mutually exclusive, possibility for the restricted contribution of N- and L-*myc* to natural tumors is that the activity of the different *myc* gene products may be effected in a tissue-specific fashion. It is notable that, to date, the only observed mechanism for activated expression of the N- and L-*myc* genes in naturally occurring tumors is by gene amplification (for review, see Alt et al. 1986) and that expression levels of N- or L-*myc* genes in REF transformants often approach those of some amplified tumors (e.g., Fig. 5). To achieve high-level amplification, a gene usually must give some selective growth advantage at low amplification (expres-

sion) levels, with the selective advantage increasing at higher levels (Schimke 1984; Alt et al. 1986). One possible rationalization for the apparently disparate transforming activities of the L- and N-*myc* genes in vivo as opposed to in vitro could be that the relatively high-level expression observed in nearly all in vitro transformants leads to a more degenerate transforming activity than would occur with small increases that initially occur in an amplification process (in vivo). In this regard, a threshold level of N-*myc* expression is necessary for regulation of MHC Class I gene expression (Bernards et al. 1986).

Methods

Genomic and cDNA cloning

Genomic DNA from human placenta was digested to completion with *EcoRI* and cloned into the *EcoRI* site of λ phage Charon 30 essentially as described previously (DePinho et al. 1985). This library was screened for clones that hybridized to exons 2 (400-bp *XhoI*–*BamHI* fragment) and 3 (1100-bp *EcoRI*–*HincII* fragment) under low stringency conditions as described by Legouy et al. 1987.

Primer-extension and oligo(dT)-primed cDNA libraries were prepared from cytoplasmic poly(A)⁺ RNA isolated from the H510 SCLC cell line (graciously provided by John Minna) and 1C.1, an L-*myc*-expressing REF transformant cell line (see text), as described previously (Kohl et al. 1986; Legouy et al. 1987). This library was screened for clones which hybridized to exons 1 and 2 L-*myc*-specific probes; probes are illustrated and described in Figure 2. Several clones were isolated and subcloned into the pUC18 plasmid vector for further analysis.

DNA sequencing

The nucleotide sequence of indicated regions of the cDNA and genomic clones was determined by the partial chemical degradation method of Maxam and Gilbert (1977).

Analysis of RNA and DNA

DNA and RNA preparation, restriction endonuclease digestions, probe preparation by the nick-translation method, blotting procedures, and hybridization conditions were performed as described previously (DePinho et al. 1985). Reduced-stringency hybridizations were performed in 35% formamide, 5 × SSCPE, 10% dextran sulfate, 1 × Denhardt's solution, and 100 µg/ml salmon sperm DNA; washing was conducted at 56°C in 2 × SSC and 0.1% SDS; all other conditions follow standard procedures as described previously (DePinho et al. 1985).

Preparation and transfection of REFs

Early-passage cultures of REFs were prepared as described elsewhere (Yancopoulos et al. 1985) from 12- to 14-day Fischer rat embryos. REFs were initially plated at near-confluent density and allowed to grow to confluence. To prepare for transfection, REFs were then replated after 1–2 days at 1.5×10^6 cells per 10-cm plate in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. DNA was introduced into REFs by calcium phosphate precipitation as described previously (Andersson et al. 1979) using 30 µg of human placental carrier DNA with 2 µg of each of the appropriate plasmid DNAs per plate. The cultures were refed 6–9 hr after transfection and split 18 hr after the transfection into either three (ex-

periments 1 and 2) or four (experiment 3) plates. Assuming that limited cell division occurs in the 24 hr after the transfection, each plate after this final passage contains independent transformants. This was verified by DNA analysis of isolated transformants (data not shown). The various plasmids used in the REF transfections are as follows: pT24-*ras* contains the mutant Ha-*ras* oncogene with (experiments 2 and 3) and without (experiment 1) a neomycin resistance gene (Fasano et al. 1983; provided by M. Goldfarb); the pKO-*myc* plasmid, kindly provided by Daniel Birnbaum, is a *c-myc* expression construct in which transcription of the second and third exons of *c-myc* is driven from a simian virus 40 promoter; the L-*myc* constructs are described below and illustrated in Table 1, bottom.

Assay for colony formation in soft agar

A single-cell suspension of approximately 1×10^4 cells in 5 ml of 0.28% agar was overlaid onto a 60-mm culture dish containing a 0.7% agar base. All agar suspensions were made in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Duplicate plates were prepared for each tested line. Normal REFs propagated in culture served as a non-transformed control for these studies.

Assay for tumorigenicity

Cell lines propagated from transformed foci (see text) were assayed for tumorigenicity by subcutaneously injecting 1×10^6 to 5×10^6 cells in 0.4 ml of PBS into 5- to 10-day-old syngeneic Fischer rats. Each line tested was injected into at least two rats. A nontransfected control was provided by propagating normal REFs in culture until sufficient numbers of cells were attained; four rats each injected with 2×10^7 normal REFs served as controls. The rats were followed until death, or for 4 weeks in the case of the normal REF injections.

Computer analysis of DNA and protein sequence

Computer analyses of the L-*myc* sequence were performed on an IBM-AT personal computer using the Microgenie Sequence Analysis Program (SciSoft, Inc.).

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