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The human L-*myc* gene is expressed as two forms of protein in small cell lung carcinoma cell lines: detection by monoclonal antibodies specific to two *myc* homology box sequences

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The L-*myc* gene is the third member of the *myc* family of proto-oncogenes. Amplification and elevated expression of the L-*myc* gene has been detected in a subset of small cell lung carcinoma (SCLC) cell lines. The biological properties and functions of the L-*myc* gene and its product have not yet been elucidated. Monoclonal antibodies against two *myc* homology boxes were used to characterize the L-*myc* gene product. These antibodies react with two groups of polypeptides of apparent masses of 60, 61 and 66 kd (the long forms), and 34 and 37 kd (the short forms) in SCLC cells expressing L-*myc* transcripts. The long form L-*myc* proteins are associated with the nuclear fraction of the cells. The short form L-*myc* proteins are present in the cytoplasmic fraction, though diffusion of the short forms from the nucleus during cell fractionation cannot be ruled out. The half-life of the long form polypeptides is ~45-90 min. The short form polypeptides have a half-life of ~120-180 min. The L-*myc* protein is not detectable in the mitotic cells, suggesting that the L-*myc* protein expression is tightly regulated during the cell cycle.

Key words: cell cycle/epitopes/*myc* boxes/nuclear oncogenes

Introduction

The *myc* gene famly includes c-, N- and L-*myc* genes and additional members may also exist (DePinho *et al.*, 1987). The c-*myc* gene, the first member of the family, was identified as the cellular homologue of the transforming sequence of avian myelocytomatosis virus (Roussel *et al.*, 1979). The N-*myc* and L-*myc* genes were subsequently identified through two stretches of limited nucleotide sequence homologous to c-*myc* (referred to as *myc* homology box sequences) (Schwab, 1985). Since the *myc* family genes are often found structurally altered in several types of malignancies by such mechanisms as chromsomal translocation, LTR insertion and gene amplification, it is generally believed that these genes play critical roles in certain step(s) of tumorigenesis of those malignancies (for review see Leder *et al.*, 1983; Cole, 1986; Alitalo and Schwab, 1986).

The gene products of c-*myc* and also N-*myc* have been identified and characterized. These proteins have similar biophysical and biochemial properties: they appear as two closely migrating polypeptides with apparent molecular masses of 58-67 kd on SDS-PAGE and are nuclear phosphoproteins with relatively short half-lives (Hann and Eisenman, 1984; Persson *et al.*, 1984; Ramsay *et al.*, 1984, 1986; Ikegaki *et al.*, 1986; Slamon *et al.*, 1986).

Structural analysis of the human L-*myc* gene has demonstrated that like the other *myc* family genes, it too is composed of three exons and two introns. Those small cell lung carcinoma (SCLC) cell lines with amplified L-*myc* DNA express relatively high levels of RNA transcripts with multiple sizes ranging from 2.2 to 3.9 kb. This complex pattern of L-*myc* gene expression was found to be due to alternative splicing of introns and use of different polyadenylation signals, generating two distinct types of RNA transcript (Kaye *et al.*, 1988). The long forms are composed of either exon 1-intron 1-exon 2-exon 3 or exon 1-exon 2-exon 3, and the short form consists of exon 1, exon 2 and a portion of intron 2. Thus unlike other *myc* family gene products, the L-*myc* gene could be expressed as two proteins of 364 and 206 amino acid residues respectively. The predicted amino acid sequence of the putative L-*myc* gene products have a high degree of similarity with the c-*myc* and N-*myc* proteins, including six regions of almost identical sequence in the long form and two regions in the short form of the putative L-*myc* proteins (Kaye *et al.*, 1988). Recently, De Greve *et al.* (1988) reported the detection of the long form L-*myc* protein in an SCLC cell line as multiple nuclear phosphoproteins with relatively short half-lives, but they were unable to detect the short form L-*myc* protein in the cell.

In this paper, we describe the identification and characterization of two forms of the human L-*myc* gene product in SCLC cells with use of monoclonal antibodies specific to two distinct *myc* homology box sequences. These antibodies, reported previously, were produced against an N-*myc*-c-*myc* fusion protein (Ikegaki *et al.*, 1986).

Results

Verification of pan-myc reactivities of the monoclonal antibodies

We have previously reported the production of monoclonal antibodies against an N-*myc*-c-*myc* fusion protein expressed in *Escherichia coli*, and described some characteristics of these antibodies (Ikegaki *et al.*, 1986). These antibodies were initially grouped in three classes based on their reactivities to different portions of the fusion protein. Class I antibodies react with the C-terminal 60% of c-*myc* protein. Class II antibodies are specific to either one of the two *myc* box sequences encoded in the second exon of *myc* family genes. Class III antibodies are N-*myc* specific.

Further epitope analyses revealed that the Class I antibodies described previously reacted with N-*myc* as well as c-*myc* proteins, suggesting that these antibodies are specific to one of the four *myc* homology sequences present within the C-terminal 60% of c-*myc* protein, namely the third to sixth *myc* boxes (Figure 3). Dissection of the corresponding c-*myc* gene segment and following expression of the resultant

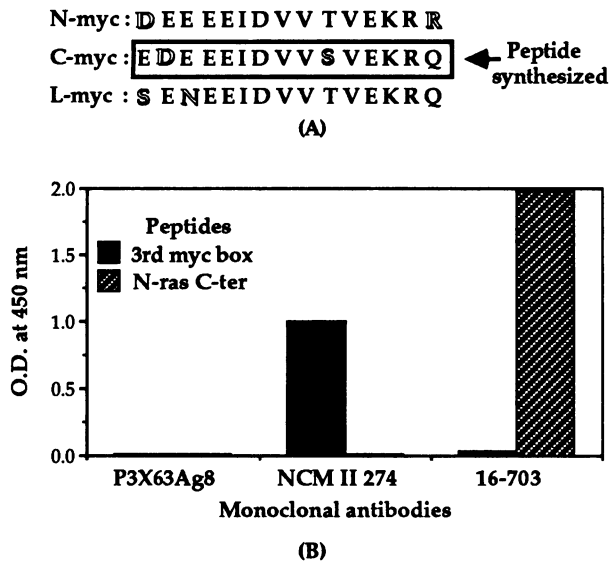

Fig. 1. The Class I monoclonal antibody, NCM II 274, is specific to the third *myc* homology box sequence. (**A**) Amino acid sequences of the third *myc* homology box of three known members of *myc* family gene products. The amino acids differing among members of the *myc* family are shown by shadowed letters. The peptide synthesized is marked by the rectangle. The sequence corresponds to the amino acid residues 257–271 of c-*myc* protein. (**B**) Specific reactivity of Class I antibody, NCM II 274, with the first *myc* homology box peptide. An ELISA was performed on the first *myc* box peptide and a control, N-*ras* C-terminus peptide, using a Class I antibody, NCM II 274. Anti-N-*ras* C-terminus peptide antibodies (16–703) were included as positive controls for the assay. P3X63Ag8 was used as a negative control.

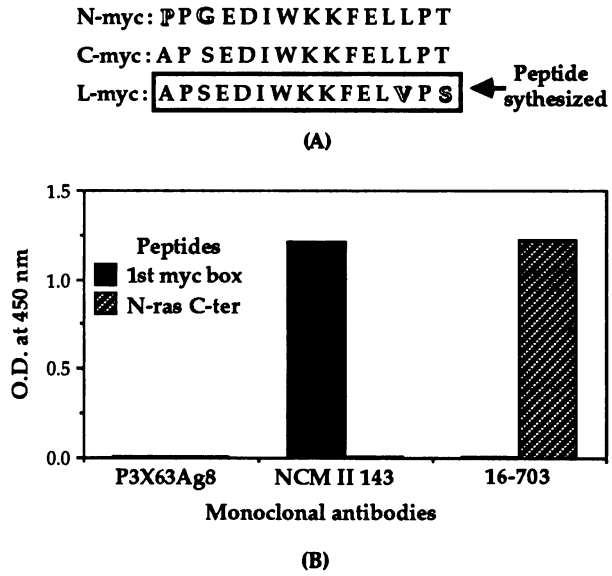

Fig. 2. The Class II monoclonal antibody, NCM II 143, is specific to the first *myc* homology box sequence. (**A**) Amino acid sequences of the first *myc* homology box of three known members of *myc* family gene products. The amino acids differing among members of the *myc* family are shown by shadowed letters. The peptide synthesized is marked by the rectangle. The sequence corresponds to the amino acid residues 24–38 of L-*myc* protein. An additional cysteine residue is attached to the N-terminus of the peptide. (**B**) Specific reactivity of Class II antibodies with the first *myc* homology box peptide. An ELISA was performed on the first *myc* box peptide and a control, N-*ras* C-terminal peptide, using a Class II antibody, NCM II 143. Anti-N-*ras* C terminal peptide antibodies (16-703) were included as positive controls for the assay. P3X63Ag8 was used as a negative control.

gene fragments in *E.coli* allowed us to identify the epitope recognized by Class I antibodies as the third *myc* homology box present to the right of the boundary of the second and third exons of the c-*myc* gene (N.Ikegaki *et al.*, in preparation). A synthetic oligopeptide corresponding to the third *myc* box sequence (Figure 1A) was prepared, and the reactivity of Class I antibodies against the peptide was then tested by an enzyme-linked immunosorbent assay (ELISA) to confirm this result. As shown in Figure 1B, one of the Class I antibodies, NCM II 274, reacted specifically to the third *myc* box peptide, but not to a control, N-*ras* C-terminal peptide. A control monoclonal antibody, 16-703 (Cheng and R.H.Kennett in preparation), specific to an N-*ras* C-terminal peptide did not react with the third *myc* box peptide. These results demonstrate the specific reactivity of the Class I antibody, NCM II 274, to the third *myc* homology box sequence.

Since Class II antibodies reacted with either the first or second *myc* box sequences, we examined which sequence encoded the epitope for each antibody. It seemed likely that all Class II antibodies were specific to the first *myc* box sequence, because they did not react with a truncated version of c-*myc* protein lacking the first *myc* box (N.Ikegaki *et al.*, in preparation). A synthetic oligopeptide representing the first *myc* box sequence was prepared to confirm this result (Figure 2A). One of the Class II antibodies, NCM II 143 was then tested against this peptide. As shown in Figure 2B, NCM II 143 reacted only with the first *myc* box peptide. A control anti-N-*ras* C-terminal peptide antibody, 16-703, did not react with the first *myc* box peptide. These results demonstrate that the Class II antibody, NCM II 143, is specific to the first *myc* homology box sequence.

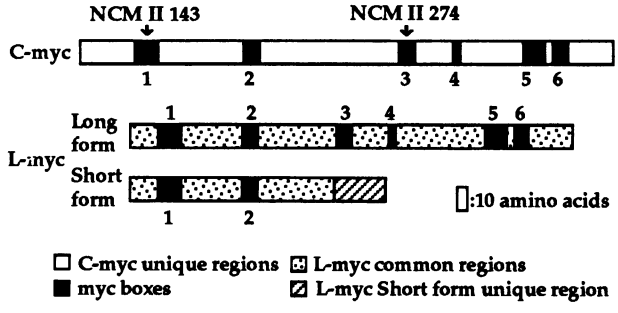

Fig. 3. Structural relationship between the c-*myc* protein and the predicted two forms of L-*myc* proteins in relation to *myc* homology boxes and epitopes recognized by NCM II 143 and 274 antibodies. The long form L-*myc* protein could contain six *myc* boxes as in the c-*myc*, whereas the short form could only contain the first and second *myc* box sequences. The order of the first to sixth *myc* homology boxes in each protein is the same.

Candidates for the L-myc gene products

Candidates for the L-*myc* gene product should satisfy at least the following conditions: the protein should be present in cells expressing L-*myc* transcripts, and there should be a positive correlation between the relative amounts of detected protein and the levels of L-*myc* transcripts. In addition, the L-*myc* gene could encode for two forms of proteins as indicated by the previous study (Kaye *et al.*, 1988). The short form could be encoded predominantly by the second exon of L-*myc* gene and therefore would only retain the first and second *myc* homology box sequences, whereas the long form, which could be encoded by the second and third exons,

would contain six regions highly homologous to other *myc* family gene products (Figure 3). A recent report, nevertheless, described the expression of only long form of the L-*myc* protein *in vivo* as multiple polypeptides with ~60 kd in molecular mass on SDS−PAGE (De Greve *et al.*, 1988).

Since NCM II 143 recognizes the first *myc* homology box and NCM II 274 is specific to the third *myc* homology box, NCM II 143 could detect both long and short forms of the L-*myc* gene products, whereas NCM II 274 could only detect the long form of L-*myc* gene product (Figure 3). To examine whether or not these monoclonal antibodies could detect both short and long forms of the L-*myc* protein *in vivo*, protein blots were prepared from several SCLC cell lines that express different levels of the L-*myc* mRNA, and the reactivity of NCM II 143 and 274 antibodies was examined.

Identification of the L-myc gene product in SCLC cells

In order to detect the L-*myc* gene product, we chose three SCLC cell lines exclusively expressing L-*myc* transcripts at different levels. NCI H 345 expresses L-*myc* transcripts at low levels even though it has amplified L-*myc* genes (J.D.Minna, unpublished data). NCI H 209 expresses L-*myc* transcripts at high levels, though it has only a single copy of the L-*myc* gene per haploid genome. NCI H 510 has amplified L-*myc* genes and expresses L-*myc* transcripts at high levels (Nau *et al.*, 1985). We also included two other SCLC lines as positive controls. NCI H 69 has N-*myc* gene amplified and expresses N-*myc* transcripts at moderate levels (Nau *et al.*, 1986). NCI H 82 has amplified c-*myc* genes and expresses c-*myc* transcripts at high levels (Little *et al.*, 1983). Three sets of protein blots were prepared from total cell extracts of these five SCLC cell lines, and the blots were subjected to immunological detection of the *myc*-related polypeptides using NCM II 274 antibody (specific to the third *myc* box) and NCM II 143 antibody (specific to the first *myc* box). An antibody produced by a mouse myeloma cell line, P3X63Ag8, which has no demonstrable reactivity, was included as a negative control. As shown in Figure 4, NCM II 274 detects multiple polypeptides with apparent molecular masses of 60, 61 and 66 kd in these SCLC lines expressing L-*myc* transcripts as well as c-*myc* and N-*myc* gene products in H 82 and H69 cells, respectively, at expected molecular mass ranges of approximately 59−63 kd. The amount of polypeptides detected in H 345, H 209 and H 510 cells correlates well with the levels of L-*myc* transcripts known to be present in these cells. NCM II 143, on the other hand detects not only the same polypeptides of 60, 61 and 66 kd detected by NCM II 274, but also reacts with 34 and 37 kd polypeptides in NCI H 209 and H 510 cells. The 34 and 37 kd polypeptides were not detectable in H 345 cells. This may be due to the low level of L-*myc* mRNA expression in this cell line and the 34 and 37 kd polypeptides expression may be below the sensitivity of the immunoblotting assay. It should be noted that the expression of 66, 34 and 37 kd species seems to vary among cell lines tested: H 209 cells express less of these species than H 510 cells.

These results demonstrate, in contrast to the previous report (De Greve *et al.*, 1988), that the L-*myc* gene is indeed expressed as two forms of polypeptide in these SCLC cell lines as predicted from the study of L-*myc* cDNA clones (Kaye *et al.*, 1988). The polypeptides with high molecular mass share two distinct epitopes present in the second and third exon of other *myc* family gene products; those with lower molecular mass share at least a single epitope present

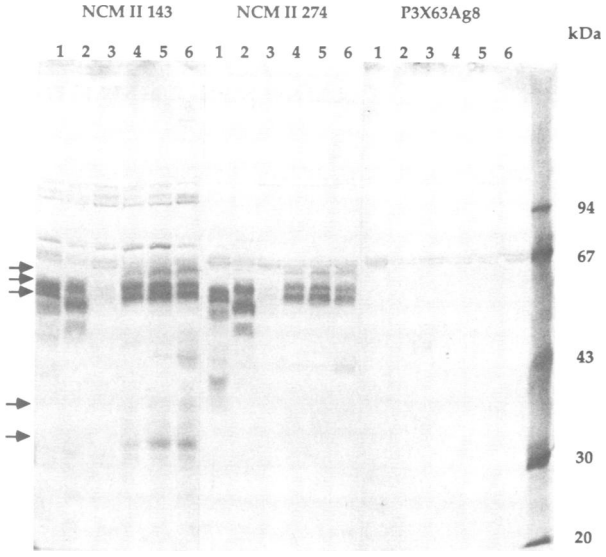

Fig. 4. Immunoblot detection of the L-*myc* gene products in SCLC lines positive for the L-*myc* mRNA by two distinct types of anti-*myc* homology box monoclonal antibodies, NCM II 143 and 274. Total cell extracts made from five SCLC cell lines, expressing c-*myc* (NCI H 82), N-*myc* (NCI H 69) and L-*myc* in different levels (low in NCI H 345, high in NCI H 209 and NCI H 510). Total cell extracts (40 μg) from each line were subjected to SDS−PAGE. Three sets of proteins blots were prepared. The blots were then subjected to immunological detection of *myc*-related proteins using NCM II 274 (specific to the third *myc* box), NCM II 143 (specific to the first *myc* box) and P3X63Ag8 (a negative control). The arrows indicate the polypeptides detected in SCLC cell lines expressing the L-*myc* transcripts with anti-*myc* box monoclonal antibodies. **Lane 1**, NCI H 69; **lane 2**, NCI H 82; **lane 3**, NCI H 345; **lane 4**; NCI H 209; **lane 5**, NCI H 510 (early passage); **lane 6**, NCI H 510 (late passage).

in the second exon with the other polypeptides. These patterns of reactivity indicate that the polypeptides with higher molecular mass species are encoded by the long form of L-*myc* transcripts and lower molecular mass species are encoded by the short form of L-*myc* transcripts. We designated the higher molecular mass species as the long forms and the lower molecular mass species as the short forms of the L-*myc* gene products. Since all the polypeptide species are detectable in NCI H 510 cells, this cell line was chosen for further characterization of the L-*myc* gene products.

Subcellular localization of the L-myc gene products

Since subcellular localizations of protein often provide some insight into possible biological functions, we next determined the distribution of the L-*myc* gene products in the cells. NCI H 510 cells were fractionated into cytoplasmic/membrane, nuclear wash, nuclear and post-nuclear fractions by a detergent lysis method. Each fraction was then subjected to immunoblotting assay using both NCM II 143 and 274 monoclonal antibodies as a mixture. As shown in Figure 5A, the long forms (60, 61 and 66 kd) are recovered in the nuclear fraction of the cell, whereas the short forms (34 and 37 kd) are found in the cytoplasmic/membrane fraction. To determine the more detailed localization of the short forms, the cytoplasmic and membrane fractions as well as the subcellular particles and nuclear fractions of NCI H 510 cells were prepared by a hypotonic shock/mechanical lysis method. These fractions were then subjected to immunoblotting assay. The result in Figure 5B strongly suggests that

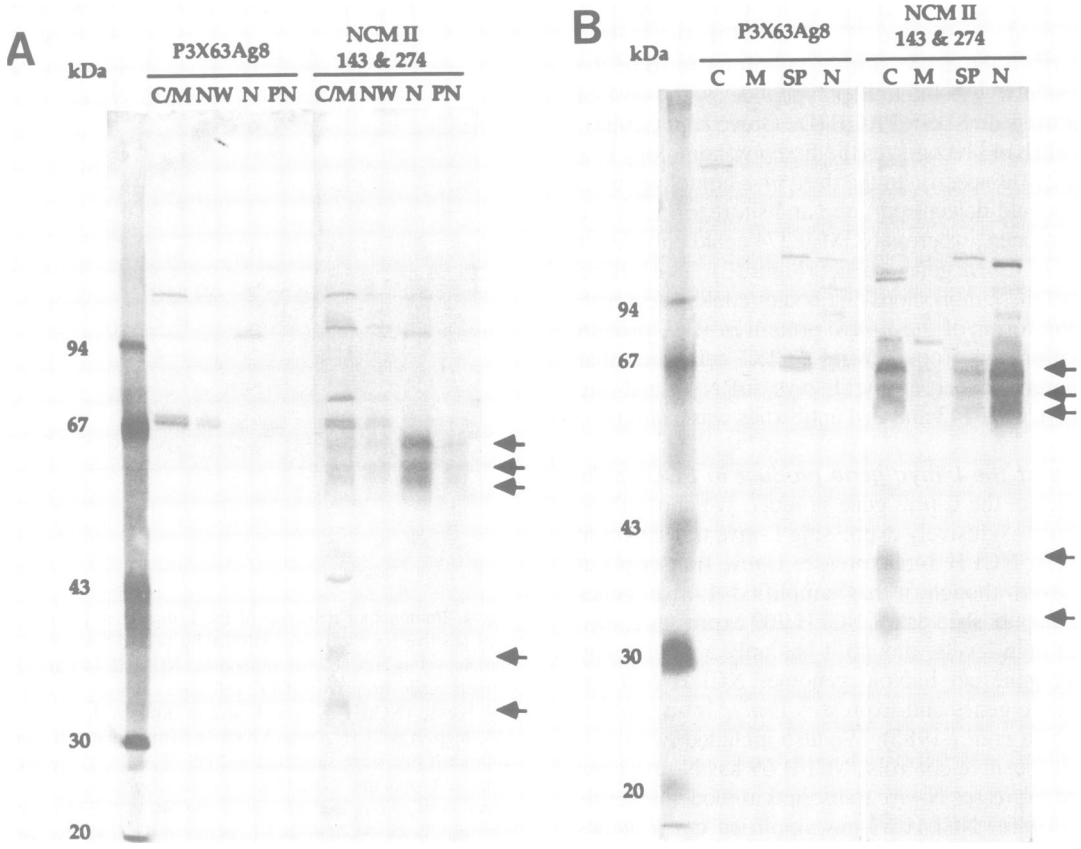

Fig. 5. Subcellular distribution of the L-*myc* gene products in NCI H 510 cells. (**A**) NCI H 510 cells were fractionated into cytoplasmic and membrane (C/M), nuclear wash (NW), nuclear (N) and post-nuclear (PN) fractions by a detergent lysis procedure. A 15-μl aliquot of each fraction was subjected to immunoblotting assay using a mixture of NCM II 143 and 274 antibodies, and a negative control, P3X63Ag8. (**B**) Cytoplasmic (C), membrane (M), subcellular particles (PS) and nuclear (N) fractions of NCI H 510 cells were prepared separately by a hypotonic shock/mechanical lysis method. A 25-μl aliquot of each fraction was subjected to immunoblotting assay using a mixture of NCM II 143 and 274, and P3X63Ag8 (a negative control). The arrows indicate the L-*myc* protein.

the short forms are present in cytoplasmic fraction of the cell, though the possibility that the short forms may have diffused out from the cell nucleus during the fractionation procedures cannot be ruled out.

Turnover rate of the L-myc gene products in the cell

It is known that both c-*myc* and N-*myc* gene products are relatively labile in the cell (Hann and Eisenman, 1984; Ikegaki *et al.*, 1986). To determine if the L-*myc* gene products also show similar characteristics, we measured the half-life of L-*myc* gene products in the cell. Extracts of NCI H 510 cells were prepared at various time points after the addition of cycloheximide, and then subjected to immunoblotting analysis (Figure 6). The half-life of each protein was estimated by comparing the intensity of bands at various time points with the band intensity of a half amount of zero time point. The half-life of 66, 61, 60, 37 and 34 kd polypeptides were estimated to be ~45, ~90, ~90, ~120 and ~180 min respectively. These results demonstrate that all the polypeptide species analysed have relatively short half-lives *in vivo*, though the short forms showed slightly longer half-lives than other *myc* family gene products.

The expression of L-myc gene products in individual cells

We next employed immunocytochemical staining methods to examine the pattern of expression of L-*myc* gene products in individual cells. Cytospin preparations of NCI H 510 cells were made and subjected to immunocytochemical detection of the L-*myc* protein using NCM II 143 (specific to the first *myc* box), NCM II 274 (specific to the third *myc* box) and P3X63Ag8 (a negative control). As shown in Figure 7B, NCM II 274, which reacts only with the long forms of the L-*myc* protein, stained nuclear structures of the cell. The stain excludes nucleoli. The levels of the staining intensity vary among the cells ranging from virtually none to very high, and interestingly no reactivity was observed in the mitotic cells (as indicated by the arrow).

Antibody NCM II 143, which reacts with both long and short forms of the L-*myc* protein, stained predominantly nuclear structures excluding nucleoli (Figure 7A and D). The intensity of the staining varied considerably among the cells. The mitotic cells (as indicated by the arrow) again did not show any detectable staining. Since NCM II 143 could detect long as well as short forms of the L-*myc* proteins and the short forms are expected to be present in the cell cytoplasm, this result was unexpected. However, it may be difficult to detect the short forms precisely by immunocytochemical methods, because the expression levels of short forms are lower than that of the long forms, and the cells expressing high levels of the L-*myc* protein are small cells with relatively low amounts of cytoplasm. Alternatively, short forms of the L-*myc* protein may be localized in the cell nucleus through weak interactions with certain nuclear components such as

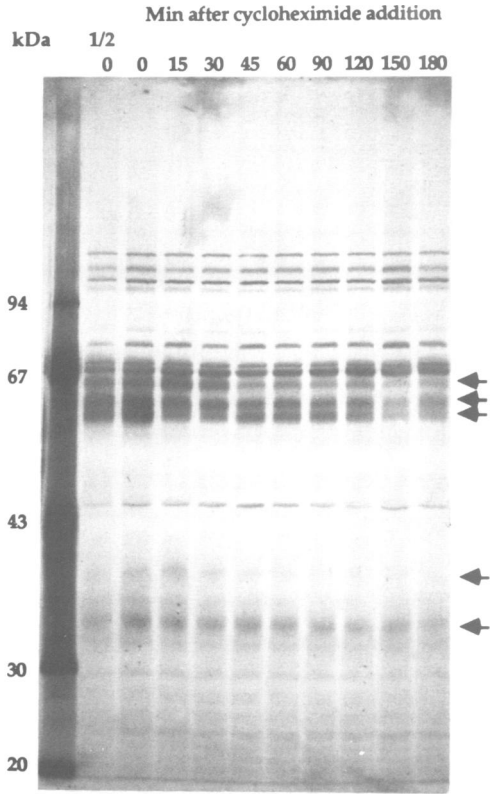

Fig. 6. Determination of half-lives of the L-*myc* gene products in NCI H 510 cells. Each aliquot of NCI H 510 cells was incubated at 37°C in the presence of cycloheximide (100 μg/ml) for the time periods indicated. At the end of each time point, the cells were immediately centrifuged in an Eppendorf centrifuge for 2 min and extracted with extraction buffer. The extract equivalent to 25 μg protein per time point was subjected to SDS−PAGE, followed by immunoblotting using NCM II 143 and 274 antibodies. The arrows indicate the L-*myc* protein.

the long forms of L-*myc* protein, but the interaction may be so weak that the short forms can easily diffuse out from the cell nuclei in the conditions used for the cell frationa-tion studies.

Discussion

The L-myc gene products in SCLC cells

Analysis of RNA transcripts and cDNA clones of the L-*myc* gene revealed that two distinct types of polypeptides could be encoded by the single L-*myc* gene (Kaye *et al.*, 1988). The predicted amino acid sequence of the larger polypep-tide shows several regions of homology to other *myc* gene family—products, suggesting that the characteristics of this protein might be different from those of other *myc* gene products.

In order to detect and characterize the L-*myc* gene product, we employed two types of anti-*myc* homology box monoclonal antibodies. One type is specific to the first *myc* homology box, and the other is specific to the third *myc* homology box. These antibodies enabled us to identify two groups of polypeptides in SCLC cell lines expressing *myc* transcripts. The long forms share both epitopes (in each of the two detected homology boxes) with other *myc* family gene products. The expression level of these species shows good positive correlation with levels of the L-*myc* transcripts present in the cell. These characteristics, as well as the similarity of the long form molecules to other *myc* gene family products in properties such as nuclear localization and short half-life strongly suggest that the long form molecules are indeed the large form of L-*myc* gene product. The long form molecules appear as at least three bands with apparent molecular masses of 60, 61 and 66 kd on SDS−PAGE, which are much larger than the predicted molecular mass for the large form of L-*myc* protein of ~40 kd. It is well known, however, that both c-*myc* and N-*myc* gene products migrate to a much larger molecular mass range on SDS−PAGE than would be expected from the predicted molecular masses, and appear also as multiple bands (Hann and Eisenman, 1984; Ikegaki *et al.*, 1986). A recent study (Hann *et al.*, 1988) demonstrated that the doublet appearance of the c-*myc* protein on SDS−PAGE is due to an alternative usage of CUG codon for the translational initiation located usptream of the major initiation codon, AUG. Expression of the L-*myc* gene may involve a similar mechanism generating multiple polypeptides of different sizes. On the other hand, the anomalous SDS−PAGE migration pattern of long form molecules might be due to some intrinsic physical property of the *myc* family proteins.

The short forms share at least one common epitope present in the second exon of the *myc* genes with the other *myc* family proteins. The multiple appearance of the short form polypeptides on SDS−PAGE may also be due to an alter-native usage of translation initiation sites as mentioned for the long form L-*myc* proteins. When a cDNA clone of the short form transcripts is transcribed by SP6 RNA polymerase and translated in a reticulocyte lysate *in vitro*, it gives rise to polypeptides of ~32 and 37 kd on SDS−PAGE, very similar sizes to those observed for the short form molecules (De Greve *et al.*, 1988). The short forms have also relatively short half-lives in the cell, though they are slightly longer than that of other *myc* proteins. The fractionation studies have shown that short form molecules are detectable in cyto-plasmic fraction of the cell, though the possibility that the short forms may have diffused from the cell nucleus during the fractionation procedures cannot be discounted. This possible cytoplasmic localization of the short forms may be due to lack of amino acid sequences encoded by the third exon of the L-*myc* gene, since the corresponding region of the c-*myc* gene has been implicated in nuclear transporta-tion of the protein (Stone *et al.*, 1987; Dang *et al.*, 1988). The low level expression of the short form molecules in the cell made it difficult for their detailed analysis, but these observations lend support to the notion that the short form molecules are indeed the short forms of L-*myc* gene product. Since the short forms of L-*myc* protein contain the unique sequence at the C-terminal (Figure 3), production of specific antibodies against the C-terminal portion of the short forms could facilitate their further characterization.

Recently, De Greve *et al.* (1988) reported the detection of the long form L-*myc* protein in an SCLC cell line as multiple nuclear phosphoproteins with relatively short half-lives. It is not clear why their antisera did not detect the short forms of L-*myc* protein *in vivo*. It could be due to differences in the method used for detection of the L-*myc* protein (i.e. their immunoprecipitation versus our immunoblotting). Nevertheless, their results on long forms of the L-*myc* protein are consistent with ours.

The L-myc gene products and the cell cycle

The immunocytochemical staining of the L-*myc* gene products revealed that expression of the L-*myc* protein is

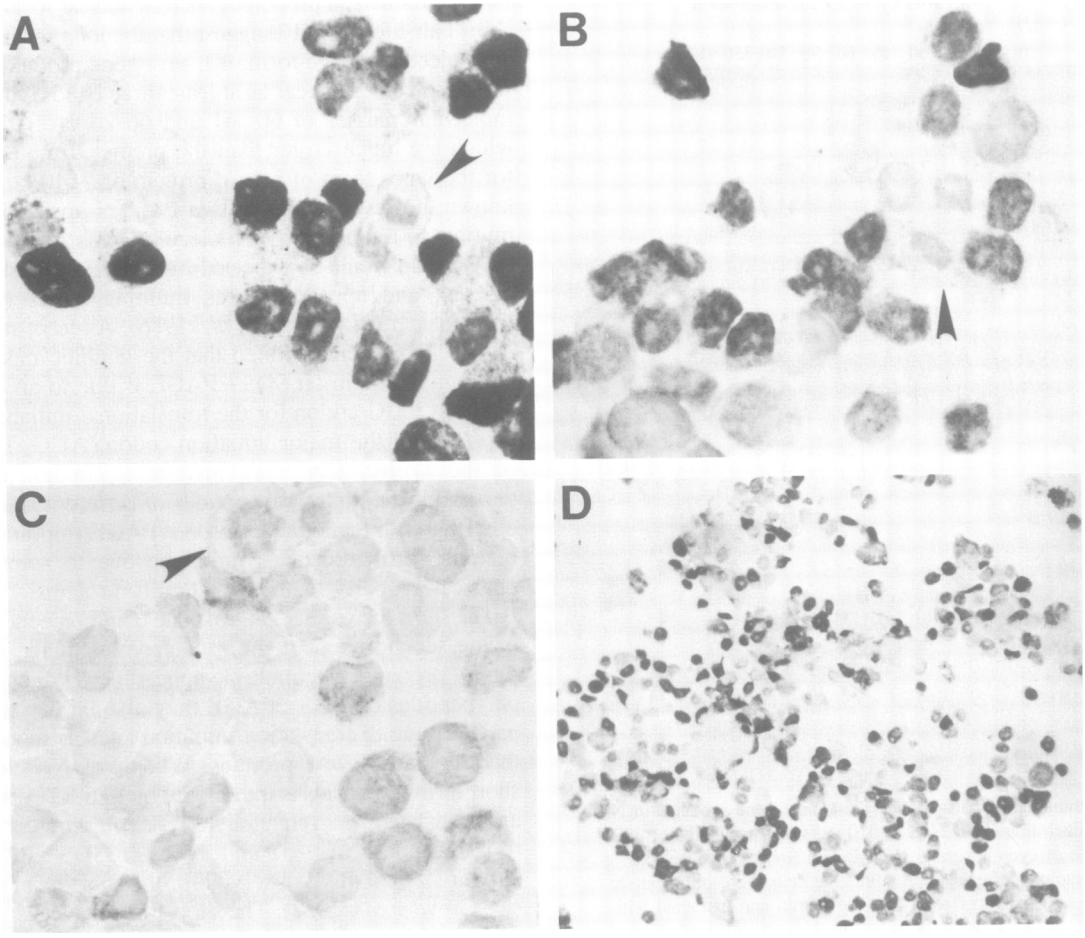

Fig. 7. Immunocytochemical detection of the L-*myc* protein in NCI H 510 cells. **(A)** NCM II 143 (specific to the first *myc* box) at high magnification; **(B)** NCM II 274 (specific to the third *myc* box) at high magnification; **(C)** P3X63Ag8 (a negative control) at high magnification; **(D)** NCM II 143 at low magnification to show clearly the heterogeneity in the L-*myc* protein expression among the cells. The arrows indicate mitotic cells.

very heterogeneous from cell to cell and it is particularly striking that the mitotic cells do not express detectable levels of long forms and probably also short forms of the L-*myc* protein. This feature of the L-*myc* protein expression is unique among the *myc* family proteins: the c-*myc* protein is expressed throughout the cell cycle (Hann *et al.*, 1985), and the N-*myc* protein is expressed in mitotic cells but it is not asociated with chromatin (N.Ikegaki, unpublished observation). This observation suggests that the L-*myc* protein expression is tightly regulated during the cell cycle, and further that the L-*myc* protein may be required in G1 period of the cell cycle since the cells expressing high levels of the L-*myc* protein are small cells with less cytoplasm.

The function of the L-myc gene products

We have demonstrated here that the L-*myc* gene is expressed as two groups of polypeptides. The long forms show a great deal of biochemical and biophysical similarities to other *myc* family gene products, including subcellular localization, *in vivo* turnover rates and migration on SDS−PAGE. Thus, the long forms of the L-*myc* gene product may have biological functions similar to those suggested for the c-*myc* gene product, including the regulation of gene expression (Kingston *et al.*, 1984; Spector *et al.*, 1987; Iguchi-Ariga *et al.*, 1988) and/or DNA replication (Classon *et al.*, 1987; Iguchi-Ariga *et al.*, 1987, 1988).

In contrast to the long forms of L-*myc* polypeptides, the short forms have characteristics differing from other *myc* proteins. The expression of these short forms seems to vary in different SCLC cell lines. It may therefore be important to analyse a number of cell lines that express different levels of the short forms and compare phenotypes of those cells in order to gain some insight into possible functions of the short forms of L-*myc* proteins.

The utility of anti-myc homology box monoclonal antibodies

The *myc* gene family proteins provided unique opportunity for utilization of mosaic of amino acid sequence homologies in order to map epitopes represented by monoclonal antibodies originally made against an N-*myc*−c-*myc* fusion protein. Through the analysis using gene dissections followed by bacterial expression of these segments and also native proteins expressed in cultured eukaryotic cells, we have identified at least two distinct types of anti-*myc* homology box monoclonal antibodies (N.Ikegaki *et al.*, in preparation, present study). Further development of anti-*myc* homology box monoclonal antibodies representing each conserved domain of the proteins would likely facilitate further analysis of the possible biological functions of the *myc* proteins *in vivo* and *in vitro*.

Materials and methods

Cell lines

All SCLC cell lines (NCI H 69, H 82, H 209, H 345, H 510) were maintained in RPMI 1640 supplemented with 5% fetal calf serum.

Monoclonal antibodies

Monoclonal antibodies described in this paper were originally produced against an N-*myc*−c-*myc* fusion protein expressed in *E.coli* (Ikegaki *et al.*, 1986). Further detailed characterization of each of the monoclonal antibodies will be reported elsewhere (N.Ikegaki *et al.*, in preparation).

Oligopeptide synthesis

Oligopeptide corresponding to amino acid residues 24−38 of the L-*myc* protein and 257−271 of the c-*myc* protein were synthesized by an automated peptide synthesizer (Applied Biosystems Model 430A) in Dr Angeletti's laboratory in the University of Pennsylvania School of Medicine. An additional cysteine residue was attached to the N-terminus of the former peptide.

ELISA

Ninety-six well microtitre plates were coated with 1−5 μg of appropriate oligopeptide per well. The plates were then blocked with 0.1% bovine serum albumin (BSA) and 0.5% fetal calf serum in phosphate-buffered saline (PBS). Monoclonal antibodies were added to the wells. After a 1 h incubation, excess antibodies were washed off with PBS containing 0.05% Tween 80, and goat anti-mouse immunoglobulins conjugated with horseradish peroxidase and substrate/chromogen mixture (H_2O_2/*o*-phenylenediamine) were added sequentially after appropriate washes. Color generated by the reaction was measured by a Multiscan spectrophotometer (Flow Lab.) at 450 nm.

Immunoblotting assay

The total cell lysate was prepared by extracting the cell pellets with 2 × PBS containing 1% Nonidet P-40 (NP-40), 0.5% deoxycholate, 0.1% SDS, leupeptin (10 μg/ml) and chymostatin (10 μg/ml) (extraction buffer). The protein concentration of the lysate was determined by the BioRad protein assay kit with BSA as a standard. A portion equivalent to 25−40 mg protein was subjected to SDS−PAGE, and then transferred to nitrocellulose paper by electrophoresis as described by Towbin *et al.* (1979) or Dunn (1986). Blots were incubated for 1 h in 2% BSA, 2% Ficol and 2% polyvinylpyrrolidone made in 50 mM Tris−HCl, pH 7.6, to block non-specific binding of antibodies. Monoclonal antibodies were then added to the blot, and the incubation was carried out overnight at 4°C. After washing off excess antibodies, the specifically bound antibodies were fixed on the blot by 0.25% glutaraldehyde in cold PBS. To visualize the specific reaction of monoclonal antibodies, the blot was incubated sequentially with biotinylated goat anti-mouse immunoglobulin antibodies, horseradish peroxidase-conjugated Streptavidin and substrate/chromogen (H_2O_2/diaminobenzidine) mixture.

Cell fractionation

Detergent lysis procedure. Cell pellet (100 mg) was homogenized in 1 ml of PBS containing 0.5% NP-40 by pippetting 20 times by an autopippetter. The homogenate was centrifuged at 100 *g* for 5 min. The supernatant was collected as cytoplasmic/membrane fraction. The pellet was resuspended in 1 ml of PBS containing 0.25% NP-40, then centrifuged at 100 *g* for 5 min. The supernatant was collected as nuclear wash fraction. The pellet was extracted with extraction buffer by vigorous vortexing. The resulting mixture was centrifuged in an Eppendorf centrifuge for 5 min. The supernatant was collected as nuclear fraction. The pellet was further extracted with 4% SDS, 10% 2-mercaptoethanol, 20% glycerol made in 0.25 M Tris−HCl, pH 6.8, and centrifuged in an Eppendorf centrifuge for 10 min. The supernatant was collected as a post-nuclear fraction.

Hypotonic shock/mechanical lysis procedure. A 100-mg portion of an NCI H 510 cell pellet was suspended in 4 ml of 25 mM Hepes, pH 7.2, containing 5 mM KCl and 1 mM $MgCl_2$, and incubated on ice for 15 min. The cell suspension was then homogenized in a Dounce glass homogenizer with an A-pestle 20 times. The homogenate was centrifuged at 100 *g* for 5 min. The pellet was extracted with 1 ml of extraction buffer. The supernatant was collected after centrifuging the extract in an Eppendorf centrifuge and termed the nuclear fraction. The 100 *g* supernatant was then centrifuged at 2000 *g* for 15 min. The pellet was extracted with 1 ml of extraction buffer and centrifuged in an Eppendorf centrifuge, and the resultant supernatant was collected and termed the subcellular particle fraction. The 2000 *g* supernatant was further centrifuged at 100 000 *g* for 30 min. The pellet was dissolved in 1 ml of extraction buffer and termed the membrane fraction. The supernatant was concentrated to 1 ml by a Centricon 30 (Amicon) and termed the cytoplasmic fraction. Leupeptin (10 μg/ml) and chymostatin (10 μg/ml) were added to all the solutions as proteinase inhibitors.

Immunocytochemical assay

NCI H 510 cells were cytofuged on glass slides coated with Concanavalin A at 4°C, fixed in 3.7% formaldehyde and 0.25% glutaraldehyde made in PBS, and permeabilized in 2% Triton X-100 and 0.05% glutaraldehyde made in PBS at room temperature. The slides were then treated with 10% goat serum, 0.5% gelatin and 0.1 M glycine in PBS to block non-specific binding of antibodies. The slides were incubated sequentially with monoclonal anti-*myc* antibodies, goat anti-mouse immunoglobulin conjugated with biotin, and streptavidin conjugated with peroxidase. The substrate/chromogen mixture containing H_2O_2 and diaminobenzidine made in Tris-buffered saline was then added to visualize the antibody reaction. The signal obtained was further intensified with the 'DAB enhancement kit' (Amersham) according to the company's instructions.

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