Jpn. J. Cancer Res. (Gann), 77, 540–545; June, 1986

# INCREASED EXPRESSION OF THE c-*myc* GENE WITHOUT GENE AMPLIFICATION IN HUMAN LUNG CANCER AND COLON CANCER CELL LINES

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**High levels of c-*myc* mRNA were observed in two human tumor cell lines, a giant cell carcinoma of the lung (C-Lu99) and a colon cancer (C1). The increased expression of c-*myc* in these cell lines, which was comparable with those in cell lines in which the c-*myc* gene is amplified, was not due to gene amplification. Run-on transcription revealed that the transcriptional rate of the c-*myc* gene was greatly increased in these cell lines.**

Key words: c-*myc* — Lung cancer — Colon cancer — Increased expression — Run-on transcription

The *myc* oncogene was first detected as the transforming sequence of avian myelocytomatosis viruses.[1] The cellular homologue, c-*myc*, is expressed in a variety of normal and tumor tissues,[2,3] and recent reports indicate that its expression is under stringent control in normal cells.[4,5] Treatment of cells with platelet-derived growth factor or mitogens leads to rapid increase in c-*myc* mRNA soon after treatment.[4] Normal regulation is lost when cells are chemically transformed.[5] Therefore, tumorigenesis may result from altered regulation of the c-*myc* gene and, in some cases, loss of its normal control. In this paper we report that the c-*myc* gene was over-expressed but not amplified in two human tumor cell lines.

## MATERIALS AND METHODS

**Cell Lines** Seven tumor-derived human cell lines were examined; a promyelocytic leukemia cell line (HL-60), a hepatoma cell line (PLC/PRF/5, Alexander), a retinoblastoma cell line (Y79), a Wilms' tumor cell line (W2), a colon cancer cell line (C1) derived from a metastatic focus in the lung (poorly differentiated adenocarcinoma) and two cell lines of giant cell carcinoma of the lung (C-Lu65 and C-Lu99).[6] All these cell lines were maintained in RPMI1640 medium supplemented with 10% fetal calf serum.

**Isolation of DNA and mRNA from Cultured Cells** High-molecular-weight DNAs from cultured cells were prepared as described by Blin and Stafford.[7] The poly(A)$^+$ RNA fraction from these cells in the late logarithmic stage of cell growth was prepared by the guanidinium thiocyanate-hot phenol method and purified by oligo(dT) cellulose column chromatography.[8]

**Probes** The recombinant pNCO501 contains the *Xho*I-*Xba*I fragment corresponding to human c-*myc* exon 1, referred to as c-*myc*(5′), and pUC19 as a vector. The plasmid pMCE2 carries the *Cla*I-*Eco*RI fragment containing human c-*myc* exon 3, referred to as c-*myc*(3′), in pBR322.

**Electrophoresis and Detection of c-*myc* mRNA and DNA** Poly(A)$^+$ RNA (3 $\mu$g) from tumor cells was separated by electrophoresis in 1% agarose gel containing 2.2*M* formaldehyde and then transferred to a Biodyne™ A membrane (PALL).[8] The filter was baked at 80° and then hybridized to the nick-translated probe. Hybridization was performed at 42° for 16 hr in 6× SSC (1×SSC is 0.15*M* NaCl/0.015*M* sodium citrate)–10m*M* EDTA–5×Denhardt's solution–0.5% SDS–100 $\mu$g/ml denatured salmon sperm DNA–10% dextran sulfate–50% formamide. The membrane was then washed with 0.2×SSC at 50°. The membrane was exposed to Kodak XRP-5 film for 2 days at −70° with an intensifying screen.

Samples of 5 $\mu$g of each DNA digested with *Eco*RI were separated by electrophoresis on 0.7% agarose gel and transferred to a Biodyne™ A membrane (PALL) as described by Southern.[9] Hybridization with $^{32}$P-labeled c-*myc*(3′) probe was carried out under the same conditions as for mRNA analysis.

**Nuclear Run-on Transcription Analysis**

The incorporation of [$\alpha$-$^{32}$P]UMP into nascent mRNAs in isolated nuclei was carried out as described by Greenberg and Ziff[10] with slight modifications. The nuclear fraction (100 $\mu$l) was prepared from cultured cells ($1\times10^7$ cells) as described previously.[10] The nuclear fraction was mixed with 100 $\mu$l of reaction buffer (10m*M* Tris-HCl, pH 8.0, 5m*M* $MgCl_2$, 300m*M* KCl, 0.5m*M* each of ATP, CTP and GTP, 100 $\mu$Ci of [$\alpha$-$^{32}$P]UTP (Amersham, 410 Ci/mmol), 2.5m*M* dithiothreitol, 2m*M* aluminon (aurintricarboxylic acid), 0.2m*M* phenylmethanesulfonylfluoride, and 0.2 mg/ml heparin) and incubated at 30° for 20 min. Under these conditions, incorporation of [$\alpha$-$^{32}$P]UMP into RNA linearly increased up to 30 min. The $^{32}$P-labeled RNA was isolated by the guanidinium thiocyanate-hot phenol method[9] and then treated with DNase I (40 $\mu$g/ml) at 37° for 30 min. Equal amounts of run-on products ($1\times10^7$ cpm) were then hybridized to nitrocellulose filters carrying dot-spots of 5 $\mu$g of alkali-denatured plasmid DNA of [1] pUC19, [2] pNCO501, [3] pBR322, [4] pMCE2 and [5] pAl containing chick $\beta$-actin cDNA.[11] Hybridization was performed at 70° for 36 hr in 10m*M* N-tris (hydroxymethyl) 2-aminoethanesulfonic acid (TES), pH 7.4, 0.2% SDS, 10m*M* EDTA, 0.3*M* NaCl, 100 $\mu$g/ml denatured salmon sperm DNA and run-on products at $1\times10^7$ cpm/ml. After hybridization, filters were washed in $0.2\times$SSC at 50°, and subjected to autoradiography.

## Results and Discussion

The level of c-*myc* mRNA was examined in seven human tumor cell lines. Figure 1A shows the level of c-*myc* mRNA in tumor cells, determined by RNA blot analysis with c-*myc*(3′) as a probe. High levels of c-*myc* mRNA were found in HL-60, C-Lu65, C-Lu99 and Cl cells, and low levels in Alexander, Y79 and W2 cells. The transcripts of c-*myc* in Y79 and W2 cells can hardly be seen in Fig. 1A. However, longer exposure of the autoradiogram showed clear 2.4 kb bands. When the values were normalized to that of C-Lu65 cells, the relative levels of the c-*myc* mRNA in the former four cell lines were 1.1,

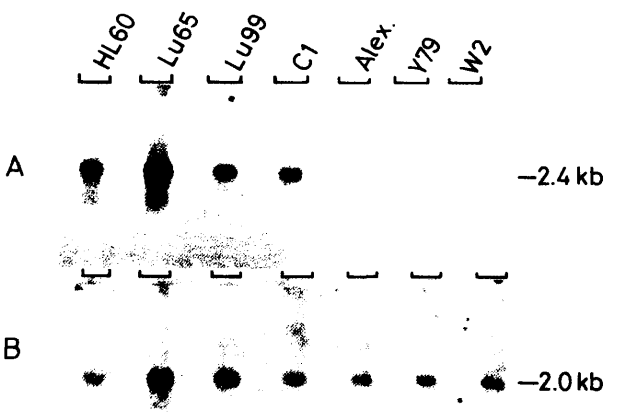


Fig. 1. Expression of c-*myc* and $\beta$-actin in human tumor cell lines. (A) Poly(A)$^+$ RNA (3 $\mu$g) from tumor cells was separated by electrophoresis in 1% agarose gel containing 2.2*M* formaldehyde and then transferred to a Biodyne™ A membrane (PALL),[9] and hybridized to the nick-translated c-*myc*(3′) probe. (B) After being allowed to undergo several half-lives of $^{32}$P decay of the initial c-*myc* probe, the same membrane as for (A) was hybridized to the chick pA1 $\beta$-actin complementary DNA probe under the same conditions as for (A).

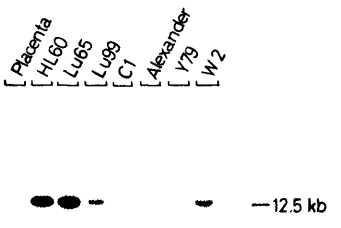


Fig. 2. Southern blot analysis of the c-*myc* gene in human tumor cell lines. High-molecular-weight DNAs from cultured cells were prepared as described by Blin and Stafford.[7] Samples of 5 $\mu$g of each DNA digested with *Eco*RI were separated by electrophoresis on 0.7% agarose gel and transferred to a Biodyne™ A membrane (PALL) as described by Southern.[9] Hybridization with $^{32}$P-labeled c-*myc*(3′) probe was carried out under the same conditions as for Fig. 1.

1, 0.7 and 1, respectively, as judged by comparing the amounts of c-*myc* mRNA with those of $\beta$-actin mRNA, shown in Fig. 1B, as an internal control. In contrast to c-*myc* mRNA, the mRNA level of N-*myc*, a c-*myc* related gene, was low in all the cell lines except Y79, in which it was high, as described by Lee *et al.*[12] (data not shown).

Since amplification of the gene is one possible reason for a high level of c-*myc* mRNA, we digested DNA from each cell line with *Eco*RI and compared the Southern blot hybridization profiles (Fig. 2). HL-60 and C-Lu65 cells gave intense bands of material of 12.5 kb. This finding is consistent with a report that the c-*myc* gene is amplified about 16-fold in HL-60 cells[13] and about 8-fold in C-Lu65 cells.[14] Therefore, the increased levels of c-*myc* mRNA in HL-60[2] and C-Lu65 cells may reflect the gene amplification in these cells. Similar high levels of c-*myc* mRNA associated with gene amplification have been demonstrated in human stomach cancers transplanted into nude mice,[15] a human APUDoma COLO320 cell line of neuroendocrine origin,[16] human cell lines of small cell carcinoma of the lung,[17] and a Morris hepatoma of rats.[18]

On the other hand, the c-*myc* gene was not amplified in C-Lu99 or Cl cells, indicating that the increased level of c-*myc* mRNA in these cells was not due to gene amplification. The slightly stronger signals in C-Lu99 and W2 DNA than those in placenta, Cl, Alexander and Y79 DNA in Fig. 2 do not indicate amplification of the c-*myc* genes. These differences were brought about by application of a larger amout of DNA to the gel for electrophoresis, because stronger signals were also observed in the cases of DNA probes for different genes on the same filter. Increased expression of the c-*myc* gene not associated with gene amplification was also observed in a Morris hepatoma of rats by Hayashi *et al.*[18] and in a human cell line of small cell carcinoma of the lung by Little *et al.*[17] Recently, extreme instability of c-*myc* mRNA (half-life, 10 min) was demonstrated in normal and transformed cells[19] and post-transcriptional regulation of the c-*myc* mRNA level by mRNA degradation was proposed.[20, 21] To distinguish whether the high levels of c-*myc* mRNA in C-Lu99 and Cl cells are the result of differences in the transcriptional rate or in post-transcriptional events, we performed nuclear run-on transcription experiments, in which the relative rates of elongation and polymerase density along specific genes can be determined.[10]

The results in Fig. 3 clearly indicate that the levels of transcripts of the c-*myc* gene in nuclei from HL-60, C-Lu65, C-Lu99 and Cl cells are high, while those in Y79 and W2 nuclei are low. Thus, the transcriptions of the gene are correlated with the levels of the mRNA in these tumor cell lines. For quantitative comparison of the transcriptional rates of the gene in these cell lines using those of the $\beta$-actin gene as an internal standard, individual dots were cut out from the blot and their radioactivities were determined in a scintillation counter. The results re-

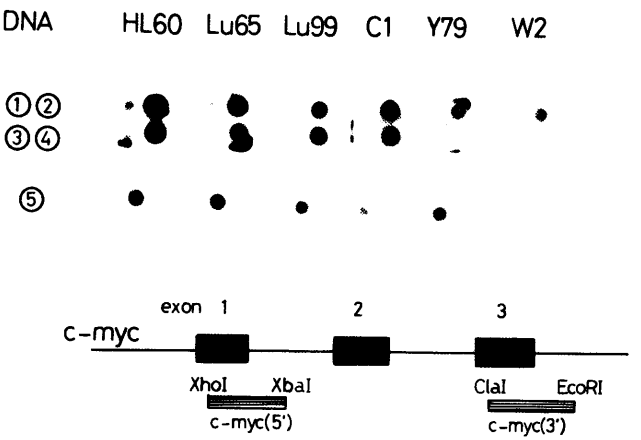


Fig. 3. Nuclear run-on transcription analysis in human tumor cell lines. The incorporation of [$\alpha$-$^{32}$P]UMP into nascent mRNAs in isolated nuclei was carried out as described by Greenberg and Ziff[10] with slight modifications. Equal amounts of run-on products ($1 \times 10^7$ cpm) were then hybridized to nitrocellulose filters carrying dot-spots of 5 $\mu$g of alkali-denatured plasmid DNA of [1] pUC19, [2] pNCO501, [3] pBR322, [4] pMCE2 and [5] pA1 containing chick $\beta$-actin cDNA. The recombinant pNCO501 contains the *Xho*I-*Xba*I fragment corresponding to human c-*myc* exon 1, referred to as c-*myc*(5′), and pUC19 as a vector. The plasmid pMCE2 carries the *Cla*I-*Eco*RI fragment containing human c-*myc* exon 3, referred to as c-*myc*(3′), in pBR322.

vealed that the relative levels of c-*myc* gene transcripts in HL-60, C-Lu99, Cl, Y79 and W2 nuclei with respect to that in C-Lu65 nuclei were 2.7, 0.6, 0.7, 0.1 and 0.3, respectively. Taking c-*myc* gene amplification in HL-60 (16-fold[13]) and C-Lu65 (8-fold[14]) cells into consideration, the results indicate that the transcriptional rates of one copy of the c-*myc* gene are 3–5 times higher in C-Lu99 and Cl cells than in HL-60 or C-Lu65 cells. Therefore, we concluded that accelerated transcription of the c-*myc* gene could be involved in the high level of c-*myc* mRNA without amplification of the corresponding genes in C-Lu99 and Cl cells.

Enhanced c-*myc* gene transcription could result from loss or disruption of regulatory elements. From this point of view, rearrangement of the c-*myc* gene in C-Lu99 and Cl cells was analyzed. Rearrangement at least in the region from 12 kb upstream of exon 1 to 0.5 kb downstream of exon 3 was eliminated by Southern blot hybridization with the c-*myc*(5′) probe after digestion of the DNAs with *Eco*RI or *Sst*I (data not shown). In the case of C-Lu99 cells, possible rearrangement beyond the region analyzed was also eliminated by karyotype analysis, which revealed no chromosomal translocation in chromosome 8.[6] Another possibility is that some unknown trans-acting factor(s) to the regulatory region of the c-*myc* gene is involved in C-Lu99 and Cl cells. Studies on this possibility are in progress.

The present observation of accelerated transcription of the non-amplified c-*myc* gene in C-Lu99 and Cl cells, which contain high levels of c-*myc* mRNA, comparable with those in HL-60 and C-Lu65 cells in which the c-*myc* gene is amplified, suggests

the possible involvement of c-*myc* gene products in the transformation of these cell lines.

## Acknowledgments

We are grateful to Drs. M. Miwa, Y. Yoshida, T. Nakajima, M. Sekiguchi and Y. Hayata for providing the HL-60, PLC/PRF/5, Y79, W2 and Cl cell lines, respectively. We are also indebted to Drs. Y. Taya, S. Noguchi, S. Sakiyama and D. W. Cleveland for gifts of the genomic clone of human c-*myc* from C-Lu65, pMCE2 and $\beta$-actin cDNA clone (pAl), respectively. This work was supported by a Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, Japan. Katsuhiko Yoshimoto was the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research.

(*Received Feb. 26, 1986/Accepted April 23, 1986*)

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