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 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 XhoI-XbaI fragment corresponding to human c-myc exon 1, referred to as c-myc(5'), and pUC19 as a vector. The plasmid pMCE2 carries the ClaI-EcoRI 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 μg) from tumor cells was separated by electrophoresis in 1% agarose gel containing 2.2M 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 0.15M NaCl/0.015M sodium citrate)-10mM EDTA-5× Denhardt's solution-0.5% SDS-100 μg/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 μg of each DNA digested with EcoRI were separated by electrophoresis on 0.7% agarose gel and transferred to a Biodyne™A membrane (PALL) as described by Southern.9) Hybridization with 32P-labeled c-myc(3') probe was carried out under the same conditions as for mRNA analysis.
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Nuclear Run-on Transcription Analysis
The incorporation of [$\alpha$-32P]UMP into nascent mRNAs in isolated nuclei was carried out as described by Greenberg and Ziff\(^{10}\) with slight modifications. The nuclear fraction (100 µl) was prepared from cultured cells (1 x 10^7 cells) as described previously.\(^{10}\) The nuclear fraction was mixed with 100 µl of reaction buffer (10mM Tris-HCl, pH 8.0, 5mM MgCl₂, 300mM KCl, 0.5mM each of ATP, CTP and GTP, 100 µCi of [$\alpha$-32P]UTP (Amersham, 410 Ci/mmol), 2.5mM dithiothreitol, 2mM aluminon (aurintricarboxylic acid), 0.2mM phenylmethanesulfonylefluoride, and 0.2 mg/ml heparin) and incubated at 30°C for 20 min. Under these conditions, incorporation of [$\alpha$-32P]UMP into RNA linearly increased up to 30 min. The 32P-labeled RNA was isolated by the guanidinium thiocyanate-hot phenol method\(^8\) and then treated with DNase I (40 µg/ml) at 37°C for 30 min. Equal amounts of run-on products (1 x 10^7 cpm) were then hybridized to nitrocellulose filters carrying dot-spots of 5 µg of alkali-denatured plasmid DNA of [1] pUC19, [2] pNCO501, [3] pBR322, [4] pMCE2 and [5] pA1 containing chick β-actin cDNA.\(^{11}\) Hybridization was performed at 70°C for 36 hr in 10mM N-tris (hydroxymethyl) 2-aminoethanesulfonic acid (TES), pH 7.4, 0.2% SDS, 10mM EDTA, 0.3M NaCl, 100 µg/ml denatured salmon sperm DNA and run-on products at 1 x 10^7 cpm/ml. After hybridization, filters were washed in 0.2 x SSC at 50°C, 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,
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1, 0.7 and 1, respectively, as judged by comparing the amounts of c-myc mRNA with those of β-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 EcoRI 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 cells13) and about 8-fold in C-Lu65 cells.14) Therefore, the increased levels of c-myc mRNA in HL-602) 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,10) 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 amount 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 cells10) 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 β-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-
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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) and C-Lu65 (8-fold) 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 EcoRI or SstI (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. 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.

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