Comparison of Amplified and Unamplified c-myc Gene Structure and Expression in Human Small Cell Lung Carcinoma Cell Lines

Eric J. Seifert, Edward A. Sausville, and Jim Battey

National Cancer Institute-Navy Medical Oncology Branch, Clinical Oncology Program/Division of Cancer Treatment/NIH, Naval Hospital, Bethesda, Maryland 20814

ABSTRACT

A human small cell lung cancer cell line (H82) demonstrates 40- to 50-fold amplification of the c-myc gene but expresses at least 250-fold more steady-state c-myc messenger RNA than an unamplified small cell lung cancer cell line (H378) with no detectable expression of c-myc. We compared the chromatin structure of c-myc in H82 to that in H378 using DNase I sensitivity and DNA methylation patterns. DNase I hypersensitivity sites were identical in H82 and H378 and were similar to the pattern seen in a B-lymphoblastoid cell line, despite extensive amplification of c-myc in H82. Methylation patterns were also very similar in H82 and H378, with hypomethylation or partial methylation at the c-myc coding regions and the flanking 5' sequences, despite the absence of detectable c-myc expression in H378. Therefore, the predominant chromatin structural patterns do not appear to correlate with observed differences in gene expression. In addition, these studies demonstrate that the patterns of DNase I hypersensitivity and of methylation can remain intact during a 40- to 50-fold gene amplification, as observed for the c-myc gene in H82.

INTRODUCTION

The c-myc gene is the homologous cellular gene to a transforming gene (v-myc) identified in the avian myelocytomatosis virus (1). There is evidence that links genetic changes involving c-myc structure and expression with a variety of cancers (2-5). Several human tumor cell lines and surgical specimens demonstrate high levels of steady-state c-myc mRNA (6, 7). Amplification of c-myc DNA with increased expression also has been found in several malignant cell lines (8-11), including lines derived from human SCLC (12). Alteration of chromatin structure represents a mechanism for modulating expression of some genes (13, 14). Transcriptionally active genes often reside in chromosomal regions characterized by increased sensitivity to DNase I and by hypomethylation of the CpG (cytosine-guanine) dinucleotide (15-19). It is unknown whether gene amplification retains or alters the pattern of DNA methylation. Similarly, it is unknown whether DNase I hypersensitivity sites are maintained during amplification. These issues have not been addressed rigorously for the c-myc gene, particularly in nonlymphoid cells. We examined DNase I hypersensitivity sites and DNA methylation patterns in the c-myc gene originating from two sources: a human SCLC cell line without amplification or clearly detectable expression of c-myc (H378) and a cell line with both amplification and enhanced expression of this oncogene (H82). We find that the amplified genes preserve a chromatin structure associated with unamplified, nonmalignant B-lymphoblastoid cells.

MATERIALS AND METHODS

Cell Culture. The cell lines H82 and H378 were established from pleural effusions obtained from patients with SCLC. Pleural fluid was collected in preservative-free heparin, and the cells were concentrated on a Ficoll-Hypaque gradient. H82 was maintained in antibiotic-free RPMI-1640 medium supplemented with 10% fetal bovine serum, while H378 grew in serum-free and antibiotic-free RPMI-1640 supplemented with hydrocortisone, insulin, transferrin, estradiol, and selenium (20). Cells were maintained and harvested at cell concentrations below 1.0 × 10⁶ cells/ml.

Isolation and Digestion of Nuclei. Nuclei were isolated from the cell lines as described previously (21) and were digested in various concentrations of DNase I (0 to 30 units/ml) for 3 min at room temperature (with each reaction containing 3 × 10⁶ cell nuclei). DNA samples obtained from nuclei digested with DNase I (21) were redissolved in 10 mM Tris-HCl:1 mM EDTA, pH 7.4. DNA concentration was measured by absorbance at 260 nm, and equal amounts of DNA were used in subsequent digestions with restriction endonucleases.

Identification of DNase I Hypersensitivity Sites. Following preparation of DNase I-digested DNA from isolated nuclei, 15-μg samples were digested with EcoRI and StII, electrophoresed on agarose gels, and transferred to nitrocellulose (22). Hypersensitive sites were visualized as subbands by the indirect end-labeling technique first described by Wu et al. (23). The Clal-EcoRI (CR) and PvuII-XbaI (PX) c-myc probes used in this study are as previously described (21, 24).

Methylation Studies. DNA samples from the H82 and H378 cell lines were prepared as described previously (25) and quantified by absorbance at 260 nm. Equal concentrations of DNA were completely digested with EcoRI, HindIII, or StII. After extraction and ethanol precipitation, the samples were digested with MspI or HpaII. The samples were then electrophoresed, transferred to nitrocellulose, and hybridized with DNA restriction fragment probes labeled by nick translation (16). Hybridized filters were washed at 52°C in 10 mM Tris-HCl, 1.5 mM sodium citrate, and 0.1% sodium dodecyl sulfate. The HindIII-Clal (HC), RsaI-XbaI (RX), and Clal-EcoRI (CR) c-myc probes have been described previously (24). As mitochondrial DNA should not contain any internally methylated 5'-CCG sequences, a mitochondrial probe was used in an attempt to ensure complete digestion with MspI and HpaII (27). To assure that identification of methylated sites was possible, we used a probe from the IgE constant region (2.6-kilobase BamHI fragment) (28), a gene which is methylated and is not expected to be expressed in SCLC cell lines.

Preparation of RNA and S1 Nuclease Mapping. Total cytoplasmic RNA was prepared as described by Chirgwin et al. (29), and the RNA concentration was measured by absorbance at 260 nm. The RNA was denatured and electrophoresed on a 1% agarose:formaldehyde gel at 120 V for 4 h. Subsequent nitrocellulose transfer, hybridization, and washing were essentially as described previously (30). Single-stranded 32PdAmass-labeled DNA probes were prepared for S1 nuclease mapping (24, 31). This method allows synthesis of a complementary DNA strand on the labeled strand was with BamHI prior to preparative electrophoresis on an 8 M urea:5% acrylamide gel. After electroelution, hybridization with 0-25 µg of total cytoplasmic RNA was performed at 59°C for 18 h as described previously (24) and was terminated by the addition of 2000 units of S1 nuclease (Boehringer-Mannheim). After incubation at 37°C for 1 h, the samples were extracted with phenol:chloroform, ethanol precipitated, and electrophoresed on 8 M urea:5% acrylamide gels.

RESULTS

Amplification of c-myc DNA in the H82 Cell Line. The human SCLC cell line H82 demonstrates amplification of c-myc DNA...
(12). A serial dilution was performed to accurately compare the number of c-myc genes in H82 DNA to a nonamplified c-myc cell line. H82 DNA was completely digested with the restriction endonuclease EcoRI, serially diluted, and then compared visually on agarose gels stained with ethidium bromide and by densitometric analysis to similarly restricted samples of H378 DNA and normal pituitary DNA (Fig. 1). The autoradiogram demonstrates a 12.5-kilobase EcoRI fragment which is the germ-line or nonrearranged human c-myc gene fragment. The human SCLC cell line H378 shows no amplification of the c-myc gene by comparison to normal pituitary DNA. Relative to the H378 band, H82 shows an equivalent intensity band at a 1:40 to 1:50 dilution. This suggests a 40- to 50-fold amplification of the relative amount of c-myc DNA in H82. Several dilutions were performed to confirm this result. An additional 7.0-kilobase band is evident in the H82 DNA digests (Fig. 1). Analyses of additional preparations of H82 digested with EcoRI, SstI, and HindIII and utilizing multiple probes suggest that changes may occur in the 2.0-kilobase region 5' to the promoter sites; however, all three exons of c-myc appear intact in the 7.0-kilobase rearranged segment (data not shown). We cannot ascertain what proportion of the enhanced expression of c-myc in H82 derives from the lesser amplified rearranged segments.

Steady-State c-myc mRNA in the H82 and H378 Cell Lines. S1 nuclease mapping was utilized to compare the amount of steady-state c-myc message in the total cytoplasmic RNA of H82 to that of H378 (Fig. 2). The normal c-myc gene directs transcripts from two promoter sites, P1 and P2, located about 160 base pairs apart (24). Total cytoplasmic RNA was hybridized to a uniformly labeled probe from the c-myc initiation region, an 860-base pair PvuII-PvuII fragment. This probe extends from 350 base pairs 5' of the first initiation site to 350 base pairs 3' of the second initiation site. The more 3' of these transcription initiation sites (related to P2) is predominantly represented in the steady-state c-myc transcripts derived from the H82 cell line. This is the same pattern of promoter utilization as described previously for B-lymphoblastoid cells (31). Thus, c-myc amplification in H82 proceeds with retention of a normal pattern of promoter utilization.

There are no detectable changes in exon 1 of the expressed c-myc genes in H82; the predicted fragments of 510 and 360 base pairs are protected from S1 nuclease digestion (Fig. 2). The S1 nuclease analysis reveals essentially no detectable c-myc mRNA in H378. Serial dilution of H82 suggests at least a 250-fold increase in c-myc mRNA as compared to H378. These results were supported by serial dilution of RNA on Northern blot analysis, which reveals the expected 2.3-kilobase hybridizing band in H82 RNA, but no detectable band in H378 RNA (data not shown). Hybridization with a human β-actin probe (32) under these conditions revealed the expected 2.1-kilobase message equally abundant in both H82 and H378 RNA (data not shown).

DNase I Hypersensitivity Sites at the myc Locus in the H82 and H378 Cell Lines. The DNase I hypersensitivity sites were visualized as subbands by the indirect end-labeling technique (15). DNA extracted from nuclei digested gently with DNase I was hybridized to a labeled probe from either end of a genomic restriction fragment. The expected genomic fragment progressively diminishes with increasing concentrations of DNase I, as the subbands gradually appear. The size of the subband fragments allows mapping of the distance between the hypersensitive sites and the end of the genomic restriction fragment as identified by the labeled probe. Fig. 3 shows the DNase I hypersensitivity sites for H378 and H82. For the samples digested with EcoRI and hybridized with the Clal-EcoRI (CR) probe, a 12.5-kilobase germ-line fragment is evident in both cell lines. A less intense 7.0-kilobase band is also seen with the H82 cell line and is derived from the rearrangement described earlier. In both cell lines, three subbands appear and map to a region 5' of the first untranslated exon. These subbands, designated I, II, and III, correspond to the hypersensitive sites described by Siebenlist et al. for c-myc in B-lymphoblastoid cell lines (21).

To display these hypersensitive sites in greater detail, the samples were digested with SstI and hybridized with a PvuII-XbaI (PX) probe (Fig. 3). This probe overlaps two germ-line fragments 13 kilobases and 600 base pairs in size. A 9.5-kilobase rearranged fragment is also observed in the H82 cell line. With increasing DNase I concentrations, we now detect additional subbands, which again correspond to the hypersensitive sites of c-myc described in B-lymphoblastoid and Burkitt lymphoma cell lines (21, 33). The earlier appearance of subbands with lower doses of DNase I may reflect any of the following: more apparent cleavage at susceptible sites due to the increased number of DNA copies in H82; intrinsically increased susceptibility to cleavage; or the presence of endogenous enzyme activity in the nuclear extracts. The III1 and III2 sites map to regions 50 to 100 base pairs 5' of the first and second c-myc promoters, respectively. The more intense band at III3 correlates with the increased amount of steady-state c-myc mRNA which is transcribed from the second promoter, as demonstrated by the S1 nuclease analysis (Fig. 2). Hypersensitivity site I maps to the 5' end of a sequence well conserved between mouse and humans, suggesting a potential functional importance (21, 24). Comparison of H378 and H82 reveals no qualitative differences; DNase I hypersensitivity sites for c-myc are identical in H82 and H378, very similar to the pattern seen in B-lymphoblastoid cell lines. In H82, the hypersensitive sites are correspondingly amplified in number, allowing visualization of bands on the autoradiogram after only a few hours. Despite the gene amplification and the presence of rearranged segments,

**DNA WILUTION H82**

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![Fig. 1. Southern blot comparison of DNA digested with EcoRI and then hybridized with the Clal-EcoRI (CR) probe for human c-myc. The H378 lane and the pituitary lane (P) contain 15 µg of DNA, while H82 has been diluted from 1:20 to 1:125 as indicated. kb, kilobases.](https://cancerres.aacrjournals.org)
no new hypersensitive sites are detected in the H82 cell line. It is possible that a few alternative hypersensitive sites might be obscured within the background of the amplified pattern.

DNA Methylation Patterns for c-myc in the H82 and H378 Cell Lines. Published sequences of the c-myc oncogene (21, 24) allow placement of predicted Hpall-MspI restriction sites over a 9.0-kilobase span (Fig. 4). DNA isolated from H82 and H378 was digested completely with HindIII and then further digested with either MspI or HpaII. 5'-CCGG sequences amount to 32 (9.1%) of the 351 potential CpG dinucleotides in the c-myc gene. Over 90% of available sites are not approached by our analysis, and many 5'-CCGG sites are not evaluable because restriction enzyme cleavage generates fragments too small for efficient transfer to nitrocellulose. By sequence analysis, the CpG sites cluster just 5' to the reported DNase I hypersensitive sites (I, II, III, I, III, II), throughout exon 1, over a 1-kilobase stretch 5' to exon 2, and in the coding regions of exons 2 and 3. Significantly fewer CpG sequences occur in the second intron and in the region 3' to exon 3.

As seen on the structural diagrams (Fig. 4), three c-myc probes were chosen to examine potential sites for methylation in the c-myc gene, including a 3-kilobase region 5' to the first promoter. This encompasses all known sites of c-myc interspecies homology as assessed by heteroduplex analysis (24). Hybridization of these DNA samples to a mitochondrial probe (27) suggested complete digestion with MspI and HpaII (data not shown). Hybridization with a probe from the IgE constant region revealed substantial methylation in a gene which is not expressed in SCLC cell lines (data not shown).

Comparison of the methylation patterns in H82 and H378 reveals few differences, most of which are attributable to rearranged bands evident in the H82 cell line (Fig. 4). Of interest, partial methylation is evident at the only site in exon 3, producing a 5.6-kilobase fragment, and at a site in the first intron, yielding a 740-base pair fragment. Partially methylated sites are located within the region 5.6 kilobases 5' of the HindIII site identified by the HindIII-C1a1 (HC) probe (data not shown). These sites also appear in both the H82 and H378 cell lines. Despite differences in c-myc expression and in gene amplification, the pattern of cleavage by MspI and HpaII is essentially the same for both cell lines.

DISCUSSION

DNase I hypersensitivity sites are located most frequently in regions of DNA adjacent to actively transcribed genes (15). We have studied several hypersensitive sites in a 2-kilobase region 5' of the first promoter of the c-myc gene in two SCLC cell lines. The sites in the H82 cell line are different than those observed in the H378 cell line or in nonmalignant, Epstein-Barr virus-immortalized B-lymphoblastoid cell lines (21, 33). Site I is located in a region of interspecies sequence homology whose functional significance remains obscure (21, 24). Site II, lies next to a DNA sequence which is thought to bind a protein in vitro extracts (21). In other systems, protein factors appear to bind preferentially to areas adjacent to hypersensitive sites (34, 35). The III and III sites are located 5' to the first and second promoters, respectively. No shift in promoter utilization analogous to Burkitt lymphoma cell lines (31) is evident in H82 by S1 nuclease analysis.

The intensities of the DNase I hypersensitive cleavage bands in H82 are amplified in accordance with the 50-fold increase in gene dosage. No new hypersensitive sites are evident despite gene amplification and the presence of less amplified, but clearly evident rearranged c-myc genes. It is possible that alternative hypersensitive sites might be obscured within the background of the amplified pattern. The H378 cell line, which contains no clearly detectable c-myc mRNA, demonstrates no changes in location or in comparable intensity of the hypersensitive sites. The sites around the transcriptionally silent c-myc gene in H378 are different from those found around c-myc in Epstein-Barr virus-immortalized B-lymphoblastoid cells, where c-myc expression is clearly evident (21, 31).

The results considered thus far do not demonstrate a pattern of altered chromatin structure that could account for the greatly increased expression of c-myc in H82. While in many cases there is correlation between nuclease sensitivity and gene expression, as with the chicken α- and β-globin genes (17, 36),

Fig. 2. S1 nuclease analysis of RNA from the two c-myc promoters comparing H82 with H378. An 800-base pair (bp) PvuII-PvuII probe extending from 340 base pairs 5' of the first promoter to 40 base pairs before the splice donor signal was hybridized with 25 μg of H378 total cytoplasmic RNA and with progressive dilutions of H82 RNA from 1:10 to 1:250 as indicated. One control lane (tRNA) contains 25 μg of yeast transfer RNA and another control lane (C) contains no RNA. The S1 protected regions were sized relative to labeled PBR327 digested with HindIII (M). The protected fragments are mapped relative to the first exon of c-myc.
in other cases there is not. For example, the human δ- and β-globin genes demonstrate hypersensitive sites in fetal liver cells that lack globin gene expression (37). The presence of hypersensitive sites in H378 may be analogous to this example.

Methylation patterns generally correlate with gene expression, with relative hypomethylation being a feature of many active genes (16, 18, 36, 38). Yet there are also numerous examples of inactive genes maintaining an undermethylated pattern (39–42). Unmethylated sites occur around globin genes found in nonerythropoietic organs (42) or in murine erythroleukemia cells which do not express the genes (40, 41). Similarly, we observe undermethylation at most sites examined in the c-myc locus, despite the absence of expression in H378. This is the same pattern of hypomethylation encountered in H82, where c-myc is expressed abundantly. DNA hypomethylation may be a general phenomenon characteristic of neoplastic cells or of any cells able to sustain growth in cell culture. Comparison of specific genes in primary human cancers to the adjacent normal tissue confirms substantial hypomethylation in malignant cells (43, 44). Conversely, the vitellogenin gene of the frog and the δ-crystallin gene of the chicken are examples of genes actively transcribed despite full methylation (45, 46).

Although most sites examined in c-myc appear undermethylated, there is evidence for partial methylation at the site in exon 3 and at a site in the first intron. Another study of growing human tumor cell lines also suggested relative hypomethylation of c-myc sites, but no correlation was made with c-myc expression (47). In this study, partial methylation at the third exon was present in some of the tumor cell lines, but not in the fibroblast strains (47). It is of interest that extensive methylation of the c-myc gene has never been described in the few cell lines examined. Perhaps the relatively hypomethylated pattern of c-myc in H378 is a feature of human adenocarcinoma cells that are able to grow in cell culture.
myc reflects the growth state in these cells, rather than a control for transcriptional regulation.

Evidence has been assembled to suggest that the DNase I hypersensitive sites in the 5' flanking region of the human c-myc gene may represent possible sites of interaction with regulatory factors (21). It has been proposed that alterations in the structure of these regions may be related to neoplasia when translocations occur vicinal to these sites (48). The data presented in this paper argue against a tissue-specific character to the elements mediating the DNase I hypersensitivity of this locus, because almost identical patterns emerge from SCLC cells as are observed in "normal" B-lymphoblastoid cells. Moreover, the data in this paper would not suggest that these chromatin structural features are necessarily linked to gene expression, as the same patterns of hypersensitivity and methylation are observed in a c-myc producer and in a nonproducer cell line. In addition, the patterns of DNase I hypersensitivity sites, DNA methylation, and promoter utilization are not altered in H82 by amplification of c-myc. Functional assays of the chromatin structure of c-myc in different states will be required to approach an understanding of how the chromatin structure relates to gene expression.

ACKNOWLEDGMENTS

We thank J. Minna, T. Bender, R. Currie, G. Wilding, and B. Brooks for reviewing the manuscript and T. Myers and E. Meyer for help in preparation of this manuscript.

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