Methylation of plasmacytoma c-*myc* genes

(Recombinant DNA; chromosomal translocation; gene regulation; kidney; liver; DNA sequence)

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SUMMARY

The chromosomal translocation associated with many tumors of immunoglobulin-producing cells frequently results in the joining of the immunoglobulin heavy-chain locus and the c-*myc* oncogene. This translocation of c-*myc* has profound structural and functional consequences for the oncogene, including loss of the 5′ end of the gene and transcriptional deregulation. We report in this communication that translocation results in a new methylation pattern of c-*myc*. In normal kidney and liver tissue, the c-*myc* gene is methylated at its 3′ end. The translocated gene in plasmacytoma DNA is extensively demethylated. On the other hand, the nonrearranged c-*myc* gene in plasmacytoma DNA (which is transcriptionally silent) is extensively methylated. In addition, we confirm the nucleotide sequence (with 19 discrepancies out of 1400 bp) 5′ to the murine c-*myc* gene, as reported by Corcoran et al. [Cell 40 (1985) 71–79].

INTRODUCTION

The characteristic chromosomal translocation found in many B cell tumors results in the joining of a c-*myc* oncogene (from murine chromosome 15) to the immunoglobulin heavy-chain locus (chromosome 12: Klein, 1983; Perry, 1983; Robertson, 1983). As a result of the reciprocal translocation process, the 5′ end of the c-*myc* gene is separated

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Abbreviations: bp, base pair(s); kb, 1000 bp; NIARD, see Fig. 2 and section c; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 0.015 M Na$_3$·citrate, pH 7–8.

from the bulk of the gene. DNA from plasmacytoma cells have parts of the c-*myc* gene in three different locations: a nonrearranged version of the gene on a normal chromosome 15; the bulk of the gene, including all of the protein-coding potential, associated with the part of immunoglobulin heavy-chain locus on a 12:15 translocation chromosome; and the 5′ end of the c-*myc* gene associated with the 5′ end of the heavy-chain locus on a 15:12 translocated chromosome (Cory et al., 1983). Translocation and truncation of c-*myc* are correlated with increased transcriptional activity of the translocated gene, but transcriptional silence of the normal c-*myc* gene (Perry, 1983; Robertson, 1983).

Eukaryotic genes that are transcriptionally active tend to be undermethylated, whereas those that are

transcriptionally less active tend to be methylated (Razin and Riggs, 1980). Prevention of methylation by 5-azacytidine results in dramatic increases in transcriptional activity of some eukaryotic genes (Groudine et al., 1981; Compere and Palmiter, 1981). Finally, recombinant DNA molecules propagated in *Escherichia coli*, and therefore carrying all of their CpG dinucleotides in the demethylated form, are transcriptionally active when inserted into eukaryotic nuclei. On the other hand, the same recombinant DNA molecules, when methylated in vitro, are now inactive in eukaryotic nuclei (Fradin et al., 1982; Busslinger et al., 1983). We have studied the effect of c-*myc* translocation on gene structure by investigation of the methylation pattern of the c-*myc* gene from plasmacytoma cells and from normal (kidney and liver) cells.

EXPERIMENTAL AND DISCUSSION

(a) Clones and methods

The recombinant DNA molecule γM52 includes the translocated c-*myc* gene from the murine plasmacytoma P3. The recombinant DNA molecule pX27-3 includes the reciprocal product of the P3 translocation; that is, half of the first exon, and all of the 5′-flanking region. The derivation and structure of these clones have been described in detail elsewhere (Dunnick et al., 1983). The clone LF11 is a *Hin*dIII subclone in M13 of a 1.5-kb *Hin*dIII fragment that includes a large portion of 5′-flanking sequences of the murine c-*myc* gene from the clone pX27-3, and ends at the *Hin*dIII site in the 5′-most exon (Fig. 1).

Methods for determining nucleotide sequences and for Southern hybridization analysis are described in the appropriate figure legends.

(b) Sequence of c-*myc* 5′ flanking region

The sequence of 1400 bp of murine c-*myc* 5′ flanking region was determined. As this sequence has been published by Corcoran et al. (1985), we present here only the 19 discrepancies (see legend to Fig. 1). This sequence will serve as a database for at least two different lines of investigation. (i) It will aid in the exact location of c-*myc* translocation sites in
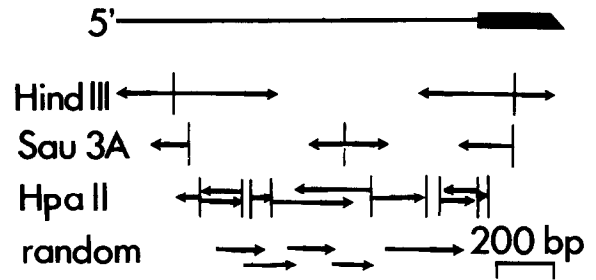

Fig. 1. Sequences in the murine c-*myc* oncogene. A schematic diagram of the 5′-flanking region and part of the first exon (darkened box) of the translocated c-*myc* gene in the murine plasmacytoma P3 is shown. The direction and extent of dideoxy sequences (Sanger et al., 1980), derived in bacteriophage M13 (Messing and Vieira, 1982) from *Hin*dIII, *Sau*3A or *Hpa*II restriction fragments, or from random fragments generated by sonications are shown. Our sequence is exactly the same as that derived from the germline c-*myc* gene by Corcoran et al. (1985), with the following discrepancies (numbering according to Corcoran et al., 1985): delete T-1392; delete G-897; insert G after −890; insert G after −761; delete G-746; substitute G for C-745; delete G-743; delete T-536; delete C-405; delete A-357; insert C after −266; substitute G for A-190; insert G after −183; insert C after −141; substitute G for A-109; delete A-100; insert two As after −95; substitute A for G-60; delete G-12.

the 5′ end of the gene and the analysis of nucleotide sequences that might be important to the translocation process. (ii) It provides the sequence of the 5′ end of the gene, a region that may play a key role in gene regulation (Perry, 1983; Robertson, 1983).

(c) Methylation of CCGG sites

We found a high concentration of *Hpa*II sites (recognition sequence CCGG) in the 5′-flanking region of this gene. Because the 5′ end of the c-*myc* gene may be important to regulation of gene expression, and because it includes so many CCGG sequences, we were interested to determine the extent and pattern of methylation of c-*myc* genes with different structures in different tissues. Using *Hpa*II cleavage, and the Southern (1975) hybridization technique, we examined the methylation state of c-*myc* in a mixture of kidney and liver DNA, and in P3 (a murine plasmacytoma cell line) DNA (Fig. 2). Of course, neither allele is rearranged in kidney and liver DNA; both c-*myc* genes are found in a 9.8-kb *Kpn*I fragment (lane 5). With the probe for the second exon, third exon, first intron, and second
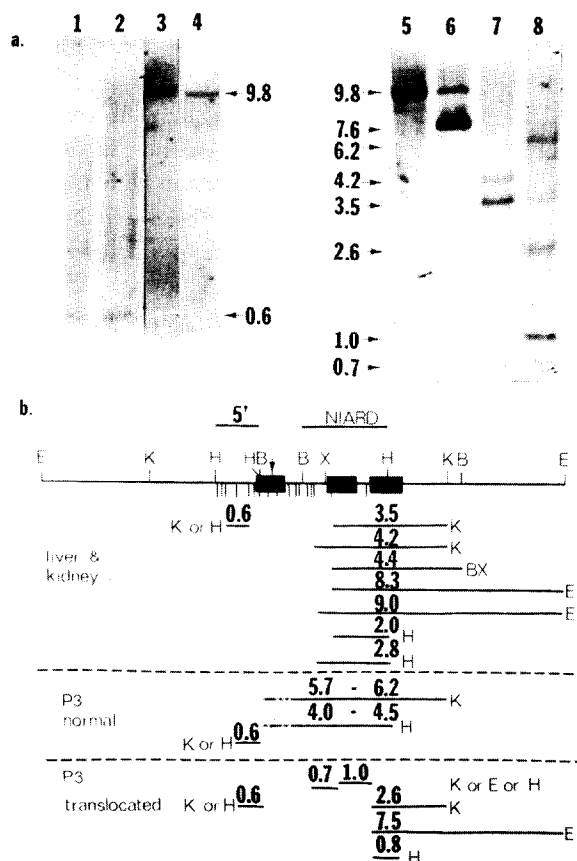
Fig. 2. Methylation of the c-myc oncogene. (a) High-M_r DNA from pooled kidneys and livers (lanes 2, 4, 5, and 7) or from plasmacytoma P3 (lanes 1, 3, 6, and 8) was digested with KpnI alone (lanes 3–6) or with KpnI + HpaII (lanes 1, 2, 7, and 8). The digested samples were fractionated on a 0.8% agarose gel and blotted onto nitrocellulose paper (Southern, 1975). Hybridization to nick-translated 5'-flanking region probe (LF11) (lanes 1–4) or the probe for the second exon, third exon, first intron, and second intron (NIARD; Harris et al., 1982; lanes 5–8) was in 6 × SSC at 68°C. The sizes (in kb) of some DNA fragments are shown. (b) A restriction enzyme cleavage site map (with relevant sites only) for the murine c-myc gene. Vertical lines below the gene: known HpaII sites. HpaII sites in the 3' end of the c-myc gene were determined by mapping of the c-myc molecular clone γM52 (Dunnick et al., 1983). Black boxes: c-myc exons. The arrow above the first c-myc exon notes the translocation site in P3 DNA (Neuberger and Cabali, 1983; Dunnick et al., 1983). We have digested plasmacytoma P3 DNA and a mixture of murine kidney and liver DNA with KpnI (K) alone, with EcoRI (E) alone, with HindIII (H) alone, or with BamHI + XbaI (BX). After the primary digestion, each sample was digested with HpaII and subjected to Southern hybridization analysis. Some of the resulting autoradiographs are shown in Fig. 2a and 3b,c. Analysis of that set of data (including those not shown) allowed us to locate the various restriction fragments within the c-myc gene. The size and location of those fragments is shown. The enzyme used in the primary digestion,

intron of c-myc (NIARD; Harris et al., 1982), we detected both the germline and translocated alleles (9.8- and 7.6-kb fragments; lane 6) in P3 DNA. (The greater hybridization signal associated with the translocated fragment is likely to reflect ploidy differences between the translocated and normal chromosomes.) KpnI + HpaII digestion of kidney and liver DNA, followed by Southern hybridization analysis with the NIARD probe, revealed three predominant fragments (0.7, 3.5 and 4.2-kb, lane 7). Primary digestion with EcoRI, BamHI + XbaI, or HindIII instead of KpnI allowed us to place these fragments at the 3' end of the c-myc gene, suggesting complete methylation of the HpaII site in the second intron, and partial methylation of the site in the second exon. Similar experiments with plasmacytoma P3 DNA revealed a different pattern of methylation. In P3 DNA at least one allele is extensively undermethylated, yielding 2.6, 1.0, and 0.7-kb fragments (lane 8). On the other hand, fragments equal to or larger than those observed in kidney and liver DNA were also detected (3.5, 6.2, and 7.6 kb).

(d) Differential methylation of two plasmacytoma c-myc alleles

To determine which allele in P3 DNA is methylated and which is demethylated, we fractionated the two alleles by centrifugation in a sucrose gradient. The normal allele is found in a 20-kb EcoRI fragment (Harris et al., 1982; Cory et al., 1983), whereas the translocated allele is found in a 14-kb fragment (Cory et al., 1983; Dunnick et al., 1983). EcoRI-digested P3 genomic DNA was fractionated on a sucrose gradient, and fractions were pooled as described in Fig. 3a. These fractionated DNA samples were fur-

with HpaII being used in every case in the secondary digestion, is indicated by the appropriate abbreviation. For example, the 3.5-kb fragment resulting from primary digestion with KpnI and secondary digestion with HpaII, and detected by the NIARD probe [lane 7, in (a)], is located in the second exon, second intron, and third exon of the kidney and liver gene. In those cases in which two or three enzymes are indicated, the same fragment resulted from digestion with any of the indicated enzymes and HpaII. The broken lines for the normal P3 gene denote partial methylation of the HpaII sites at the 5' end of this gene. In P3 DNA, fragments were assigned to the normal or translocated gene by the analysis described in Fig. 3.
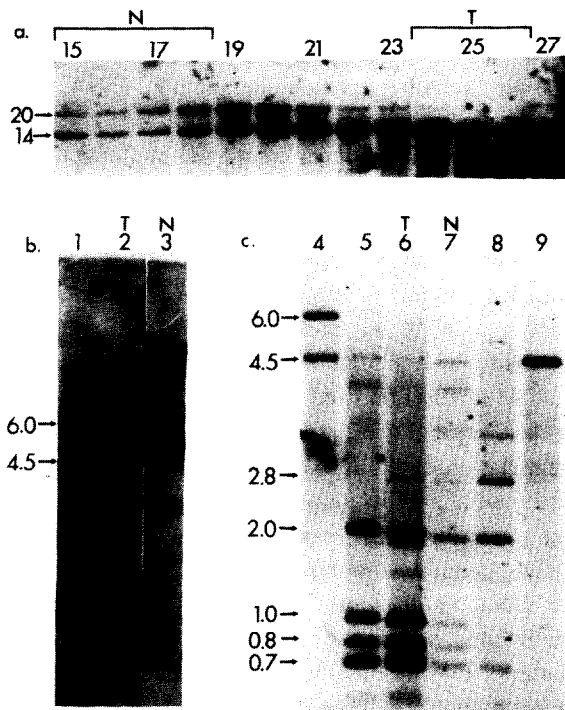
Fig. 3. Methylation of c-*myc* in plasmacytoma P3 DNA. (a) High-M_r DNA from P3 cells was digested with *Eco*RI and fractionated on a sucrose gradient. Fractions 15 to 27 were concentrated by ethanol precipitation, and aliquots were fractionated on an agarose gel. The DNA samples in the gel were blotted onto nitrocellulose by the Southern (1975) procedure and hybridized to radiolabeled c-*myc* NIARD probe. Fractions 15 to 18, containing the nonrearranged c-*myc* gene in a 20-kb *Eco*RI fragment, were pooled. Fractions 24 to 26, containing the translocated c-*myc* gene in a 14-kb *Eco*RI fragment, were also pooled. (b) DNA samples of P3 DNA (lane 1), fractions 24 to 26 (lane 2), or fractions 15 to 18 (lane 3) were digested with *Hin*dIII, fractionated on an agarose gel, blotted onto nitrocellulose paper, and hybridized to the radiolabeled NIARD (see Fig. 2) probe. (c) DNA samples of P3 DNA (lanes 4 and 5), of sucrose gradient fractions 24 to 26 (T, lane 6), of sucrose gradient fractions 15 to 18 (N, lane 7), or of kidney and liver DNA (lanes 8 and 9) were digested with *Hin*dIII (lanes 4 and 9) or *Hin*dIII + *Hpa*II (lanes 5, 6, 7, and 8). The digested samples were then fractionated on a 1.1% agarose gel, blotted onto nitrocellulose paper, and hybridized with the NIARD probe in 6 × SSC at 68°C. Sizes of fragments in kb are indicated on the left-hand margin of the autoradiographs.

ther digested with *Hin*dIII (Fig. 3b). The fraction including the translocated allele contained a large amount of the 6.0-kb *Hin*dIII fragment associated with the translocated gene, and a small amount of the 4.5-kb fragment associated with the normal gene (Fig. 3b, lane 2). The fraction including the normal

allele was a mixture of about equal amounts of the 6.0-kb translocated fragment and 4.5-kb normal fragment (Fig. 3b, lane 3).

These P3 DNA fractions, along with genomic DNA from P3 plasmacytoma DNA and a mixture of kidney and liver DNA, were digested with *Hin*dIII and *Hpa*II and analyzed by the Southern hybridization procedure (Fig. 3c). The c-*myc* genes in kidney and liver DNA are found in a (non-rearranged) 4.5-kb *Hin*dIII fragment (lane 9) and are methylated to an intermediate extent (lane 8). The c-*myc* genes in plasmacytoma P3 DNA seem to fall into two sets: one is extensively demethylated, the other extensively methylated (lane 5). The translocated allele appears to be the demethylated c-*myc* gene, as the fraction including this allele yielded the small fragments associated with extensive demethylation of the gene (Fig. 3c, lane 6). On the other hand, the fraction which included both alleles yielded 4.5- and 4.0-kb *Hin*dIII-*Hpa*II fragments (lane 7). These larger fragments indicate that the normal allele is extensively methylated, *Hpa*II cutting the *Hin*dIII fragment once or not at all. This fraction also yielded a small amount of the 0.7–1.0-kb fragments associated with demethylation. The intensity of hybridization of these small fragments is consistent with the level of contamination by the translocated allele. The hybridization signal of the small fragments (lane 7) is much reduced compared to either the purified translocated fragment (lane 6) or the total genomic DNA (lane 5), even though the hybridization signal of the 4.0 and 4.5-kb fragments is much greater than that for the translocated allele (lane 6) and equal to that for the total plasmacytoma DNA (lane 5). P3 DNA, and DNA from both fractions, yields a 2.0-kb *Hin*dIII-*Hpa*II fragment. From the relative intensity of hybridization of this fragment in the various lanes, it appears that the translocated allele is partially methylated at the *Hpa*II site in the second intron, resulting in a 2.0-kb fragment. Nevertheless, it is also possible that some of this fragment is derived from the population of normal c-*myc* genes.

At least two *Hpa*II sites at the 5′ end of the c-*myc* genes in kidney and liver DNA, and in P3 DNA, appeared to be undermethylated. With a 5′-flanking region probe, we detected a small (0.6-kb) fragment after *Hpa*II digestion of either P3 or kidney and liver DNA (Fig. 2, lanes 1 and 2). After digestion with *Kpn*I only, this same probe detected the non-

rearranged c-*myc* gene in kidney and liver DNA (lane 4), the nonrearranged gene in P3 DNA (lane 3, 9.8-kb), and the translocated reciprocal product in P3 DNA (lane 3, 11 kb). From our limited analysis, we detected hypomethylation of some sites in the 5′ flanking region of the c-*myc* gene, but could not distinguish between translocated and normal c-*myc* genes.

(e) Conclusions

We have defined three different patterns of methylation for murine c-*myc* genes. (i) The translocated c-*myc* gene in plasmacytoma DNA is largely demethylated and is transcriptionally active. It has been previously shown that the adjoining immunoglobulin gene is also demethylated (Dean et al., 1983). (ii) The nonrearranged gene in plasmacytoma DNA is extensively methylated. In most plasmacytomas, this normal allele is transcriptionally silent (Stanton et al., 1983; Adams et al., 1983). (iii) The c-*myc* genes in a mixture of kidney and liver DNA are methylated to an intermediate extent; that methylation takes place in the second exon and intron. The transcriptional activity of these normal kidney and liver genes might be very low. Nevertheless, at least in liver DNA, the genes are capable of induction to very high rates of transcription (Makino et al., 1984).

Genes that encode 'housekeeping functions' tend to be undermethylated in their promoter regions, but methylated at their 3′ ends (Stein et al., 1983). The kidney and liver c-*myc* gene seems to be methylated at its 3′ end (Figs. 2 and 3). Our limited analysis suggests undermethylation of the promoter region of the c-*myc* gene, but more extensive analysis will be necessary to confirm this point.

The murine c-*myc* gene has more CpG dinucleotides in its 5′ end than in its 3′ end (CpG dinucleotides are slightly underrepresented in the second and third exons; Stanton et al., 1983; Bernard et al., 1983). Even though the sequence of the second intron is not available, the presence of a single *Hpa*II site in this intron suggests few CpG dinucleotides. Thus, c-*myc* seems to fit the observation of Tykocinski and Max (1984) that eukaryotic genes tend to have many CpG dinucleotides at their 5′ ends, but few at their 3′ ends. Tykocinski and Max also suggested that CpG dinucleotides are in low abundance in the 3′ end of eukaryotic genes because, in their methylated state, CpG dinucleotides tend to mutate to CpA or TpG. The methylation pattern and the CpG content of the 5′ and 3′ ends of the c-*myc* gene in kidney and liver DNA are consistent with this hypothesis.

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