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Chromatin Structure Changes Suggest a Compensatory Response to c-myc Gene Amplification in Malignant Fibrous Histiocytoma

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Abstract Changes in chromatin structure as determined from DNAse I hypersensitive site analysis are associated with c-myc amplification and increased transcript/protein levels in malignant fibrous histiocytoma (MFH) cell lines. A DNAse I hypersensitive site near the PO promoter region was observed in one MFH cell line (UR HCL 1), and in normal fibroblasts (HFF), but not in an MFH cell line with an amplified c-myc gene (P3C). A DNAse I hypersensitive site exclusive to P3C amplified c-myc was identified slightly 3′ of exon one. No alterations in c-myc DNAse I hypersensitive site patterns were observed in HFF fibroblasts following serum release, when peak levels of c-myc transcript were induced. DNAse I hypersensitive site patterns associated with gene amplification may reflect a compensatory response by P3C cells to an abundance of c-myc transcript. Furthermore, elevated levels of protein in P3C cells provide additional evidence that amplified c-myc is an oncogene in MFHs. © 1992 Wiley-Liss, Inc.

Key words: c-myc transcription, DNAse I hypersensitivity, oncogenes, sarcomas, tumor cell lines

Amplification of myc family genes and increases in transcript production have been documented for a variety of tumor cell types (Alitalo et al., 1983; Alt, et al., 1986; Dalla-Favera, et al., 1982b; Johnson et al., 1987). Multi-copy oncogenes have been associated with tumor pathogenesis (Seeger et al., 1985; Slamon et al., 1987), suggesting that gene amplification is one possible mechanism through which proto-oncogenes may acquire oncogenic properties.

DNAse I hypersensitive sites are thought to signify DNA sequences of regulatory importance and are presumably sites of binding for regulatory proteins. Data from lymphocyte studies provide convincing evidence that DNAse I hypersensitive site pattern changes in presumed regulatory regions of the c-myc gene signify alterations in transcriptional control (Fig. 1): Siebenlist et al. (1984) have identified distinct DNAse I hypersensitive site patterns which are associated with translocated and non-translocated c-myc alleles in Burkitt's lymphoma and non-malignant lymphoblastoid cells. Other studies have revealed changes in DNAse I hypersensitive site patterns which accompany decreased transcription of amplified c-myc in HL-60 cells induced to differentiate (Dyson et al., 1985; High et al., 1987; Siebenlist et al., 1988).

Specific DNA sequences thought to mediate transcription limiting activities have been identified in the c-myc exon 1/intron 1 border region, and may be inactive in Burkitt lymphoma cells (Cesarman et al., 1987; Zajac-Kaye et al., 1988) (Fig. 1). An area associated with a block to c-myc transcript elongation in differentiating HL-60 cells has also been assigned to this region (Bentley and Groudine, 1986a; Eick and Bornkamm, 1986). These studies have provided valuable insight as to what mechanisms may be in place when abnormal c-myc transcription accompanies neoplasia, and they suggest that loss of ability to limit transcript production may be one characteristic of an oncogenic c-myc gene.

We have found amplification of the c-myc gene in 26% of examined malignant fibrous histiocytomas (MFH), whose cells of origin are thought to be of mesenchymal lineage (Iwasaki et al., 1987). This is accompanied by increases in transcript and frequency of cell division, suggest-

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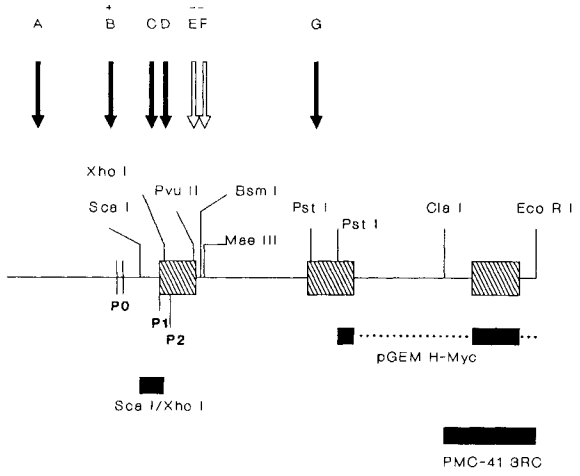

Fig. 1. Schematic representation of the c-myc gene noting previously documented nuclease sensitive regions and areas thought to be associated with transcription limiting elements. A, B, C, and D represent DNAse I hypersensitive areas described by Siebenlist et al. (1984, 1988). Loss of intensity of B has been reported in HL-60 cells induced to differentiate with DMSO (Siebenlist et al., 1988), and this site may be associated with the P0 promoter (Bentley and Groudine, 1986a, 1986b; + symbol). E and F represent sequences thought to be associated with c-myc transcription suppressing mechanisms (Cesarman et al., 1987; Zajac-Kaye et al., 1988) and are marked by a (−) symbol. G indicates an S-1 nuclease sensitive area previously reported in the intron 1/exon 2 region (Grosso and Pitot, 1985). Also shown are the c-myc probes used in DNAse I hypersensitive site (PMC 41 3RC, Sca I/Xho I) and Northern blot (pGEM H-myc) analyses. The PMC 41 3RC probe is a Cla I/Eco R I human c-myc fragment (Dalla-Favera et al., 1982a) obtained from Dr. R.C. Gallo. The Sca I/Xho I probe was generated by restriction digests of a human c-myc clone obtained from Oncor, Inc. (Gaithersburg, MD). The pGEM H-myc probe is a human c-myc cDNA in pGEM 1 (Watt et al., 1983).

ing some role of c-myc in the progression of these tumors (unpublished data). We now report that changes in DNAse I hypersensitive site patterns accompany amplification of the c-myc gene in MFH cell lines, but are not associated with a physiologically induced increase in c-myc transcript in normal fibroblasts. Comparison of our data with previously reported studies of c-myc in lymphoid cells suggests that these changes may represent a compensatory response by P3C cells to an abundance of transcript resulting from gene amplification. Furthermore, increased translation in these cells provides additional evidence that amplified c-myc is an oncogene in MFHs.

MATERIALS AND METHODS

Cell Culture

The UR HCL 1 MFH cell line was obtained from American Type Culture Collection (Rockville, MD), and P3C cells were derived from an MFH cultured in this laboratory. These cells and HFF fibroblasts were grown in Dulbecco's MEM (DMEM) supplemented with 10% fetal bovine serum at 37°C. Analysis of c-myc chromatin structure and transcript levels following serum release of quiescent fibroblasts (presumably G0/G1 transition) was accomplished by growing HFF cells to 70–80% confluency in media and conditions described above. At this level of confluency, cells were actively cycling as confirmed by Northern blot hybridization with the thymidine kinase (TK) probe (data shown below). Fibroblasts were then made quiescent by addition of DMEM/0.1% serum and incubation for 3 days at 37°C. DMEM/10% serum was then re-introduced to release the cells.

Polyacrylamide Gel Electrophoresis (PAGE)

The discontinuous system for PAGE as described by Laemmli (1970) was used in this analysis. Stacking gels consisted of 4% acrylamide in 0.125 M Tris-HCL, pH 6.8, and 0.1% SDS. Separating gels were 8.5% acrylamide in 0.375 M Tris-HCL, pH 8.8, and 0.1% SDS. Twenty micrograms of protein were combined with an equal volume of treatment buffer (0.125 M Tris-HCL, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol), incubated at 90°C for 1.5 min, ice-quenched, then loaded onto the gel. Gels were electrophoresed at 30 ma/1.5 mm gel thickness in 0.025 M Tris-HCL, pH 8.3, 0.192 M glycine, and 0.1% SDS.

Western Blotting and Immunoperoxidase Assay

Western blotting of proteins onto 0.2 μm pore size nitrocellulose (Schleicher & Schuell) was carried out at 0.6 amps for 45 min at 4°C using methods described by Towbin et al., (1979). Following transfer, blots were air-dried, and incubated for 3 hr in PBST (1× phosphate buffered saline (PBS), 0.05% Tween 20) and 2% bovine serum albumin (BSA). An anti-human c-myc monoclonal antibody obtained from Dr. H.L. Niman (IGG 1, ascites purified by protein A chromatography) was diluted (0.1 mg/ml) in PBST/2% BSA, added to one blot, and allowed to incubate at room temperature for 1 hr with gentle agitation. As a control for non-specific binding, an identical blot was incubated with an anti-met 72 monoclonal antibody (IGG 1, ascites purified by protein A column), (0.1 mg/ml in PBST/2% BSA) obtained from Dr. A. Kimura. The blots were then washed 3 times for 5 min

each with PBST. This was followed by incubation with a 1 μg/ml solution of a horseradish peroxidase conjugated goat-anti-mouse Ig (Southern Biotechnology Associates) diluted in PBST/2% BSA for 1 hr at room temperature. Blots were then washed 3 times with PBST as before, incubated with a 180 μg/ml solution of the substrate diaminobenzoate (DAB) in PBST/0.01% H_2O_2, and the reaction stopped with excess H_2O. Quantification of c-myc protein bands was carried out by reflectance densitometry. As a control for quantification, a third identical blot was stained with a 0.1% solution of india ink in PBST.

Preparation of Radiolabeled Probes

Probes were nick translated by adding 250 ng of DNA to a reaction mixture which contained 80 μCi 32P (dATP), 5.0 μl of 10X (dCTP, dGTP, dTTP), 1.25 μl of 1 mg/ml BSA, 2.5 μl of nick translation buffer (0.5 M Tris HCL, pH 7.8, 0.1 M 2-mercaptoethanol, and 0.05 M $MgCl_2$), 1.5 μl DNAse I/polymerase I (BRL), and H_2O to a final volume of 25 μl. The reaction was run at 15°C for 45 min. Labeled DNA was separated from unincorporated nucleotides using a Biogel A-15M (Bio-Rad) column. Probes (200 ng of DNA) labeled by random primer extension were first denatured at 90°C for 2 min. Following denaturation, 5 μl of primer extension buffer (1M hepes, pH 6.6, 25 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 0.25 M Tris HCL, pH 8.0, 0.1 mM dCTP/dGTP/dTTP, 2 mg/ml BSA, 15 mg/ml primer), 5 units Klenow, 100 μCi 32P (dATP), and H_2O were added to a final volume of 25 μl. This reaction mixture was allowed to sit at room temperature for 16 hr. Unincorporated nucleotides were separated from labeled DNA as described for nick translations.

DNAse I Hypersensitive Site Analysis

Mapping of DNAse I hypersensitive sites was accomplished by DNAse I digestion of nuclei followed by indirect end-labeling of the DNA (Wu, 1980). Approximately 2.0 × 10 E8 cells from P3C, UR HCL 1, and HFF cell lines were washed in 100 ml 1X PBS and pelleted by centrifugation at 2,000 rpm for 3 min at 4°C. Pellets were resuspended in 10 ml ice-cold 1X RSB (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$), 0.5% nonidet P40, and 10 μl 0.1 M phenylmethylsulfonyl fluoride (PMSF), then incubated 5 min at 0°C. Nuclei were recovered by centrifugation at 2,000 rpm within 3 min at 4°C. Pellets were washed 3 times with 100, 50, and 20 ml of ice-cold 1X RSB respectively, followed by centrifugation at 2,000 rpm, 3 min at 4°C. Nuclei were resuspended in 1X RSB, and digested with varying concentrations of DNAse I (Boehringer Mannheim) for 10 min at 37°C. Controls for endogenous nuclease activity were 0 μg/ml DNAse I at both 0°C and 37°C. The reaction was stopped by addition of 1/10 volume 0.25 M EDTA, 1/20 volume 10% SDS, 1/20 volume proteinase K (10 mg/ml), then incubated overnight at 37°C. DNA was recovered by repeated organic extractions and ethanol precipitation after treatment with RNAse A, and resuspended in 10 mM Tris, 1 mM EDTA. Locations of DNAse I hypersensitive regions were determined following electrophoresis and hybridization methods described by Southern (1975).

RNA Analysis

Total cellular RNA was isolated by guanidine isothiocyanate extraction procedures described by Chirgwin et al. (1979), separated by size using formaldehyde agarose gel electrophoresis (Rave et al., 1979), and transferred to Zetabind.

RESULTS

Mapping of c-myc DNAse I Hypersensitive Sites From the 3′ Direction

DNA from DNAse I treated nuclei of P3C, UR HCL 1, and HFF cell lines was restricted with Eco R I and evaluated for locations of DNAse I hypersensitive sites by Southern blot hybridization with PMC 41 3RC (Fig. 2). Optimum DNAse I concentrations for P3C, UR HCL 1, and HFF cell lines were 0.2, 0.5, and 1.0 μg/ml DNAse I. Locations of DNAse I hypersensitive areas were determined from comparison of bands to lambda DNA digested with Eco R I and Hind III. Controls of 0 μg/ml DNAse I at 0°C and 37°C (shown in Fig. 2) were run for each cell line. A band seen in the P3C Control lane (P3C 0 μg/ml) may be due to endogenous nuclease activity, and its presence may reflect the overall susceptibility of that region of DNA to cleavage by nucleases. Regardless, this control indicates that observed differences in banding patterns between P3C and the other cell lines (sites 2 and 5) involve DNAse I specific bands.

A schematic representation of the c-myc gene and DNAse I hypersensitive sites for the P3C, UR HCL 1, and HFF cell lines as determined from Southern hybridization is illustrated in Figure 3. Five identical DNAse I hypersensitive sites are apparent for UR HCL 1 and the fibro-

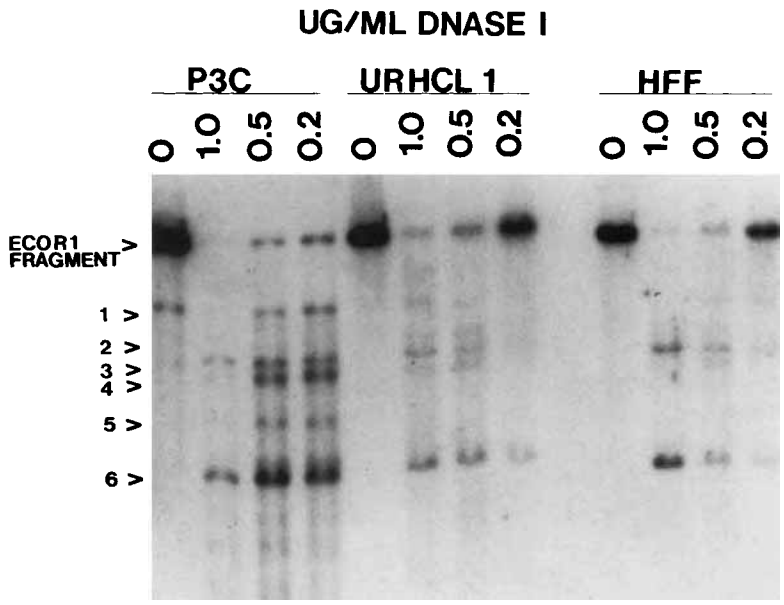

Fig. 2. Southern blot of P3C, UR HCL 1, and HFF genomic DNAs from DNAse I treated nuclei. The concentrations of DNAse I shown for each cell line were the ones which gave optimal visualization of DNAse I bands. Seven micrograms of P3C and fifteen μg of UR HCL 1 and HFF DNA from DNAse I treated nuclei were restricted with Eco R I, electrophoresed through 0.8% agarose gels, blotted, and hybridized with the PMC 41-3RC probe labeled by nick translation (10 E 8 cpm/μg). Reduced amounts of P3C DNA were used to assure that increased signal intensity of amplified c-myc in P3C cells did not overshadow other bands of interest during the length of exposure time necessary to detect single copy c-myc bands in UR HCL 1 and HFF. Controls shown for each cell line were 0 μg/ml DNAse I at 37°C degrees. Band sizes and locations of DNAse I hypersensitive sites (1, 2, 3, 4, 5, and 6) were determined by comparison to lambda DNA marker bands produced by restriction with Eco R I and Hind III. Locations of these sites relative to c-myc demographic structure are shown in Figure 3.

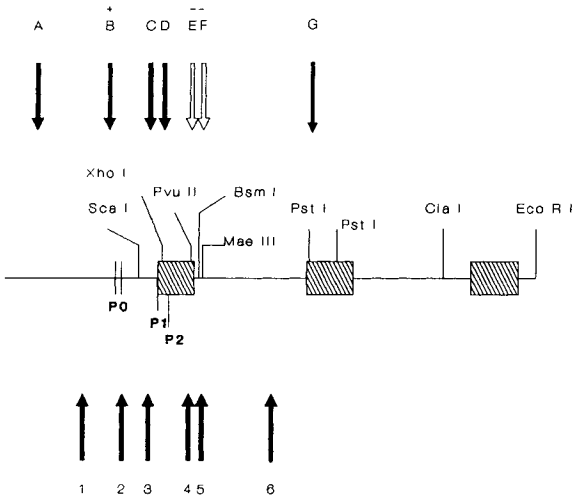

Fig. 3. Schematic representation of the c-myc gene as shown in Figure 1, indicating DNAse I hypersensitive site locations for P3C, UR HCL 1, and HFF cell lines. C-myc DNAse I hypersensitive sites 1, 2, 3, 4, and 6 were observed in the HFF-fibroblast and UR HCL 1-MFH cell lines containing single copy c-myc genes. Sites 1, 3, 4, 5, and 6 were present in an MFH cell line with an amplified c-myc gene (P3C). This diagram is a composite of data obtained from Southern blot hybridization analysis with the PMC 41-3RC (Sites 1, 2, 3), and Sca I/Xho I probes (Sites 4, 5, 6) (Fig. 5).

blast line HFF (sites 1, 2, 3, 4, and 6). The amplified c-myc gene in the P3C cell line also had four of these sites (1, 3, 4, and 6); however, a site which mapped to an area near the P0 promoter region was not present (site 2), and a new site which mapped to the 5′ region of the first intron was observed (site 5).

These analyses indicated that site 1 mapped to a region 5′ of the P0 promoter. Sites 2 and 3 were located in similar regions as B and C in lymphocytes. Site 2, not seen in P3C amplified c-myc, mapped to a region near B whose loss of intensity has been shown to precede decreased c-myc transcription in differentiating HL-60 cells (Siebenlist et al., 1988). Site 4 in HFF, UR HCL 1, and P3C cells was located in a region of the gene previously associated with a c-myc transcript elongation block through studies with Burkitt lymphoma cells (Site E, Fig. 3) (Cesarman et al., 1987). Site 5 was observed exclusively in P3C cells and mapped to the 5′ area of the first intron which may also be associated with transcript limiting activities (Site F, Fig. 3) (Zajac-Kaye et al., 1988). Site 6 in the HFF, UR HCL 1, and P3C cell lines is located near the second exon.

Chromatin Structure of the c-myc Gene Following Serum Release of Quiescent Fibroblasts

c-myc DNAse I hypersensitive sites in normal fibroblasts were mapped during quiescence and following serum release (presumably G0/G1), when increased levels of c-myc transcript are physiologically induced. Transcript levels of c-myc and thymidine kinase (TK) from serum deprived HFF fibroblasts (G0) and those post-serum release were evaluated by Northern blot analysis to ascertain that desired phases of the cell cycle were represented in accordance with expected c-myc and TK expression during the cell cycle (Blanchard et al., 1985; Johnson et al., 1982; Thompson et al., 1985) (Fig. 4). Hybridization with c-myc demonstrated a profile of transcript levels which peaked 1 hr after serum release, then returned to levels comparable to those of G0 during log phase growth. TK transcript levels were highest during log phase. Hybridization with beta-actin demonstrated consistent actin transcript levels and therefore RNA quantitation.

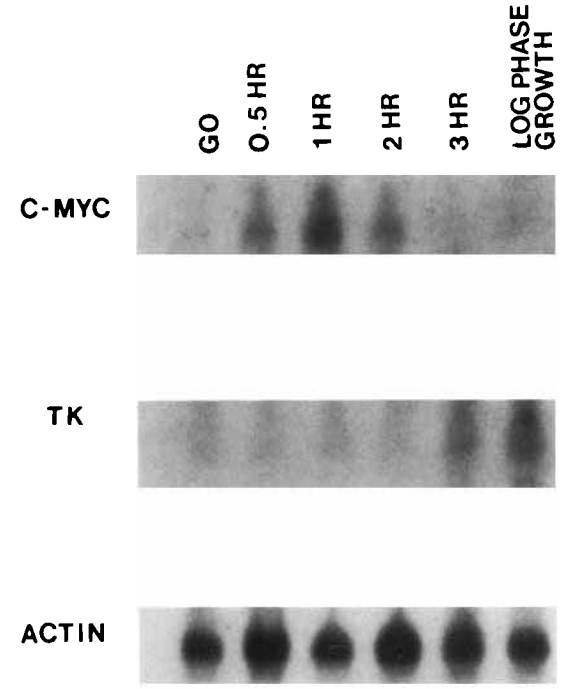

Fig. 4. Northern blot hybridization showing c-myc, TK, and actin transcript levels in HFF fibroblasts. Quiescent HFF fibroblasts (G0) in Dulbecco's MEM containing 0.1% fetal bovine serum (37°C degrees, 3 days), were released by addition of MEM supplemented with 10% fetal bovine serum. Total cellular RNA was extracted at G0, 0.5, 1, 2, and 3 h after serum release, and during log phase growth. Fifteen micrograms of total cellular RNA were electrophoresed through formaldehyde agarose gels, blotted, and hybridized with pGEM H-myc, pTK 11 human thymidine kinase (Bradshaw and Deininger, 1984), and human beta-actin (Gunning et al., 1983) probes. The pGEM H-myc probe was labeled by nick translation (10 E8 cpm/μg), while PTK 11 and human beta-actin were labeled by random primer extension (10 E8 cpm/μg).

Fifteen micrograms of genomic DNA isolated from HFF DNAse I treated nuclei at each time point were restricted with Eco R I and analyzed by Southern hybridization with PMC 41 3RC (data not shown). Locations of c-myc DNAse I hypersensitive sites at each time point examined were found to be identical to those described for HFF during previous experiments (Figs. 2, 3, sites 1, 2, 3, 4, and 6). Despite an observed peak level of c-myc transcript 1 hr after serum release, no associated changes in DNAse I hypersensitive site patterns were observed.

c-myc Protein Levels in P3C, UR HCL 1, and HFF Cells

A comparison of c-myc protein levels was made between tumor cells with amplified (P3C) and single copy (UR HCL 1) c-myc genes, and normal HFF fibroblasts during periods of observed peak c-myc transcript levels (1 hr post serum release). Twenty micrograms of protein from P3C, UR HCL 1, and HFF (G0, 0.5, 1, 2, and log phase) cells were evaluated for relative c-myc protein levels to determine if the increased levels of c-myc transcript seen in P3C cells affected cellular levels of c-myc protein.

Western blot analysis showed that the c-myc monoclonal antibody bound to a band in the 65 kd size range area of the gel. An identical control panel incubated with anti-met 72 showed no non-specific binding, and staining of a third identical panel with India ink indicated that protein quantitation was consistent between samples (data not shown). Relative levels of c-myc protein were determined by reflectance densitometry, and values normalized to those of fibroblasts in G0 (Table I). Fibroblasts 0.5 and 2 hr after serum release, and during log phase

TABLE I. Relative Levels of c-myc Protein From Western Blot Analysis of P3C, UR HCL 1, and HFF Cell Lines as Determined by Reflectance Densitometry[a]

P3C	UR HCL 1	HFF/ G0	HFF/ 0.5	HFF/ 1	HFF/ 2	HFF/ L
6	2	1	1	2	1	1

[a]Values were normalized to c-myc protein levels of HFF cells in G0 which were given a value of 1.

growth (L) had the same amounts of c-myc protein as during G0. c-myc protein levels in UR HCL 1 cells and fibroblasts 1 hr after serum release (presumably G0/G1 transition) were twice this level, while P3C cells had 6 times the amount of protein.

Mapping Analysis of c-myc DNAse I Hypersensitive Sites 4, 5, and 6 From the 5′ Direction

Mapping of P3C c-myc DNAse I hypersensitive sites 4, 5, and 6 in the exon 1/intron 1 region from a 5′ direction lended additional reliability for assessment of their locations. Fifteen micrograms of HFF DNA and 5 μg of P3C DNA from DNAse I treated nuclei were restricted with Sca I. These samples along with 5 μg aliquots of P3C genomic DNA restricted with Sca I, and either Mae III, Bsm I, or Pvu II were analyzed by Southern blot hybridization with the Sca I/Xho I probe (Fig. 5). Resolution of DNAse I generated bands on this blot was found to be reasonably good as exemplified by the ability to easily resolve fragments generated from restriction of P3C genomic DNA with Sca I/Mae III and Sca I/Bsm I, which differ by approximately 50–60 base pairs. Lambda DNA digested with Eco R I/Hind III was run on either side of the gel as size markers, and to control for uniform migration. Two different samples of P3C DNA from DNAse I treated nuclei (0.2 and 0.5 μg/ml—P3C A and P3C B, respectively) were run because both produced optimal visualization of DNAse I generated bands, as did DNA from HFF nuclei digested with 0.5 μg/ml DNAse I. As with earlier analyses, bands corresponding to sites 4 and 6 were observed in both the P3C and HFF cell lines, while site 5 was present exclusively in P3C cells. Evaluation by this method more reliably placed sites 4 and 5 in regions thought to be associated with transcript limiting activity in lymphocytes (Sites E and F, Figs. 1, 3). Site 6 was confirmed to be slightly 5′ of exon 2.

This analysis also indicates that site 3, as shown in Figure 3, is actually located 5′ to the Sca I site. If this site had been located within the Sca I/Xho I probe region, bands of appropriate fragment size would have been observed.

DISCUSSION

We have demonstrated differences in DNAse I hypersensitive site patterns between single copy and amplified states of the c-myc gene in MFH cell lines. The nature of DNAse I hypersensitive site changes associated with amplified c-myc in P3C cells suggests that similar events which may restrict transcriptional activity in lymphocytes may also be employed by mesenchymal cells, possibly in an attempt to shut-down or limit abnormal transcript production.

More specifically, a site near the P0 promoter (site 2, Fig. 3), which is observed in UR HCL 1 and HFF cells, but not in P3C cells, is *located in* the same region as a previously documented DNAse I hypersensitive area (B, Fig. 3) (Siebenlist et al., 1988) which has presumed involvement with maintaining transcriptional activity of amplified c-myc in HL-60 cells. The observation that site 2 and B map to different sides of P0 does not preclude the possibility that they are in the same DNAse I sensitive region, considering these areas may include as many as 200 base pairs, or about the size of a nucleosomal repeat (Gross and Garrard, 1988). Furthermore, a site observed exclusively in P3C cells maps to an area in the first intron which may possess c-myc transcription limiting capabilities (Sites F and 5, Fig. 3) (Zajac-Kaye et al., 1988). These data suggest a compensatory response by P3C cells to an abundance of transcript produced by an amplified c-myc gene. Despite the suggested outcome of such an action by these cells, elevated amounts of c-myc transcript and protein prevail, indicating that such measures are insufficient to protect the cell from potential consequences of gene amplification.

A six-fold increase in P3C c-myc protein levels compared with those of normal fibroblasts (Table I) indicates that this mutation is reflected at the cellular protein level, potentially impacting normal cell function. c-myc protein levels have been reported to remain constant throughout the cell cycle in normal and transformed cells (Hann et al., 1985), while other studies have shown that c-myc transcript levels are inducible by such stimuli as platelet derived growth factor (PDGF) (Kelly et al., 1983). The observed transient increase in c-myc transcript levels following serum addition to quiescent HFF fibroblasts may explain the two-fold increase in protein observed after 1 hr. A two-fold increase in protein was also seen in UR HCL 1 cells, and may be tumor cell associated.

Studies with quiescent fibroblasts following serum release indicate that DNAse I patterns observed for P3C amplified c-myc are not associated with increased c-myc transcript levels in

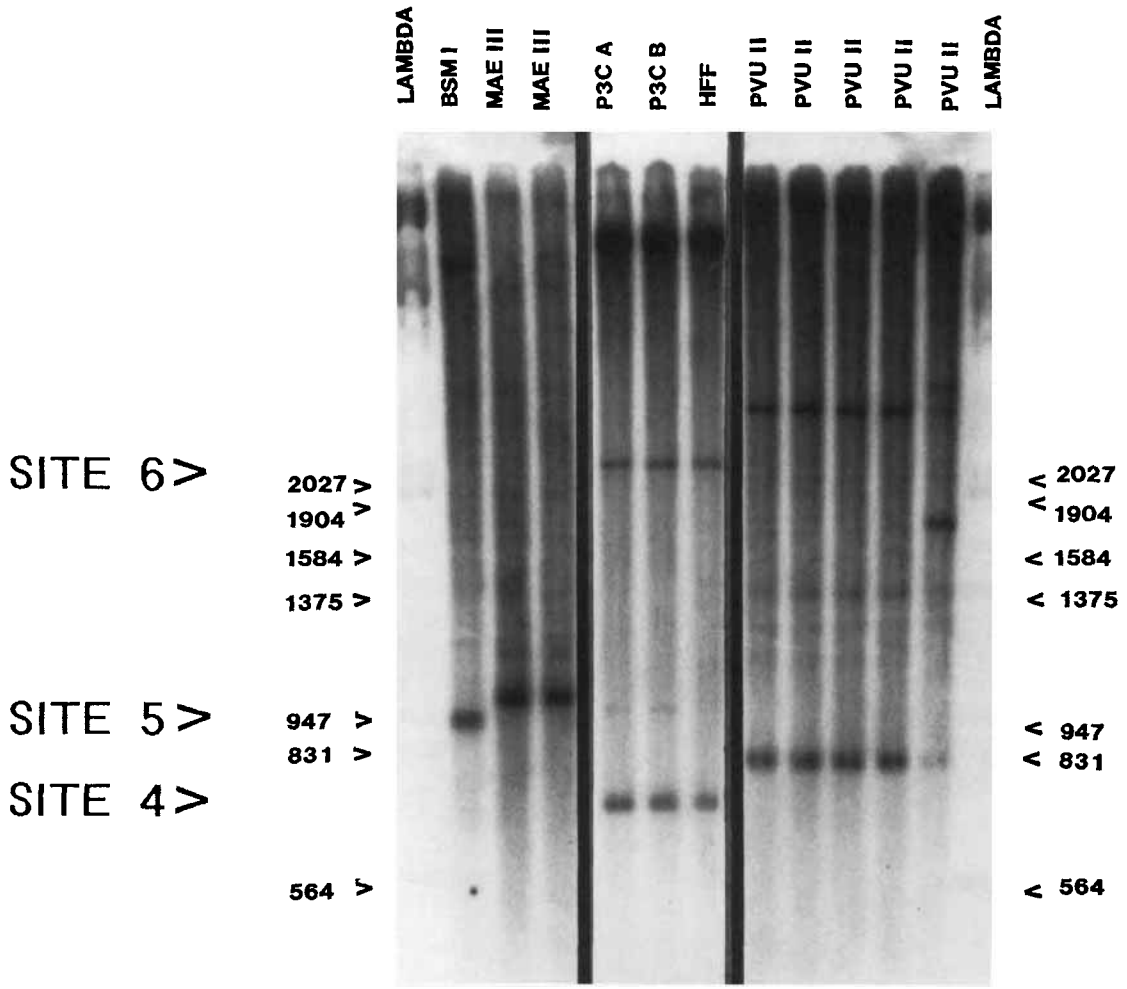

Fig. 5. Southern blot hybridization of P3C and HFF DNAs from DNAse I treated nuclei used in mapping DNAse I hypersensitive sites 4, 5, and 6 from the 5′ direction. This was accomplished by comparison of c-myc DNAse I generated bands in P3C and HFF cells to DNA fragments of known sizes generated by double restriction of P3C genomic DNA. Five micrograms of P3C DNA from DNAse I treated nuclei (P3C A and P3C B were treated with 0.2 and 0.5 μg/ml DNAse I, respectively) and 15 μg of HFF DNA from DNAse I treated nuclei (treated with 0.5 μg/ml DNAse I) were restricted with Sca I (Boehringer Mannheim), and were the actual samples used to map sites 4, 5, and 6. These were compared with aliquots of untreated P3C genomic DNA restricted with Sca I and either Pvu II (BRL), Bsm I (New England Biolabs), or Mae III (Boehringer Mannheim). This generated fragments corresponding to known distances between the Sca I site and selected restriction sites in the same region as the DNAse I sites of interest (Fig. 3). The digestions were conducted for 30 min using various restriction enzyme concentrations. This allowed partial digestion of the DNA, for the purpose of selecting fragments of desired length. Restricted DNAs which yielded optimum visualization of the desired marker band sizes were used as markers in the actual mapping analysis. DNA samples were electrophoresed through a 1.5% agarose gel and hybridized with a Sca I/Xho I c-myc genomic fragment labeled by random primer extension (10 E8 cpm/μg). Bands corresponding to sites 4, 5, and 6, are indicated, as are P3C genomic DNA marker fragments ScaI/Bsm I (labeled BSM I), Sca I/Mae III, (labeled MAE III), and ScaI/Pvu II (labeled PVU II). Also shown are lambda DNA size markers produced by digestion with Eco RI and Hind III which were run on either side of the gel to control for uniformity of electrophoresis.

normal mesenchymal cells, and support a post-transcriptional model of c-myc regulation (Blanchard et al., 1985). Perhaps when c-myc is amplified in MFHs, normal post-transcriptional controlling mechanisms are insufficient to compensate for the abundance of transcript produced. Therefore, the cell may implement changes involving regulatory regions of the gene. Our data suggest the nature of these changes to be the implementation of a transcription limiting entity mediated through the 5′ region of intron 1, and the removal of a factor near the P0 promoter region, presumably associated with maintaining transcription. Despite this infer-

ence, increased levels of transcript and protein are observed in P3C cells, and the possibility cannot be ignored that these tumor cells have responded to transcription limiting adjustments by either somehow enhancing message stability or maintaining transcription by some other mechanism. In any case, changes in chromatin structure suggest a compensatory response to gene dosage effects mediated through key regulatory sites.

The questions of why c-myc amplification occurs and what processes would select for this trait remain only partially answered. It would seem that c-myc gene amplification must be a mechanism by tumor cells to obtain selective growth advantages over surrounding cells. Since c-myc produces a DNA binding protein which may ultimately mediate cell replication (Persson and Leder, 1984; Wickstrom et al., 1988), increased quantities of protein may provide a means for more rapid growth. This is supported by patient studies which show an association between c-myc gene amplification, increased transcript production, and increased cell division in MFHs (unpublished data). Since transformation is a multistep process, perhaps c-myc amplification in P3C cells represents one stage in a transformation cascade involving a series of transforming steps and subsequent responses by the cell to counteract their effects. In a scheme of this nature, it seems reasonable to suspect that when the cell can no longer respond effectively, transformation ensues.

In summary, changes in c-myc DNAse I hypersensitive site patterns which accompany amplification in MFH cells suggest an attempt by these cells to compensate for surplus amounts of gene product. The amplified gene appears to lack a DNAse I hypersensitive site which may be associated with the maintenance of transcript production. An additional site is seen in the first intron which may signify the implementation of a transcription limiting mechanism. The observation that DNAse I hypersensitive site pattern changes are not seen in normal fibroblasts following serum release supports the proposed importance of post-transcriptional regulation in controlling cellular levels of c-myc. Despite any cellular attempts to counteract abnormal gene activities, increased amounts of transcript and protein are seen. This may provide a selective growth advantage for MFH tumor cells, since the c-myc gene may participate in regulation of cell division. Changes in DNAse I hypersensitive site patterns are associated with c-myc gene amplification in MFHs and may reflect oncogenic properties of this gene.

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