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Biochemistry

A plasmacytoma-specific factor binds the c-*myc* promoter region

(oncogene/B-cell development/DNA-binding protein/repressor)

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ABSTRACT **We used an electrophoretic mobility-shift assay to study proteins that bind to sequences in the 5′ flanking region of the murine c-*myc* gene. By comparing the DNA–protein complexes formed with extracts from cells representing earlier stages of B-cell development with those from plasmacytomas, we identified a plasmacytoma-specific protein that binds to a region within the c-*myc* promoter. Five other regions of this promoter show extensive sequence-specific binding, but the binding is not clearly B-cell stage-specific. Methylation-interference and *o*-phenanthroline/copper-protection experiments identified a single plasmacytoma-specific protein binding site 290 base pairs 5′ of the transcription start site P1. Homologues of a core sequence, d(AGAAAGGGAAAGGA), within the 25-base-pair binding site are found at three additional sites in the murine c-*myc* locus. The plasmacytoma-specific occurrence of this protein suggests that it may play a role in the transcriptional repression of the normal c-*myc* gene observed in plasmacytomas.**

The c-*myc* protooncogene is consistently involved in reciprocal chromosomal translocations with the immunoglobulin loci in human Burkitt lymphomas and murine plasmacytomas (1, 2). Translocation results in deregulated expression of truncated c-*myc* mRNA, whereas mRNA from the normal (untranslocated) c-*myc* allele is undetectable in these tumors (1, 2). Deregulated expression of the translocated c-*myc* gene has been studied extensively because it is probably one causal element in the malignant transformation of these cells (2–4). A putative role for myc protein in DNA synthesis is consistent with its deregulated expression in rapidly dividing tumor cells (5). However, the repressed level of expression from the normal c-*myc* gene in plasmacytomas is also of interest. It may represent the normal state of c-*myc* in terminally differentiated plasma cells and therefore allow us to study key controls on c-*myc* expression in nondividing cells. Expression of c-*myc* in normal plasma cells has not been studied.

Several lines of evidence suggest that repression of normal c-*myc* expression in plasmacytomas and some Burkitt lymphomas is related to the differentiated state of these cells. Cell lines representing earlier proliferative stages of B-cell development do transcribe the normal c-*myc* gene, unlike plasmacytomas, which are transformed counterparts of terminally differentiated, nondividing plasma cells (6, 7). Somatic-cell hybrid studies showed that the mature B-cell environment of a Burkitt lymphoma is able to repress transcription of an active c-*myc* gene derived from splenic B cells, suggesting a trans-dominant negative factor in the Burkitt cells (8). Furthermore, somatic-cell hybrid studies showed that expression of a translocated c-*myc* gene is not sufficient to repress transcription of the normal c-*myc* gene in early B cells (9). Finally, in several hematopoietic cell lines that can be induced to differentiate *in vitro*, terminal differentiation is accompanied by a decrease in c-*myc* expression (10–12).

Run-on transcription experiments have shown that the normal c-*myc* gene is not transcribed at detectable levels in plasmacytomas (ref. 13 and unpublished work); thus c-*myc* repression occurs at the transcriptional level. This is consistent with the inactive chromatin structure of the normal c-*myc* gene in plasmacytomas as demonstrated by DNase I sensitivity (14). The expressed c-*myc* gene in the pre-B-cell line 18-81 has relatively open chromatin structure; it is more sensitive to DNase I than either the active immunoglobulin heavy-chain enhancer region or the inactive immunoglobulin α-heavy-chain constant region (E.K., unpublished results). The transcriptional repression of c-*myc* in plasmacytomas suggests that cis-acting sequences within the normal c-*myc* gene may be involved in preventing transcription at the terminal stage of differentiation.

A large region 5′ of c-*myc* is highly conserved between the murine and human genes, suggesting that multiple regulatory elements may be located there (15). Indeed, recent deletion and *in vivo* competition studies suggested that both positive and negative regulatory elements reside 5′ of the c-*myc* gene (16–20). Since the occurrence of closely spaced positive and negative regulatory elements can confound precise identification of individual elements in a deletion analysis, we have used a different strategy to identify c-*myc* regulatory elements. Using a sensitive electrophoretic mobility-shift assay for DNA-binding proteins (21), we studied *in vitro* protein binding to 5′ flanking sequences from four regions of high homology between murine and human c-*myc* (15). We compared the DNA–protein complexes formed with crude nuclear extracts from three pre-B- or B-cell lines, representing proliferative B-cell stages at which c-*myc* is transcribed, to the complexes formed with extracts from three plasmacytomas, representing terminally differentiated cells that do not transcribe normal c-*myc*. c-*myc* regions recognized by proteins specific for the earlier or the terminal B-cell stage could be involved in stage-specific regulation of c-*myc* transcription. We have identified a DNA-binding protein found only in plasmacytomas and mapped its binding site in the c-*myc* promoter region. This protein may be involved in repression of c-*myc* transcription in plasmacytomas.

MATERIALS AND METHODS

Cell Lines and Tumors. Cell lines and tumors were maintained by standard methods.

Nuclear Extracts. Nuclear extracts were prepared and stored as described (22), with slight modifications. Buffers A, C, and D were modified by addition of 0.2 mM EGTA and leupeptin, pepstatin, and aprotinin (each at 2 μg/ml; Sigma). The step using buffer B was omitted. Most extracts contained 2–3 mg of protein per ml, as determined by the Bradford assay (23) with bovine serum albumin standards. Heparin-Sepharose fractionation was performed by loading extract in 100

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Abbreviations: *o*-Phen/Cu, *o*-phenanthroline/copper; myc-PCF, plasmacytoma-specific factor.

mM NaCl/50 mM Tris·HCl, pH 7.5/0.2 mM EDTA/0.2 mM EGTA, followed by step elutions with 0.3 M and 0.6 M NaCl in the same buffer.

Mobility-Shift Assay. The mobility-shift assay was performed as described previously (37).

Methylation Interference. Methylation interference was performed as described (24), except that DE81 paper (Whatman) was used for electroelution.

***o*-Phenanthroline/Copper "Footprinting."** A preparative-scale mobility-shift reaction mixture [25–50× in probe cpm (≈300,000 cpm); 10× in extract, poly(dI-dC)·poly(dI-dC), and volume] was run in a 6% polyacrylamide gel. The whole gel was then treated with 1,10-phenanthroline/copper (*o*-Phen/Cu) reagent, which cleaves DNA within the gel, using a procedure developed by M. Kuwabara and D. Sigman (personal communication). The DNA bands were isolated by electroelution onto DE81 paper as described for methylation interference (24), and isolated DNA was run in 8 M urea/8% polyacrylamide denaturing gels.

Computer Analysis. GenBank* was searched using the Nucleic Acid Query (NAQ) software from the Protein Identification Resource.

RESULTS

A B-cell stage-specific protein in plasmacytomas binds in the c-*myc* promoter region. To identify putative positive or negative regulators of c-*myc* transcription, we compared the DNA-binding activity of extracts from cells representing earlier proliferative stages of B-cell development with that of extracts from terminally differentiated B cells secreting antibody at high levels. 18-81 and 70Z/3 are pre-B-cell lines that transcribe the normal c-*myc* gene (6, 25). WEHI-279 is an immature, low-level-secretor B-cell line with a translocated c-*myc* gene, but it produces only normal c-*myc* RNA (6, 26). The plasmacytomas M603, J558L, and P3X63-Ag8 (P3X) represent terminally differentiated plasma cells. They express a translocated c-*myc* gene but not the normal c-*myc* gene (refs. 13 and 27–29; unpublished results). The restriction fragments used as probes and competitors in the mobility-shift assays are shown in Fig. 1. Each probe covers a region of >80% homology between mouse and human c-*myc*.

The regions immediately 5′ to the transcription initiation sites P1 (137-bp probe, Fig. 1) and P2 (113-bp probe) should contain binding sites for common transcription factors (16, 19), and we compared the DNA–protein complexes formed by all six extracts using these probes (Fig. 2 *a* and *b*). Although some quantitative differences were observed, all six extracts gave similar patterns of DNA–protein complexes. The P3X extract did not show complex 137C in this experiment (Fig. 2*b*), although it did in other experiments (Fig. 3*b*). Since all the extracts formed similar complexes with these two probes, we conclude that the preparations and conditions of the different extracts are comparable. These results also show that no B-cell stage-specific proteins bind the regions covered by the two probes under our conditions.

Two upstream probes (163 bp and 141 bp, Fig. 1) also

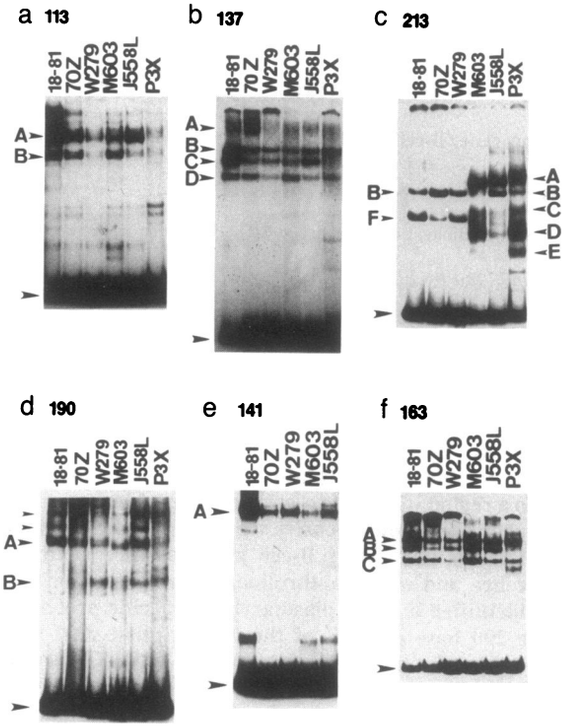

FIG. 2. Comparison of DNA-binding activity in different extracts for six probes. Each probe used is indicated above each panel, and above each lane is the source of the extract. Each autoradiogram of a mobility-shift experiment has letters that mark retarded complexes. The position of free probe is indicated by the lowest arrowhead in each panel. Reaction conditions: probe 113, extract 18-81 (3.2 μg), 70Z/3 (3.5 μg), WEHI-279 (4.2 μg), M603 (1.4 μg), J558L (3.2 μg), P3X (6.3 μg) with 4 μg of poly(dI-dC)·poly(dI-dC); probe 137, half the quantities for 113, with 5 μg of poly(dI-dC)·poly(dI-dC); probes 190, 163, and 223, the same extract quantities as for 113, with 4, 4, and 5 μg of poly(dI-dC)·poly(dI-dC), respectively; probe 141, 18-81 (3.2 μg), 70Z/3 (1.8 μg), WEHI-279 (3.1 μg), M603 (1.4 μg), J558L (3.2 μg) with 4 μg of poly(dI-dC)·poly(dI-dC). P3X extract is not shown for probe 141 because we did not obtain clear results.

*EMBL/GenBank Genetic Sequence Database (1987) GenBank (Bolt, Beranek, and Newman Laboratories, Cambridge, MA), Tape Release 50.

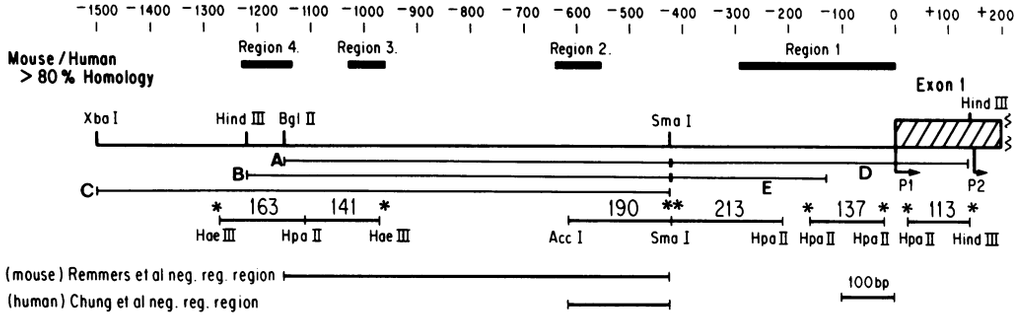

FIG. 1. Map of c-*myc* 5′ flanking sequences. Hatched box represents the 5′ part of exon 1 with the two major transcription start sites, P1 and P2. Above the map are horizontal bars that indicate regions of high homology (>80%) between human and murine c-*myc* (15). Below the map, the probes are shown with their length in base pairs (bp) and an asterisk to indicate the position of the radiolabel. At the bottom, two negative regulatory regions identified by others (16, 17) are indicated. Horizontal bars A–E indicate competitor fragments used in this study (see Fig. 3 legend).

formed nearly identical complexes with all extracts (Fig. 2 *e* and *f*). No stage-specific binding proteins are apparent. An additional lower complex was seen with the 141-bp probe in some extracts (Fig. 2*e*), but since its presence does not correlate with normal c-*myc* expression, we did not investigate it further.

When protein binding was compared using the 190-bp probe, we found two major complexes, 190A and -B, and no striking differences between all of the earlier stage cells and the plasmacytomas (Fig. 2*d*). 18-81 extracts did consistently lack the 190B complex, but this complex was seen in 70Z/3 and WEHI-279. The 190B band of 70Z/3 is not sharp in the experiment shown but was seen more clearly in other experiments (data not shown).

In striking contrast, the 213-bp probe showed several strong complexes specific to plasmacytoma extracts (Fig. 2*c*; complexes 213A, -C, -D, and -E). A common complex (213B) and a complex apparently specific to earlier B cells (213F) were also found. The different plasmacytoma-specific complexes occurred in varying quantities in different extract preparations. All of the 213-series complexes were sensitive to treatment with 100 ng of proteinase K but not RNase A, demonstrating that the complexes were DNA–protein complexes (data not shown).

To determine whether the complexes observed with the six different probes correspond to sequence-specific binding, competition experiments were performed. The extracts were preincubated with unlabeled competitor fragments to compare the ability of homologous and unrelated DNAs to compete with the labeled probe for binding to extract components. All labeled complexes seen with the 113-, 137-, 141-, 163-, and 190-bp probes showed sequence-specific competition (Fig. 3 *a–d* and *h*). The plasmacytoma-specific complexes 213A, -C, -D, and -E were also shown to result from sequence-specific binding (Fig. 3 *e* and *f*). Although *Hpa* II-cut pBR322 does compete to some extent for binding with M603 extract (Fig. 3*e*), addition of small amounts of specific fragment inhibited binding more completely. Complete inhibition of 213B complex formation required a 110-fold molar excess of specific fragment (Fig. 3*g*), while only a 5-fold molar excess of competitor was necessary to prevent formation of the 213A, -C, -D, and -E complexes (Fig. 3*f*), suggesting that the protein responsible for complex 213B may be present in vast excess. Sequence-specific competition of the 213F binding activity was not observed (data not shown).

Plasmacytoma-Specific Complexes Have a Common Binding Site. To map the binding sites for plasmacytoma-specific complexes 213A and -D, we performed methylation-interference experiments (24). Fig. 4*a* shows the results obtained using M603 crude nuclear extract. Free probe (Fr) was run adjacent to DNA samples derived from the indicated complex. Bands corresponding to six closely spaced deoxyguanosine residues were decreased or absent in DNA derived from the A and D complexes of both extracts. Thus, methylation of these deoxyguanosine residues interferes with the binding of a protein (Fig. 4*a*). These deoxyguanosines are located near position −290 (Fig. 4*e*) and occur at the 5′ border of human/mouse homology block 1 (Fig. 1). Similar results were obtained for complexes 213A, -D, and -E using extract from P3X, although two of the six methylated deoxyguano-

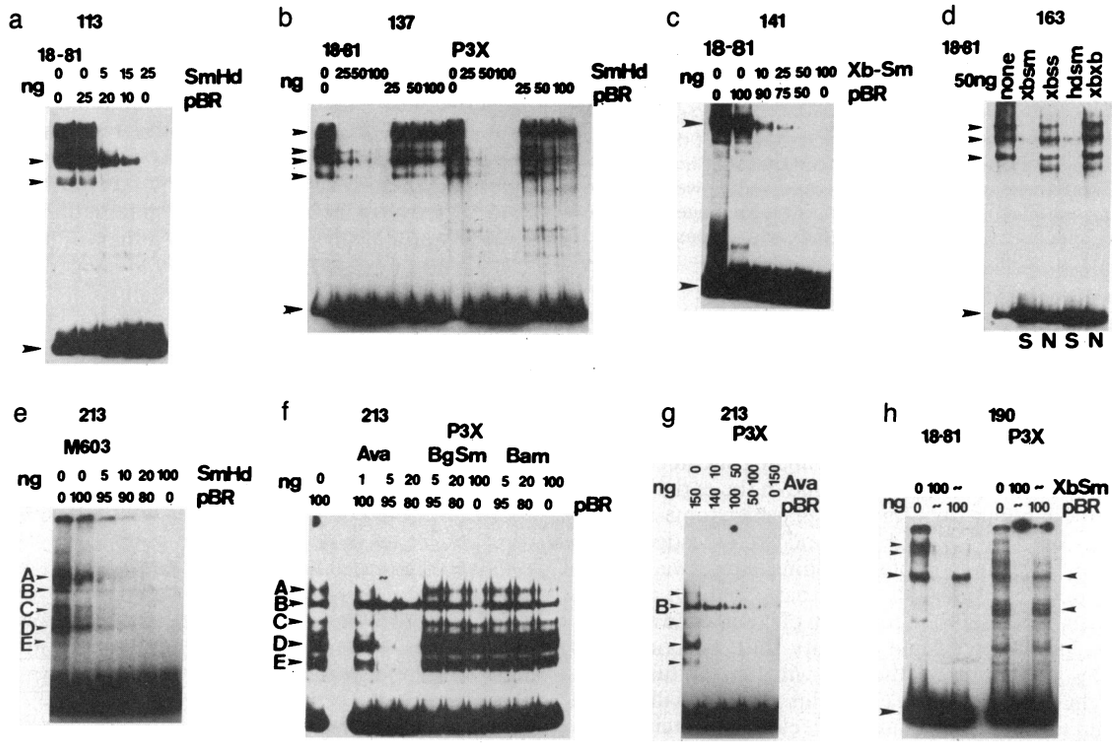

FIG. 3. Competition experiments. Each panel shows competition for one probe; probes and extract sources are shown above each panel. In *a*, *c*, *e*, *f*, and *g*, varying amounts of nonspecific and specific competitor were added together; in *b*, *d*, and *h*, individual reaction mixtures contained specific or nonspecific competitor. Directly above each lane are the nanograms of specific (upper numbers) or nonspecific (lower numbers) competitor DNA added. Binding conditions for the lanes with no competitor are the same as in Fig. 2; reaction mixtures with competitor contained 1 μg less poly(dI-dC)·poly(dI-dC) and were incubated 5–10 min before labeled probe was added. Nonspecific competitor in *a*, *b*, *c*, *e*, *f*, *g*, and *h* is *Hpa* II-cut pBR322; nonspecific competitors in *d* are XbSs, a 900-bp *Xba* I–*Sst* I fragment that covers the second coding exon of c-*myc*, and XbXb, a 1.9-kb *Eco*RI–*Xba* I fragment from the 5′ region of the murine immunoglobulin α constant gene segment (14). Sequence-specific competitors are SmHd, a 562-bp *Sma* I–*Hin*dIII fragment (Fig. 1, bar D); XbSm, a 1079-bp *Xba* I–*Sma* I fragment (Fig. 1, bar C); HdSm, a 797-bp *Hin*dIII–*Sma* I fragment (Fig. 1, bar B); *Ava*, a 286-bp *Ava* I fragment (Fig. 1, bar E); BgSm, a 726-bp *Bgl* II–*Sma* I fragment (Fig. 1, bar A); Bam, a 1.1-kb *Bam*HI fragment covering the *myc* first exon, intron, and site-1 homology. The letters S and N below lanes in *d* indicate whether the competitor was specific or nonspecific, respectively.

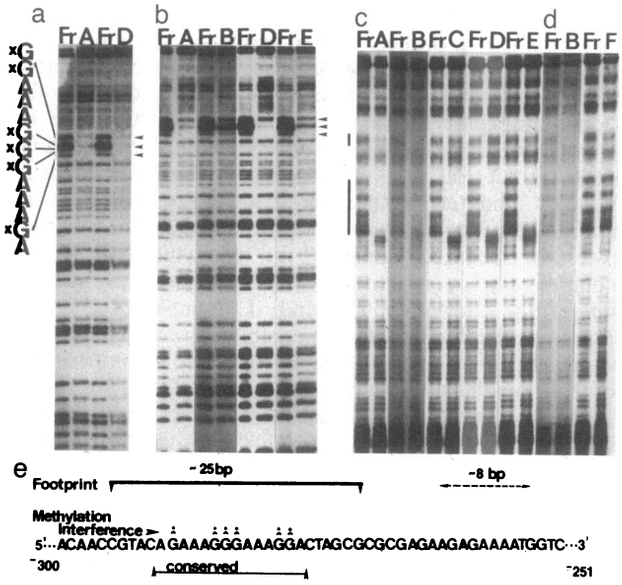

FIG. 4. Methylation interference and *o*-Phen/Cu footprinting. See ref. 24 for an explanation of methylation interference. (*a*) Methylation-interference experiments performed with the 213-bp probe and M603 crude extract. Above each lane is either "Fr," for DNA derived from free probe, or a letter indicating the complex from which the DNA was derived, as labeled in Fig. 2*c*. Arrowheads point to the position of deoxyguanosine residues decreased or absent in complex lanes A and D. (*b*) Methylation-interference experiments with P3X heparin-Sepharose 0.6 M NaCl fraction. (*c*) *o*-Phen/Cu footprinting of complexes formed with the 213-bp probe and the P3X 0.6 M NaCl fraction are shown. The large vertical bar indicates the position of a protected region in complexes 213A, -C, -D, and -E. The small vertical bar indicates the small region that was partially protected in complexes 213A and -B. (*d*) *o*-Phen/Cu footprinting of the 213B and 213F complexes of 18–81 extract. (*e*) The footprinted sequence and close-contact deoxyguanosine residues of the plasmacytoma-specific complexes (213A, -C, -D, and -E) are shown on the left part of the sequence. The sequence conserved between murine and human c-*myc* is indicated (see Fig. 5). The 8-bp sequence on the right is the region that may be protected in the common 213B complex and the specific 213A complex.

sine residues interfere only partially with protein binding (Fig. 4*B*). Methylation-interference experiments with P3X and 18-81 extracts did not demonstrate any binding sites in the 213B and -F complexes using the same end-labeled probe (Fig. 4*b* and data not shown). Thus, three plasmacytoma-specific complexes have one protein binding site in common that is not observed in complexes found in the early B cells.

To map the binding sites for each plasmacytoma-specific complex (213A, -C, -D and -E) and to investigate the possibility of additional protein binding sites, we used a procedure developed by M. Kuwabara and D. Sigman (personal communication). Separation of complexed and free probe in a preparative-scale mobility-shift experiment is followed by treatment of the gel with the diffusible *o*-Phen/Cu chemical nuclease (30). This reagent will cleave DNA within a gel except at sites protected by proteins, thus forming "footprints" of each DNA–protein complex stably maintained within the acrylamide gel. Free and bound DNA is then extracted from the nondenaturing gel and analyzed in a denaturing gel. *o*-Phen/Cu footprinting experiments with the P3X 0.6 M NaCl fraction showed identical 25-bp protected regions for complexes 213A, -C, -D, and -E (Fig. 4*c*); these regions include the six close-contact deoxyguanosine residues identified by methylation interference for 213A and -D (Fig. 4*e*). The 213B complex showed a partially protected region of 8–10 bp centered at −258; the same region was partially protected in the 213A complex with P3X extract (Fig. 4*c*) and in the 213B complex with 18-81 extract (Fig. 4*d*). We did not observe any additional protected regions for complexes 213C, -D, and -E, suggesting that binding at only one site is responsible for these different shifted bands. The 213F complex showed no discernible protected regions (Fig. 4*d*), consistent with competition results suggesting that it is not sequence-specific.

In summary, both methylation interference and *o*-Phen/Cu footprinting identified one plasmacytoma-specific binding site on the 213-bp probe. We will refer to the plasmacytoma-specific protein that binds at this site as myc-PCF (for "plasmacytoma-factor"). The multiple mobility-shifted complexes that show binding to the myc-PCF site may result from oligomerization of myc-PCF, association of myc-PCF with other proteins, or degradation of a larger protein.

A Sequence Within the myc-PCF Binding Site Is Repeated Four Times in the c-*myc* Locus. The central portion of the plasmacytoma-specific *o*-Phen/Cu-protected sequence was compared to mouse sequences in the GenBank sequence library.* Surprisingly, 3 additional good matches were found within the c-*myc* gene out of a total of 22 matches found in GenBank (Fig. 5). These sites have been numbered 1–4; site 2 corresponds to the myc-PCF binding site. The core conserved sequence is d(RGAAAGGGRRAGGA), where R represents A or G. Sites 3 and 4 are contained within the two probes 190 and 163, but no plasmacytoma-specific binding was found with those probes (Fig. 2 *d* and *f*). Site 1 is within the first intron just 3′ of exon 1 and has not been tested directly for binding.

To test further for plasmacytoma-specific binding to sites 1, 3, and 4, we assayed their ability to compete for plasmacytoma-specific binding to site 2 on the 213-bp probe. A 30-fold molar excess of a DNA fragment containing either site 3 or 4 and a 20-fold molar excess of a fragment containing site 1 were not sufficient to compete for binding, although a 3- to 4-fold molar excess of a homologous site-2 fragment was able to compete (Fig. 3*f*). If sites 1, 3, and 4 do bind the plasmacytoma-specific factor, they must either be of lower affinity or require conditions or interactions that are not easily reproduced *in vitro*. Perhaps other *o*-Phen/Cu-protected sequences flanking the conserved sequence at site 2 are important for myc-PCF binding. Analysis of the human c-*myc* promoter demonstrated that murine sites 3 and 4 are not conserved. However, murine sites 1 and 2 are conserved in the human c-*myc* sequence (12/15 and 11/13 bp; Fig. 5; ref. 31).

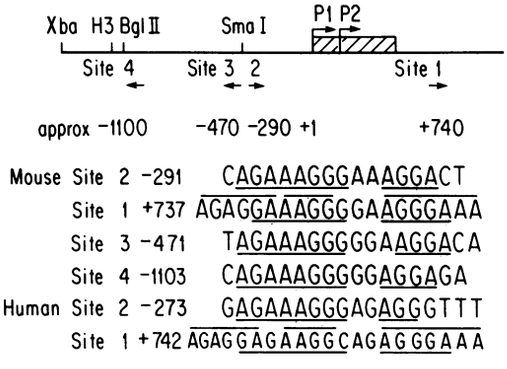

FIG. 5. A map and sequence comparison of the binding site of myc-PCF. At top is shown a map of the c-*myc* locus with the four homologous sites indicated below as horizontal arrows. Site 2 is bound by myc-PCF. Below each site is the approximate position of the sites relative to the transcription start site P1. (H3 indicates a *Hin*dIII cleavage site.) Below the map, the sequences of the different sites are compared. Underlining indicates the homologous sequences between murine and human sites. The overline on mouse and human site 1 indicates the extended homology between the site-1 sequences.

DISCUSSION

We wish to understand how c-*myc* transcription is repressed in terminally differentiated B cells. We have compared DNA-binding activity for c-*myc* 5′ flanking sequences in nuclear extracts from pre-B- and B-cell lines, which transcribe c-*myc*, with that in extracts from plasmacytomas, which do not transcribe normal c-*myc*. We have identified a DNA-binding protein present in plasmacytomas but not in cell lines representing earlier stages of B-cell development, mapped the binding site for this protein, and shown that similar sequences occur three additional times within the c-*myc* gene.

Regulation of the normal c-*myc* gene is clearly complex, as suggested by previous studies (32) and by the multiple protein binding sites detected 5′ of c-*myc* in this study. However, the similarity of DNA–protein complexes formed with five out of six DNA probes used in this study suggests that a limited number of DNA-binding factors may be responsible for repression of the c-*myc* gene in plasmacytomas. The plasmacytoma-specific activity of myc-PCF suggests it may be one of the important factors in c-*myc* transcriptional repression, although verification awaits functional studies.

If myc-PCF represses c-*myc* and is present only in mature, nondividing B cells, then one would expect to find translocations that delete the myc-PCF binding site primarily in mature cells rather than earlier B cells. c-*myc* translocations in plasmacytomas nearly always remove the myc-PCF binding site (2), which would allow the transcription of translocated c-*myc* observed in these cells. In contrast, such translocations rarely occur in B-cell lines from earlier stages (6). A recent comparison of endemic and sporadic forms of Burkitt lymphoma also supports this idea (33). The sporadic tumors were consistently of a more mature phenotype (IgM secretors) and had translocation breakpoints within or immediately 5′ to c-*myc*, whereas the endemic tumors were less mature (little or no secretion) and had more distant translocations. Thus, deregulation of the c-*myc* gene at different stages of B-cell development may have different prerequisites. In murine plasmacytomas and mature Burkitt lymphomas, the deletion of the myc-PCF binding site by translocation may be required. It is also possible that other important 5′ flanking sequences are removed by translocation.

Previous studies have demonstrated cis-acting negative regulatory regions that do not include the myc-PCF binding site. Remmers *et al.* (17) and Chung *et al.* (16) demonstrated negative regulatory regions in the mouse and human c-*myc* 5′ flanking regions (Fig. 1) that were 5′ of the myc-PCF binding site. In one case, no deletions of the myc-PCF binding region were made (17), and the *in vivo* competition study (16) gave no evidence of a negative element, but this result was complicated by the presence of positive elements in the tested region (19). We did not observe clear differences between all pre-B- and B-cell vs. all plasmacytoma extracts for proteins binding to part or all of the negative regions defined in these earlier studies.

It has been suggested that increased levels of myc protein from the translocated allele in plasmacytomas might inhibit transcription of the normal allele (1). In tumors derived from cells that constitutively express activated c-*myc* gene constructs, the endogenous c-*myc* allele is repressed (4, 34). However, when cells are transfected with constitutively expressed c-*myc* constructs, high levels of myc expression do not repress endogenous c-*myc* transcription (34–36). Thus, the question of an autoregulatory effect for myc protein remains unresolved. It is possible that myc-PCF is myc protein; however, the cell lines COLO-320 (colon carcinoma) and HL-60 (promyelocytic leukemia), which express high levels of c-*myc* RNA due to the presence of amplified c-*myc* genes, do not show myc-PCF activity although they do contain 213B activity (E.K., unpublished results). Thus, we think it more likely that myc-PCF is a factor involved in c-*myc* repression in terminally differentiated cells that do not express c-*myc*, and we expect that it may be found in other terminally differentiated tissues.

The occurrence of additional copies of the "GAAAGGG" sequence within the c-*myc* locus is intriguing, although our data show no evidence that myc-PCF binds to sites 1, 3, and 4. Mutational analysis should determine the functional role of each site or combination of sites.

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