A Myc-associated Zinc Finger Protein-related Factor Binding Site Is Required for the Deregulation of c-myc Expression by the Immunoglobulin Heavy Chain Gene Enhancers in Burkitt’s Lymphoma*

Hsien-Ming Hu, Magdalena Arcinas, and Linda M. Boxer†

From the Center for Molecular Biology in Medicine, Veterans Affairs Palo Alto Health Care System, and the Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

The deregulation of expression of the c-myc gene in Burkitt’s lymphoma results from the translocation that links one c-myc allele to one of the immunoglobulin genes. This physical linkage promotes interactions between c-myc and immunoglobulin gene regulatory elements that affect c-myc transcription initiation and elongation. We have located a region in the c-myc promoter that is required for the complete activation by the immunoglobulin heavy chain gene enhancer. This regulatory element contains a core sequence, GGGAGG, similar to the GA box recognized by the transcription factor Myc-associated zinc finger protein (MAZ). UV cross-link analysis indicated that the mass of this protein did not correspond to that of MAZ, suggesting that a protein related to but distinct from MAZ bound to this site. Mutation of this regulatory element resulted in a loss of promoter activity induced by the immunoglobulin heavy chain gene enhancer. This site was also required for the c-myc promoter shift from P2 to P1. In vivo footprinting revealed that this site was occupied on the translocated c-myc allele but not on the untranslocated allele. Taken together, these findings suggest that this regulatory element is required for the full activation of c-myc promoter activity by the immunoglobulin heavy chain gene enhancer.

Burkitt’s lymphoma is characterized by specific chromosomal translocations that juxtapose the proto-oncogene c-myc on chromosome 8 to one of the Ig loci on chromosome 2, 14, or 22. The most common form of the translocation is t(8;14) where the c-myc gene is covalently linked to the immunoglobulin heavy chain gene (IgH)1. The translocated c-myc gene is highly expressed, whereas the normal allele is silent. Furthermore, the transcripts initiated from the c-myc P1 promoter, which normally contribute to a minor (10–20%) population of c-myc mRNA, increase to a level greater than the transcripts initiated from the P2 promoter, a phenomenon known as promoter shift (1–3). It is assumed that the physical linkage of the c-myc gene to one of the immunoglobulin loci promotes the interactions between c-myc and Ig regulatory elements that affect c-myc transcriptional initiation and elongation. In support of this view, it has been demonstrated that linkage of the murine IgH 3’ enhancer region, MHS1234, to a 2.3-kb region of the human c-myc promoter in an episomal vector is sufficient to reproduce the activation of c-myc transcription and the promoter shift in stably transfected Raji cells (4). Similar results have been obtained with the Igκ (5) and Igα (6) light chain enhancers. Despite these findings, most of the cis-acting enhancer and promoter elements that contribute to the deregulation of expression of the c-myc gene remain unidentified.

We have shown previously that an NF-xB site in the MHS4 region of the IgH enhancer is required for the transcriptional activation of the translocated c-myc gene and is involved in inducing the c-myc promoter shift from P2 to P1 (7). Others have found that NF-xB and PU.1 sites are critical for the deregulation of expression of the c-myc gene by the Igκ enhancer (8). It has been shown that the IgH enhancer MHS1234 activates expression from the c-myc P2 promoter by a mechanism involving increased histone acetylation along the c-myc promoter region (9). This finding, however, fails to explain the increased expression from the P1 promoter, suggesting that the c-myc P1 and P2 promoters are activated through somewhat different mechanisms by the Igκ enhancer.

Several cis-acting elements in the c-myc promoter that have been identified and implicated in the basal and activated expression of the c-myc gene. Transcription factors such as Sp1 (10, 11), Myc-associated zinc finger protein (MAZ) (12, 13), and E2F (14) have been reported to bind to three cis-elements located between the P1 and P2 start sites. These sites are required for the basal activity of the P2 promoter in proliferating cells; however, their roles in the deregulation of the c-myc gene in Burkitt’s lymphoma cells have not been fully studied. Core promoter elements, including an Sp1 site and a TATA box located within 100-bp upstream of the c-myc P1 promoter, are essential and sufficient for Igκ enhancer-activated P1 expression (15). Two NF-xB sites are active in the regulation of c-myc in normal cells as well as in Burkitt’s lymphoma cells (16–18). These sites contribute to activation of the P2 promoter with very little effect on the P1 promoter (16). Studies with the t(8;22) translocation have shown that c-myc promoter sequences upstream of the immediate P1 promoter (−101 to −1056) are required for full activation by the Igκ enhancer and for the induction of the promoter shift (6). The specific transcription factor binding sites that mediate this activity were not identified.

In this report, we show that a positive regulatory element
Deregulation of c-myc Expression

located −523 bp upstream of the P1 transcription start site is required for the transcriptional activation of the translocated c-myc gene and that it is involved in inducing the promoter shift mediated by the IgH enhancer. This element contains a GA box binding core, which is similar to that of a zinc finger transcription factor, MAZ. We also evaluated the effect of the Sp1 site in the P1 core promoter on the activation of c-myc transcription by the IgH enhancer.

EXPERIMENTAL PROCEDURES

**Plasmid Constructs**—The plasmid pHSH1234EP contains the human c-myc promoter (−2328 to +936), the firefly luciferase gene, and the murine IgH enhancer MHS1234 with the Epstein-Barr virus origin of replication and the hygromycin B resistance gene. Its construction has been described previously (7). Reporter constructs containing serial 5′ deletions of the c-myc promoter were derivatives of pHSH1234EP. These constructs were used for transient and stable transfections (Fig. 1, A and B).

**Site-directed Mutagenesis**—Mutagenesis of the MAZ-related factor binding site was performed by the oligo-directed mutagenesis method with the Quick Change kit from Stratagene. The following primer was used for mutagenesis, GAAGGGAATTCAGGGA

**Electrophoretic Mobility Shift Assay**—Oligonucleotides were synthesized with 5° end labeling and were used for EMSA. Wild-type (WT) refers to the sequence containing the promoter regulatory elements required for the activation of c-myc transcription by the IgH enhancer, reporter constructs containing serial 5′ deletions of the c-myc promoter region were generated (Fig. 1, A and B). These constructs contain the murine DNase I-hypersensitive sites (MHS1234) derived from the IgH, which have been shown to be sufficient for inducing the deregulation of c-myc expression (4). Transfection of these constructs into Raji cells revealed that a 30-bp region (−540 to −510) located between deletion constructs D8 and D9 was responsible for approximately half of the activity observed with the full-length promoter (Fig. 1C). The rest of the activity was mediated by sequences downstream of the SmaI site at −102 of the c-myc promoter (Fig. 1C, lane D11).

**RESULTS**

**Identification of a Regulatory Element in the c-myc Promoter That Is Required for Activated Expression of c-myc**—To search for the promoter regulatory elements required for the activation of c-myc transcription by the IgH enhancer, reporter constructs containing serial 5′ deletions of the c-myc promoter region were generated (Fig. 1, A and B). These constructs contain the murine DNase I-hypersensitive sites (MHS1234) derived from the IgH, which have been shown to be sufficient for inducing the deregulation of c-myc expression (4). Transfection of these constructs into Raji cells revealed that a 30-bp region (−540 to −510) located between deletion constructs D8 and D9 was responsible for approximately half of the activity observed with the full-length promoter (Fig. 1C). The rest of the activity was mediated by sequences downstream of the SmaI site at −102 of the c-myc promoter (Fig. 1C, lane D11).

**The Sp1 Site Located in the P1 Core Promoter Is Required for the Activated Expression of the c-myc Gene by the IgH Enhancer**—The Sp1 site and the TATA box in the P1 core promoter have been shown to be essential and required for the activation of the c-myc P1 promoter by the IgH enhancer because the mutation of either site completely abolishes transcription from P1 (15). To investigate whether either or both of these sites contributed to the activation of c-myc by the IgH enhancer and accounted for the activity downstream of −102 of the c-myc promoter, reporter constructs with deletions encompassing these sites were generated as shown in Fig. 1B. Deletion of the Sp1 site further decreased the c-myc promoter activity by another 50%, leaving only 25% of the activity of construct
pMHS1234EP (Fig. 1C, lane D13). No additional reduction of promoter activity was observed when the TATA box was deleted after deletion of the Sp1 site. The residual activity was likely from the activation of the P2 promoter. Our results are consistent with the results observed with the IgH enhancers. The activity of the TATA sequence was observed in the presence of the Sp1 site (15). These elements account for the activity of the P1 core promoter in the presence of the IgH enhancers.

EMSA Scanning Mutagenesis Identifies a Protein Binding Site in the 30-bp Upstream Region of the c-myc Promoter—Although a region extending 1 kb upstream of the c-myc promoter was required for full activation of c-myc by the IgH enhancers, no elements that mediated this activity were defined in this region (6). Our results with the IgH enhancer identified a region of 30 bp from −510 to −540 that demonstrated significant activity in the presence of the enhancers. Sequence analysis of this 30-bp region revealed possible transcription factor binding sites between −520 and −540. EMSA was performed with a radiolabeled oligonucleotide corresponding to the sequences from −520 to −540 upstream of the P1 promoter to determine whether protein-DNA interactions could be detected with nuclear extract from Raji cells. Several protein-DNA complexes were observed (Fig. 2A). One slowly migrating species was formed in a sequence-specific manner, as this complex was competed by unlabeled DNA of the same sequence as the probe but not by an unrelated Myb binding site (Fig. 2A, lanes 2 and 3, respectively). This region contains a GA-rich binding core with the sequence AGGGAGGAA on the noncoding strand that may potentially bind a transcription factor of the zinc finger family. Double-stranded oligonucleotides encompassing this region with sequential 4-base pair mutations (M1–M6) were used as competitors in EMSA to locate a protein binding site. Two oligonucleotides (M2 and M3) failed to compete well for the specific complex at a 100-fold molar excess (Fig. 2B, lanes 3 and 4). Because oligonucleotide M2 competed for the complex to some extent, the sequence of oligonucleotide M3 was selected for functional mutagenesis studies (see below).

A MAZ-related Factor Binds to the 23-bp Upstream Site—To study the identity of the protein interacting with the regulatory element, competitive EMSA was performed to test the ability of various consensus binding sites for members of the zinc finger protein family to compete with the specific protein-DNA complex. As shown in Fig. 3A, lanes 2 and 3, the complex was competed by the addition of a 100-fold molar excess of unlabeled probe and by a 100-fold molar excess of the binding site for MAZ. The competition was abolished when a mutated MAZ binding site was used as a competitor (Fig. 3A, lane 4). The complex was, however, not competed by the presence of a
100-fold molar excess of the binding sites for the zinc finger proteins myeloid zinc finger protein 1 (MZF-1) or Sp1, both of which recognize a core sequence very similar to that of the active c-myc promoter element (Fig. 3A, lanes 5 and 6, respectively). In addition, the binding sites for Octamer and NF-κB transcription factors, which resemble the GA core, failed to compete (Fig. 3A, lanes 7 and 8, respectively).

To further analyze the interaction between the MAZ-like protein and the active element, we studied the ability of the MAZ consensus sequence to disrupt the protein-DNA complex at different concentrations by EMSA. Although the MAZ consensus was able to compete off the specific complex at molar concentrations of 100- to 200-fold (Fig. 3B, lanes 5 and 6), it did so less efficiently than the wild-type oligonucleotide (Fig. 3B, lanes 2, 3, and 4). Increasing concentrations of the mutated site or a consensus sequence for Sp1 showed no competition at high concentrations (Fig. 3B, lanes 8–10 and 11–13, respectively).

**Molecular Mass Determination Identifies a Unique Protein, MAZ-R**—UV cross-linking followed by SDS-polyacrylamide gel electrophoresis of the EMSA complex was performed to determine the molecular mass of the protein that interacted with the 23-bp region. A major band with a molecular mass of 90 kDa along with two less abundant species of 50 and 42 kDa were identified on the gel (Fig. 4). After correction for the bound oligonucleotide, the predicted molecular mass of each protein was 75, 35, and 27 kDa, respectively. The molecular mass of MAZ was estimated at 60 kDa (20). These results suggest that the active c-myc promoter element binds a transcription factor that is distinct from MAZ, but which recognizes a core sequence with great similarity to that of MAZ. Hence, we designated the promoter element a MAZ-related (MAZ-R) protein binding site.

**The MAZ-related Protein Binding Site Demonstrates Functional Activity—Mutation of four base pairs** (oligonucleotide
M3) in the core sequence of the MAZ-R binding site prevented the protein from binding to DNA. This mutation was introduced into the reporter construct to test its effect on c-myc promoter activity. As shown in Fig. 1C, lane mutant, the activity of the c-myc promoter decreased by 50% in agreement with the results from the deletion study.

The MAZ-R Site Is Occupied on the Translocated c-myc Allele—To determine the relevance of this site for the endogenous c-myc gene, we examined the occupancy of the site on each c-myc allele in Raji cells. In vivo footprinting by ligation-mediated PCR was performed after separation of the translocated and untranslocated c-myc alleles as described previously (16, 21). Protection of five guanine residues was observed on the noncoding strand in this region (Fig. 5). These five guanine residues form the core sequence of the MAZ-R site. Examination of the coding strand was not informative because there are no guanines located in this region.

The MAZ-R Element Is Involved in the Promoter Shift Induced by the IgH Enhancer—We and others have shown previously that the linkage of the murine IgH enhancer to the 2.3-kb human c-myc promoter induced the full promoter shift (4, 7). We were interested in assessing the role of the MAZ-R element in the induction of the promoter shift. Stable cell lines containing various episomal vectors (Fig. 1A) were generated. Total RNA from the transfected pools was analyzed by S1 protection analysis with the probe shown in Fig. 6A. The ratio of transcripts initiated at the P1 and P2 promoters was unchanged with the deletion of sequences upstream of −540 (Fig. 6, B and C, pMHS1234EP and lane D8). When the MAZ-R element was deleted, the promoter shift decreased significantly from 1.7 to 0.78 (Fig. 6, B and C, lane D9). Transcription from the P1 promoter decreased by 60%, whereas transcription from the P2 promoter decreased by only 2%.

Mutation of the MAZ-R site also resulted in a significant decrease in the promoter shift when analyzed in two independently obtained cell lines (Fig. 6, B and C, mutant1 and mutant2). Although the level of the P2 transcript was essentially unchanged, the level of the P1 transcript was significantly decreased. The ratio of P1 to P2 was reduced from 1.7, the full promoter shift in pMHS1234EP, to 0.85 for mutant 1 and 0.9 for mutant 2 (Fig. 6C).

**DISCUSSION**

The breakpoints on the c-myc gene in t(8;14) Burkitt’s lymphoma can be internal to the transcription unit or up to several hundred kilobases 5’ of the c-myc gene. The regulation of expression of the translocated c-myc allele in these variants may involve different sets of transcription factors that bind to the
c-myc promoter region and interact with IgH enhancer elements. It has been shown that four enhancer regions, MHS 1, 2, 3, and 4, located 3' of the murine IgH are sufficient to activate transcription and induce the promoter shift when linked to the c-myc promoter region (4). However, the molecular mechanisms by which the IgH enhancer deregulates c-myc expression are not well understood. In this study we analyzed the upstream region of the c-myc gene for elements that are required for deregulation by the IgH enhancers using both transient and stable transfections into Raji cells. We identified a novel regulatory element located −523 bp upstream of the c-myc P1 transcription start site. Functional studies demonstrated that this element was required for the full activation of the c-myc promoter by the IgH enhancers. In vivo footprinting revealed that the MAZ-R site was occupied on the translocated c-myc allele but not on the untranslocated allele. The MAZ-R site also played a role in inducing the promoter shift from P2 to P1 that is observed in Burkitt’s lymphoma cells. These results are in agreement with the findings that the Igλ enhancer requires sequences 1 kb upstream of the c-myc P1 promoter for the full activation and induction of the promoter shift. Our study with the IgH enhancers extends these results by identifying the element that is responsible for this activity.

The transcription factor that interacts with this promoter element is most likely a novel factor. Analysis of its DNA binding site suggested that it might be a member of the zinc finger transcription factor family. The competition EMSA showed that the protein binding to the element also recognized, to a lesser extent, the binding site for MAZ. UV cross-linking studies revealed that the molecular masses of the proteins that interacted with this element did not correspond to that of MAZ, suggesting that a different DNA-binding protein that recognizes a similar binding sequence interacts with this element. Hence, we designated this novel regulatory c-myc element a MAZ-related binding site. It is not clear whether three proteins bind to the MAZ-R site or whether the two smaller proteins represent degradation products.

The MAZ-related protein binding site appears to exert its effect by activation of the P1 promoter rather than the P2 promoter. S1 protection analysis showed that deletion of this site caused a 50% drop in the P1/P2 ratio, mostly because of a large decrease in the level of the P1 transcripts with very little change in the level of the P2 transcripts. Previous studies have suggested that the P1 and P2 promoters are activated through different mechanisms by the IgH enhancers. Increased histone acetylation is one of the possible mechanisms through which the IgH enhancer activates expression from the P2 promoter, but this mechanism does not account for the activated transcription from the P1 promoter (9). In addition to chromatin structure remodeling, the IgH enhancer may facilitate the assembly of the transcriptional machinery on the promoters through direct protein-protein interactions between the enhancer binding proteins and the transcription initiation complex. The IgH enhancer also increases the elongation efficiency by removing the block near the P2 promoter (2, 4). The MAZ-related factor binding site may contribute to the formation of the initiation complex on the P1 promoter or enhance P1 transcription elongation or both. Our results do not provide an explanation for why the MAZ-R site appears to influence transcription from the P1 promoter as opposed to the P2 promoter.

We also showed that the P1 promoter core elements, including the Sp1 site located upstream of the P1 promoter, are critical for the IgH enhancer-mediated activation of the c-myc promoter. S1 analysis showed that the IgH enhancer activated the expression of the c-myc gene when linked to the core promoter, and a P1/P2 ratio of −1.0 was achieved. This result is consistent with the findings with the Igκ enhancers, which demonstrated the requirement of these sites for enhancer-mediated activation of the P1 promoter (15). Our study showed that inclusion of the MAZ-R site in the promoter region further increased the total promoter activity by 50% and also increased the P1/P2 ratio to 1.7. An analysis of our results with those of Geltigner et al. (15) suggests that at least three elements (the MAZ-R site, the Sp1 site, and the TATA sequence) are required for interaction with the Ig enhancers. These studies provide additional insights into the mechanisms involved in the deregulation of expression of the c-myc gene by translocation into one of the Ig loci.

REFERENCES
1. Strobl, L. J., and Eick, D. (1992) EMBO J. 11, 3307–3314
2. Strobl, L. J., Kohlhuber, F., Mautner, J., Polack, A., and Eick, D. (1993) Oncogene 8, 1437–1447
3. Taub, R., Kelly, K., Battey, J., Latt, S., and Lenoir, G. M. (1984) Cell 37, 511–520
4. Madisen, L., and Groudine, M. (1994) Genes Dev. 8, 2212–2226
5. Polack, A., Feederle, R., Klobeck, G., and Hortnagel, K. (1995) EMBO J. 12, 3913–3920
6. Gerbitz, A., Mautner, J., Geltlinger, C., Hortnagel, K., Christoph, B., Asenbauer, H., Klobeck, G., Polack, A., and Bornkamm, G. W. (1999) Oncogene 18, 1745–1753
7. Kanda, K., Hu, H.-M., Zhang, L., Grandemamps, J., and Boxer, L. M. (2000) J. Biol. Chem. 275, 32538–32546
8. Wittekindt, N. E., Hortnagel, K., Geltlinger, C., and Polack, A. (2000) Nucleic Acids Res. 28, 800–808
9. Madisen, L., Krumm, A., Heber, T. R., and Groudine, M. (1998) Mol. Cell. Biol. 18, 6281–6292
10. DesJardins, E., and Hay, N. (1993) Mol. Cell. Biol. 13, 5710–5724
11. Majello, B., De Luca, P., Suske, G., and Lania, L. (1995) Oncogene 10, 1841–1848
12. Bossone, S. A., Asselin, C., Patel, A. J., and Marucu, K. B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7452–7456
13. Pyer, J. J., Moberg, K. H., and Hall, D. J. (1992) Biochem. J. 31, 4102–4110
14. Moberg, K. H., Logan, T. J., Tyndall, W. A., and Hall, D. J. (1992) Oncogene 7, 411–421
15. Geltlinger, C., Hortnagel, K., and Polack, A. (1996) Gene Expression 6, 113–127
16. Ji, L., Arcinas, M., and Boxer, L. M. (1994) Mol. Cell. Biol. 14, 7967–7974
17. Kessler, D. J., Spicer, D. B., La Rosa, F. A., and Sonenshein, G. (1992) Oncogene 7, 2447–2453
18. La Rosa, F. A., Pierce, J. W., and Sonenshein, G. E. (1994) Mol. Cell. Biol. 14, 1039–1044
19. Mueller, P. R., and Wold, B. (1989) Science 246, 780–786
20. Song, J., Murakami, H., Tsutsui, H., Tang, X., Matsumura, M., Itakura, K., Kanazawa, I., Sun, K., and Yokoyama, K. K. (1998) J. Biol. Chem. 273, 20603–20614
21. Ji, L., Arcinas, M., and Boxer, L. M. (1995) J. Biol. Chem. 270, 13392–13398