NF-κB Activity Is Required for the Deregulation of c-myc Expression by the Immunoglobulin Heavy Chain Enhancer*

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The c-myc gene is translocated to one of the immunoglobulin genes in Burkitt’s lymphoma resulting in deregulated expression of c-myc. Several enhancers have been shown to be important for expression of the immunoglobulin heavy chain gene. Four enhancer regions (murine-hypersensitive sites (MHS) 1, 2, 3, and 4) located 3′ of the murine immunoglobulin heavy chain gene play a role in activating expression of the translocated c-myc gene. The enhancer regions also result in a shift in transcriptional initiation from the P2 promoter to P1 that is characteristic of the translocated c-myc allele. We found that the most 3′ enhancer region (MHS4) activated the c-myc promoter by 46-fold in the Raji Burkitt’s lymphoma cell line, and it was the most active enhancer in these cells. The addition of enhancer regions MHS1,2 and 3 to MHS4 increased c-myc transcription by an additional 3-fold and resulted in the full promoter shift from P2 to P1. By deletion analysis of enhancer region MHS4, we located a region that was critical for the transcriptional activity of MHS4. Electrophoretic mobility shift assay analysis revealed that NF-κB/Rel family members bound to this region. Mutation of the NF-κB binding site abolished both the enhancer activity and the promoter shift activity of MHS4. An active NF-κB site was also identified in the human HS4 enhancer. Inhibition of c-myc promoter activity driven by the immunoglobulin enhancers was observed with expression of a super-repressor IκBα construct. These results indicate that the NF-κB/Rel transcription factors play an important role in the deregulation of the translocated c-myc gene in Burkitt’s lymphoma and suggest that interference with NF-κB function may represent a new approach to the treatment of Burkitt’s lymphoma.

A characteristic feature of Burkitt’s lymphoma cells is the presence of reciprocal translocations between the c-myc locus on chromosome 8 and one of the immunoglobulin gene loci on chromosomes 2, 14, or 22. The most common translocation is the t(8;14). In this translocation, the c-myc gene is covalently linked to the immunoglobulin heavy chain (IgH) gene. As a result of this translocation, the transcription of the translocated c-myc gene is deregulated, whereas the normal c-myc 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 transcripts initiated from the P2 promoter (1–3). These findings support a model in which sequences present in the IgH gene locus deregulate expression from the cis-linked c-myc allele by promoting interactions between c-myc and IgH gene regulatory elements that affect c-myc initiation and elongation. It should be noted, however, that the translocation breakpoint in many sporadic Burkitt’s lymphomas separates the c-myc promoter from the coding region (4, 5). In these cases, the regulatory elements of the IgH enhancers apparently activate c-myc transcription without interaction with the c-myc promoter elements. Transcription often initiates in the first intron of c-myc in these sporadic Burkitt’s lymphomas.

We found that the transcription factors, Nm23H2 and NF-κB, activated the c-myc promoter (6, 7). Others have also shown that NF-κB is an important regulatory factor for the murine c-myc promoter (8, 9). Because the IgH 3′ enhancers are linked to the c-myc gene in every Burkitt’s lymphoma with the t(8;14) translocation, we sought to identify the transcription factors that bind to sequences in the enhancer region and activate the translocated c-myc gene.

Several enhancers have been shown to be important for expression of the IgH gene. Four B cell-specific and cell stage-dependent DNase I-hypersensitive sites, MHS1 to MHS4, are located 10–35 kilobases 3′ of the Ca gene (10–13). The activity of individual enhancer elements varies during B cell differentiation (10, 14, 15), and these regions have been shown to function as a locus control region in B cells (10). Recently, it has been shown that MHS1–MHS4 increase expression from the c-myc P2 promoter by an increase in histone acetylation. However, this increase in acetylation does not explain the MHS1–4 activation of transcription from the P1 promoter (16). Enhancers have been located downstream of two human Ca genes (17–19), and these regions share some homology with the murine HS1–4, but only limited functional studies have been performed on the human enhancers.

The 3′ region of the IgH locus is linked to the translocated c-myc gene in all t(8;14) translocations in Burkitt’s lymphoma, and it is likely that this region plays a role in the deregulated expression of the translocated c-myc gene. In this study, we show that an NF-κB site in the MHS4 enhancer is required for the transcriptional activation of the translocated c-myc gene and that it plays a major role in the induction of the P2-mediated transcription of the translocated c-myc allele. Furthermore, we show that NF-κB/Rel proteins are expressed at high levels in the nuclei of Burkitt’s lymphoma cells and that interference with their function decreases c-myc expression.

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1 The abbreviations used are: IgH, immunoglobulin heavy chain gene; MHS, murine-hypersensitive site(s); EMSA, electrophoretic mobility shift; HH5, human-hypersensitive site; bp, base pair(s).
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Plasmid Constructs—Mouse genomic DNA fragments containing MHS1–4 (see Fig. 1A) were subcloned from murine genomic DNA by the polymerase chain reaction amplification method. The sequences of the primers used were: MHS12 forward, CCTCAGTCATATGAGAGAAGATGAGACAGA; MHS12 reverse, GGCTGCGTCAGACAAAAACCTTGAGTTGACAACTTGACAACTTGACAGC; MHS3 forward, CTGGGTGCGACACTAAAG; MHS3 reverse, GGCTGCGTGCAAGACAG; CATTGGCTCCGGTCTAAACAC; MHS4 forward, CTTCGGCGGGCATGGTCCAG; and MHS4 reverse, GGTCTCACCGGTTGGATTGTTGGTGAAG. The amplified fragments were ligated into pBluescript KS− with the firefly luciferase reporter gene and the human c-myc promoter (−2238 to +936). These constructs for transient transfections are shown schematically in Fig. 1B.

The c-myc promoter and MHS1234 episomal transfections were generated by inserting a 4.2-kilobase DNA fragment that contains the EBV by inserting a 4.2-kilobase DNA fragment that contains the EBV expression vector (21) consisting of 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, and incubated at 70 °C for 30 min. The blots were washed twice in Tween for 10 min at room temperature before blocking and incubation with antibody. For the supershift experiments, the nuclear extract was incubated with a 1–2 μg of antibody at 4 °C for 1 h prior to the addition of the labeled probe. The monoclonal antibodies against p50, p65, c-Rel, and CREB-2 were obtained from Santa Cruz Biotechnology.

UV Cross-linking and SDS-Polyacrylamide Gel Electrophoresis—EMSA was performed as described above. The wet gel was exposed to film to locate the EMSA complexes. UV cross-linking was performed as described (6) except that the cross-linking was done at 4 °C for 1 h. Blot Analysis—After the S1 protection assay was generated and labeled by unidirectional polymerase chain reaction. The T7 primer was annealed to a linearized human c-myc plasmid and extended in the presence of [α-32P]dCTP in a 20-cycle polymerase chain reaction reaction. The resulting single-stranded c-myc probe was purified on a 6% acrylamide-urea gel. The 730-bp c-myc probe covers DNA sequences from the Smal site in the c-myc promoter to the PvuII site near the end of exon I. The S1 protection assay was performed with the Ambion S1 assay kit following the instructions of the manufacturer. Briefly, 40 μg of total RNA was hybridized with 5 × 106 cpm of labeled probe for 1 h, followed by digestion with 250 units of S1 nuclease at 37 °C for 30 min. The protected S1 products were resolved on a 6% acrylamide-urea gel. Quantitation of the S1 signals was performed with a PhosphorImage system. The studies were repeated six times, and the average values with the standard deviations were calculated. The loading control for the S1 assay was performed separately by Northern analysis of 20 μg of total RNA hybridized with a human GAPDH probe.

RESULTS

MHS4 Is the Strongest Enhancer in Raji Cells—A map of the murine IgH gene is shown in Fig. 1A. We focused on the murine IgH 3’ enhancers initially because these had been cloned before the human IgH enhancers, and some data were available on the active sites in the murine enhancers. To determine which MHS regions were the most active, we cloned these regions into the promoter of the human c-myc promoter (+2238 to +936). These constructs are shown schematically in Fig. 1B.

MHS4 showed the greatest enhancer activity (46-fold) in Raji cells in transient transfections (Fig. 1C). Both MHS1,2 and MHS3 displayed relatively weak activity; however, they were able to interact with MHS4 to increase its enhancing activity synergistically. MHS1,2 increased c-myc promoter activity by 3.5-fold, and MHS3 also increased the promoter activity by 3.5-fold. The combination of MHS1,2,3 resulted in an increase in promoter activity of 46-fold, the combination of MHS1,2,4 led to an increase of 71-fold in promoter activity, and MHS4,5 showed an 88-fold increase in c-myc promoter activity. The

MHS4 enhanc...
The greatest activity was seen with a combination of all four MHS regions (124-fold increase). All of these MHS regions were active in either orientation relative to the c-myc promoter. The MHS regions were also active in the mature B cell line DHL-9, but the activity of these elements was less than in Raji cells.

**Identification of the Active Region in MHS4**—Serial deletions were constructed in the MHS4 region to determine which sequences were responsible for the enhancer activity. These constructs contain full-length MHS1–3, as shown in Fig. 2A. Transfection of these plasmids into Raji cells revealed one major region of activity (Fig. 2B). A decrease in enhancer activity of approximately 3-fold was observed between constructs pMHS\(_D\)_7 and pMHS\(_D\)_8. The deleted region corresponds to nucleotides 650–674 in MHS4.

**p50 and p65 Bind to the MHS4 NF-κB Site**—A potential NF-κB binding site was located in the region between nucleotides 650 and 674. Double-stranded oligonucleotides of this region were synthesized and used in EMSA to determine whether Rel family proteins bound to this sequence. Four specific protein-DNA complexes (A, B, C, and D) were observed with Raji cell nuclear extract and the oligonucleotides (Fig. 3A). Complexes B and C have similar mobilities and are not always clearly separable. The four complexes were competed by the addition of a 100-fold molar excess of cold oligonucleotide but not by a 100-fold molar excess of mutated probe. EMSA with the labeled mutated probe revealed no specific EMSA complexes. The four EMSA complexes were also competed by a 100-fold molar excess of the HIV-κB site. An unrelated sequence, the CRE consensus binding site, had no effect on the complexes.

Supershift studies were performed with antibodies against three members of the Rel family to try to identify the proteins in the EMSA complexes (Fig. 3A). An antibody against p50 diminished the intensity of all four complexes and resulted in a supershifted complex. Addition of the anti-p65 antibody resulted in a consistent decrease in intensity of complex B, but no supershifted complex was observed. The anti-c-Rel antibody had no effect, and an antibody against CREB also had no effect on any of the EMSA complexes.

To further study the proteins interacting with the NF-κB site in MHS4, UV cross-linking and SDS-polyacrylamide gel electrophoresis of the EMSA complexes in Raji cells were performed. EMSA complexes A and D were too weak in intensity to allow identification of proteins involved in binding to the oligonucleotides, so EMSA complexes B and C were examined together. As shown in Fig. 3B, two proteins were observed on SDS gel analysis. The larger one (I) had a molecular mass of 77 kDa. After correction for the bound oligonucleotide, the predicted molecular mass of this protein was 65 kDa. A more cell stage-specific DNase I-hypersensitive sites. Two sites, MHS1 and MHS2, are located within the 3' Ca enhancer. These are separated from MHS3 and MHS4 by 13 and 17 kilobases, respectively. The drawing is not to scale. Restriction sites located near the sites used for cloning are indicated. H1, HindIII; P, PstI; X, XbaI; S, SacI; H3, HindIII. B, map of the c-myc promoter luciferase MHS constructs. The human c-myc promoter (−2328 to +936) was ligated to MHS1–4 in a head-to-head orientation. P1 and P2 are the two major promoters of the c-myc gene. The size of MHS1.2 is 1564 bp, MHS3 is 1182 bp, and MHS4 is 1380 bp. C, activity of MHS1–4 with the c-myc promoter in Raji cells. Transient transfections were performed as described under “Experimental Procedures” with 10 μg of each reporter gene and 100 ng of pRL-TK. Each column represents the mean of 6–10 different transfections. The error bars show the standard error. The luciferase activity is relative to the activity of the c-myc promoter without any enhancers. This was assigned a value of 1.

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2 K. Kanda and L. M. Boxer, unpublished data.
intense band at 62 kDa (2) was also observed. The predicted molecular mass of this protein was 50 kDa after correction for the bound oligonucleotide. The results from EMSA competition and supershift studies and the UV cross-linking results support the conclusion that the Rel family proteins, p50 and p65, recognize and bind to the NF-κB site in MHS4.

Mature B cells constitutively express nuclear NF-κB/Rel proteins. We wished to determine whether NF-κB/Rel family proteins were expressed in the nuclei of Burkitt’s lymphoma cells at levels comparable with those found in mature B cells. As shown in Fig. 3C, substantial amounts of p50, p65, and c-Rel were present in the nuclei of Burkitt’s cell lines and patient samples. In addition, strong complexes were observed with these nuclear extracts and the MHS4 NF-κB site in EMSA.²

The NF-κB Site in MHS4 Demonstrates Functional Activity—Mutation of three base pairs in the NF-κB site in MHS4 prevented protein binding in EMSA. To study the functional activity of this NF-κB site, this mutation was engineered into the c-myc promoter MHS1–4 construct, and transient transfections were performed. The results are shown in Fig. 2B, column pMHS-κBm. The activity of the c-myc promoter was decreased by approximately 3-fold. These results agree with the results from deletion of this region.

An octamer site is active with the immunoglobulin λ light chain and the 5′ VH promoters (23, 24). We showed by EMSA that octamer proteins in Raji nuclear extracts bound to this site.² A mutation that prevented protein binding was engineered into the c-myc promoter MHS1–4 construct. As shown

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**Fig. 2. Deletion and mutation analysis of the MHS4 region.** A, map of the MHS4 deletion constructs and the construct with the mutated NF-κB and octamer binding sites. All of these constructs contain full-length MHS1–3 and the c-myc promoter. pMHS-κBm is the same as pMHS1234 except that the NF-κB site has been mutated as indicated, and pMHS-Octm has a mutated octamer site. B, transient transfection of the MHS4 deletion constructs into Raji cells. Each column represents the mean of 8–13 different transfections.
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Fig. 3. EMSA and UV cross-link analysis of the NF-κB site in MHS4. A, EMSA with an oligonucleotide containing the NF-κB sequence from MHS4 and Raji nuclear extract. Lane 1 contains no competitor oligonucleotide, and lanes 2, 3, and 4 contain a 100-fold molar excess of cold wild type (Wt), mutant (Mt), and consensus (HIV-B) NF-κB oligonucleotides, respectively. Lane 5 contains a 100-fold molar excess of a consensus CRE binding site oligonucleotide (irrelevant competitor). Lanes 6, 7, 8, and 9 contain 1 μg of antibodies against p50, p65, c-Rel, and CREB, respectively. The four specific complexes are labeled A, B, C, and D. The arrow on the right marks the supershifted complex. B, denaturing SDS-polyacrylamide gel of the EMSA complexes formed with Raji nuclear extract and the MHS4 NF-κB site. EMSA complexes B and C were analyzed. Two proteins, labeled 1 and 2, were observed. The migration of the molecular mass markers is shown on the right. After correction for bound oligonucleotide, the molecular mass of protein 1 was approximately 65 kDa and that of protein 2 was 50 kDa. C, Western analysis of p50, p65, and c-Rel in nuclear extracts from mature B cell lines (DHL-4 and DHL-9), endemic and sporadic Burkitt’s lymphoma lines (Raji, Ramos, Daudi, DG75, and Jijoye), and two sporadic Burkitt’s samples (Sample 1 and Sample 2). Equal amounts of nuclear protein were used for each sample.

In Fig. 2B, column pMHS-Octm, there was no loss of transcriptional activity.

Because MHS4 has very strong enhancer activity in the absence of MHS1–3, we wished to determine whether the NF-κB site was active in this setting also. The mutation of the NF-κB site was engineered into pMHS4, the construct with the c-myc promoter and only MHS4. As shown in Fig. 4, mutation of this site decreased the activity by more than 20-fold (pMHS4-κBm). The activity of pMHS4-κBm was essentially the same as the construct that lacks all of the enhancers (pHS0). Taken together, these results demonstrate that the NF-κB site is required for the enhancer activity of MHS4 with the c-myc promoter.

The NF-κB Site Is Responsible for the Promoter Shift Activity of MHS4—Because the MHS1–4 elements have been shown to confer a promoter shift from P2 to P1 on a cis-linked c-myc gene (10), we wished to determine what role the MHS4 NF-κB site played in the promoter shift. S1 analyses were performed with cell lines stably transfected with the episomal vectors shown in Fig. 5A. All four MHS regions were required for the full promoter shift (P1/P2 of 1.2 ± 0.05). In agreement with the results of a previous study (10), we found that none of the MHS regions alone resulted in a significant promoter shift. We wished to examine the role of the NF-κB site in the promoter shift. Mutation of the NF-κB site in the construct with all four MHS regions resulted in a decrease in P1/P2 to 0.75 ± 0.05 (Fig. 5B, pMHS-κBmEP). The promoter shift of the construct with MHS1,2,3 was 0.75 ± 0.08 (Fig. 5B, pMHS123EP). Although the level of transcription is low in the absence of the intact MHS4 region, these studies have been repeated six times, and the values are quite reproducible. We conclude that the entire promoter shift activity of MHS4 is dependent on an intact NF-κB site. After correction for copy number of the episomal plasmid, the transcriptional activity of each construct was determined (Fig. 5C). The transcriptional activity of the construct with the mutated NF-κB site was reduced to the level of activity of the construct containing only MHS1,2,3 (Fig. 5C). These results are in good agreement with the results from the transient transfection experiments, and they support our conclusion that the MHS4 NF-κB site is required for the transcriptional enhancing activity of MHS4 as well as the promoter shift activity.

The Human HS4 Region Contains an Active NF-κB Site—Recently, the human IgH gene enhancers have been identified (17–19). In contrast to the murine locus, there are two copies of
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Fig. 6. EMSA analysis of the NF-κB sites in the human and murine HS4 enhancer with Raji nuclear extract. Lane 1 contains no competitor oligonucleotide. Lanes 2–6 contain a 50-fold molar excess of cold wild type (Wt), mutant (Mt), murine HS4 NF-κB (MHS4 Wt), murine HS4 NF-κB mutant (MHS4 Mt), and consensus (HIV kB) NF-κB oligonucleotides, respectively. Lane 7 contains a 100-fold molar excess of a Myb site oligonucleotide (irrelevant competitor). Lanes 8, 9, 10, and 11 contain 1 μg of antibodies against p50, p65, c-Rel, and CREB, respectively. The five specific complexes are labeled A, A’, B, C, and D. The arrow on the right marks the supershifted complex.

HS1–4 in the human IgH locus. An NF-κB site was located in the human HS4 (HHS4) sequence. The NF-κB site in HHS4 differed from the MHS4 NF-κB site by one nucleotide: GGAAGCCCCA (MHS4) and GGAACCCCCA (HHS4). Oligonucleotides of the HHS4 NF-κB sequence were synthesized and used in EMSA. As shown in Fig. 6, specific complexes were formed with this sequence with Raji nuclear extracts. Both the MHS4 NF-κB site and the HIV kB site competed with the HHS4 NF-κB site (Fig. 6, lanes 4 and 6). An antibody against p50 produced a supershifted complex, and an antibody against p65 decreased the intensity of complex B (Fig. 6, lanes 8 and 9). Therefore, it appeared that Rel family members recognized the HHS4 NF-κB site. Further studies demonstrated that a 2–3-fold lower concentration of the MHS4 NF-κB site compared with the HHS4 NF-κB site competed for the complexes that formed with the HHS4 NF-κB oligonucleotide (Fig. 6, lanes 2 and 4).

The human HS4 region was cloned and ligated into the c-myc promoter-luciferase vector (Fig. 7A). The presence of the HHS4 region increased c-myc promoter activity by 14-fold, and the HHS4 region showed orientation independent activity (Fig. 7B, pHHS4F and pMHS4R). Mutation of the NF-κB site decreased the promoter activity to a level similar to that seen with mutation of the NF-κB site in the murine HS4 (Fig. 7B, pHHS4F-Mt). These results suggest that the NF-κB site is required for maximal activity of the human HS4, and these results are similar to those obtained with the murine HS4.

The human HS4 region demonstrated less activity than that of the murine HS4 enhancer. To determine whether the lower enhancer activity was due to the difference in the sequence of the NF-κB site, we changed the sequence of the murine HS4 NF-κB site to match the sequence of the human HS4 NF-κB site. Although this mutated MHS4 construct increased c-myc promoter activity, it was less active than the wild type MHS4 sequence (Fig. 7B, pHHS4-Mt). The activity of the mutated MHS4 construct was similar to that of the human HS4 construct. These results suggest that the lower activity of the human HS4 enhancer is due to the difference in sequence of the NF-κB site and that other sequences in the enhancer cannot compensate for this difference. These results are consistent with the finding that Rel proteins bind with a slightly higher

Fig. 5. Role of the MHS4 NF-κB site in induction of the c-myc promoter shift. A, map of the c-myc promoter luciferase MHS constructs in the episomal vector. B, S1 analysis of the c-myc promoter MHS constructs in stably transfected Raji cells. Stable cell lines were prepared as described under “Experimental Procedures.” Results with two independently transfected cell lines for each construct are shown. P1 and P2 mark the c-myc transcripts initiated from the transfected construct. P1d and P2d mark the transcripts initiated from the trans-located c-myc allele in the Raji cells, which has a deletion in exon 1. C, relative activity of each of the c-myc promoter MHS constructs in stably transfected Raji cells. After quantitation of the S1 signals, the relative activity of each construct (the sum of P1 and P2 initiated transcripts) was determined per copy number of the episomal plasmid. Each column represents the mean of four experiments.
The affinity to the murine HS4 NF-κB site compared with the human NF-κB site.

Because there are two copies of the HS4 enhancer in the human IgH locus, we tested the effect of two copies of HS4 with the c-myc promoter. As shown in Fig. 7B, the activity of the c-myc promoter increased by 77-fold (pHHS4F2). The activity of two copies of HS4 was greater than the activity of a single copy of HS4.

Interference with NF-κB Activity—To further assess the importance of NF-κB proteins in the expression of c-myc, a dominant negative (super-repressor) form of IkBα was used in transient transfection studies. Co-transfection of the dominant negative IkBα expression vector with the c-myc promoter MHS4 construct at a ratio of 0.5–1 resulted in a 4-fold reduction in activity (Fig. 8, column 2). The activity was reduced by 20-fold at a ratio of 4:1 (Fig. 8, column 4), and this is similar to the activity of the MHS4 construct with a mutated NF-κB site. Repression of the activity of the c-myc promoter MHS1–4 construct was also observed in co-transfection experiments (Fig. 8, columns 9–12). A reduction in activity of 3-fold was observed with a ratio of 4:1. These results are similar to the results we obtained with constructs with mutated NF-κB sites. When the NF-κB site was mutated in MHS4, there was very little effect with co-transfection of the IkBα super-repressor construct (Fig. 8, columns 5–8 with MHS4 alone and columns 13–16 with MHS1234). Although two NF-κB sites are active in the human (6) and murine (8, 9) c-myc promoters, in the presence of the IgH enhancers (MHS1234), mutation of the promoter NF-κB sites has very little effect on the transcriptional activity (Fig. 8, column 17). Co-transfection of the super-repressor IkBα construct with the c-myc promoter with mutations in both NF-κB sites linked to wild type MHS1234 resulted in a dose-dependent decrease in activity that was very similar to that seen with the wild type c-myc promoter linked to MHS1234 (Fig. 8, compare columns 17–20 with columns 9–12).

**DISCUSSION**

We have shown that the IgH gene MHS4 enhancer is the most active enhancer in Raji Burkitt's cells. MHS4 is a relatively weak enhancer in the mature B cell line, DHL-9. Other investigators have observed that MHS4 is active in pre-B cells as well as in murine plasmacytoma cell lines (10).

Our studies demonstrate that the NF-κB site in MHS4 is required for the enhancer activity of this region when it is linked to the c-myc promoter. It also induces a shift in transcription initiation from P2 to P1, and the NF-κB site is required for this activity of the MHS4 region. We showed that Rel family members were expressed in the nuclei of Burkitt's cells and that p50 and p65 bound to the NF-κB site in MHS4 by EMSA and EMSA supershift studies as well as by UV cross-linking SDS-polyacrylamide gel electrophoresis. Both the transient transfection studies and the studies with the stably transfected cell lines revealed that enhancer activity was lost when the NF-κB site in MHS4 was mutated. The promoter shift caused by MHS4 was also lost when the NF-κB site was mutated, although it is clear that regions of MHS1,2, and 3 also contribute to the full promoter shift.

It is of interest to compare our results with MHS4 and the
c-myc promoter with those of other investigators with MHS4 and the λ light chain gene promoter. The NF-κB site in MHS4 is an important positive regulatory site for the λ gene promoter at all stages of B cell development (11, 15, 23). However, two other transcription factor binding sites have been shown to be required for maximal enhancer activity with the λ gene promoter. In B cells, an octamer site and a site for BSAP (B cell-specific activator protein) function as positive regulatory elements, whereas the BSAP site is inactive in plasma cells. In pre-B cells, the octamer site is active, but the BSAP site is a negative regulatory element. We find no activity in either of these regions when MHS4 is linked to the c-myc promoter. Our preliminary results with the bel-2 promoter and MHS4 suggest that the NF-κB site is active but that an additional site is required for maximal activity. These results suggest that promoter-specific interactions between transcription factor complexes formed on the promoter and transcription factors bound to MHS4 occur and influence the ability of the enhancer to increase transcriptional activity. In support of this, our preliminary data suggest that maximal activity of the c-myc promoter with the IgH enhancers requires the presence of the full-length c-myc promoter. Deletions into the full-length c-myc promoter decrease transcriptional activity somewhat even in the presence of the IgH enhancers, suggesting that interactions are occurring between the IgH enhancers and transcription factors bound to the c-myc promoter region. It is clear, however, that the IgH enhancers are the critical components in the deregulation of c-myc expression and that elements in the c-myc promoter are of less importance or not required at all as observed in some cases of sporadic Burkitt’s lymphoma.

Further evidence to support the important role of p50 in the regulation of IgH gene expression comes from studies of mice with a targeted disruption of p50 (25, 26). These mice demonstrated defects in germ-line IgH gene transcription and class switching to constant region genes. Decreased serum levels of several IgH isotypes were seen in these mice. Further studies showed that in vitro activated B cells from these mice had reduced levels of germ-line transcripts from γ3 and ε constant region genes. NF-κB sites have been identified in the germ-line promoters of several constant region genes and also in the murine MHS1,2 enhancer (23). Mice with a homozygous knockout of the 3′αE (MHS1,2) enhancer also showed a marked reduction in γ3 and ε constant region germ-line transcripts in activated B cells (27). There were differences in the responses of B cells to LPS between the p50−/− mice and the 3′αE−/− mice, however. B cells from the p50−/− mice failed to proliferate and secrete IgM in response to LPS, whereas the response of B cells from the 3′αE−/− mice was normal. This difference may be due to the fact that p50 regulates IgH gene expression through the NF-κB binding site in MHS4 as well as through the NF-κB site in MHS1,2.

Our studies with the c-myc promoter and the IgH enhancer regions were initiated with the murine IgH enhancers because these enhancers are the best characterized of any species. More recently, the human 3′ IgH enhancer region was identified (17–19). We demonstrated that the NF-κB site in the human HS4 region was active and that Rel family members bound to this site. Because of a difference of a single nucleotide, the activity of the human HS4 NF-κB site was less than that of the murine HS4 NF-κB site. The significance of this is not clear. In the c-myc translocation, other enhancer elements from the human IgH region are also present, and their presence may compensate for the difference in activity of HS4. In addition, the entire human HS1–4 region is present in two copies, one downstream of the Co1 gene and the other downstream of the Co2 gene (18). We showed that two copies of the human HS4 enhancer increased c-myc promoter activity more than a single copy of the murine HS4 region. As far as we are aware, our studies with the c-myc promoter are the first functional studies with the human HS4 region, and its activity with the immunoglobulin promoter has not been investigated.

Further demonstration of the importance of NF-κB factors in the deregulation of the c-myc promoter by the IgH enhancers is provided by our studies with the super-repressor IκBa expression construct. The super-repressor form of IκBa contains serine to alanine substitutions at amino acids 32 and 36, which inhibit signal-induced phosphorylation and subsequent proteasome-mediated degradation of IκBa (21, 28–31). This mutant IκBa protein acts as a super-repressor because it binds to NF-κB and inhibits DNA binding as well as nuclear translocation. Our results show that inhibition of NF-κB activity decreased c-myc promoter activity by 3-fold in the presence of MHS1–4. A greater decrease in promoter activity (20-fold) was observed with MHS4 alone. It is likely that other sites present in MHS1–3 contribute to the activation of c-myc promoter activity in addition to the NF-κB site in MHS4. When the NF-κB site in MHS4 was mutated, there was essentially no effect of coexpression of IκB. In contrast, when the two NF-κB sites in the c-myc promoter were mutated in the presence of the wild type MHS enhancers, the effect of IκB was very similar to that seen with the wild type c-myc promoter linked to the enhancers.

We showed that NF-κB/Rel proteins are present in the nuclei of Burkitt’s lymphoma cells at levels comparable with those observed in mature B cells. Our studies demonstrate that the NF-κB site in HS4 is a major positive regulator of a linked c-myc gene. Interference with NF-κB function by co-transfection of the super-repressor IκBa leads to a substantial decrease in c-myc transcription. Several studies have demonstrated that a decrease in c-myc levels results in decreased proliferation and colony formation of Burkitt’s lymphoma cells. Most of these studies have been performed with antisense oligonucleotides targeted to c-myc, for example (32–34). Studies on animal models of lymphomas with deregulated c-myc expression revealed that c-myc antisense oligonucleotides could prevent tumor formation (35) or decrease tumor growth (36). Because it is clear that deregulated c-myc expression is important for the pathogenesis and continued growth of Burkitt’s lymphoma cells, our results suggest that therapeutic modalities that interfere with NF-κB function may be useful and novel approaches to the treatment of Burkitt’s lymphoma.

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