Regulatory Elements in the Immunoglobulin Heavy Chain Gene 3’-Enhancers Induce c-myc Deregulation and Lymphomagenesis in Murine B Cells

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Burkitt’s lymphoma is invariably associated with chromosomal translocations that juxtapose the c-myc proto-oncogene with regulatory elements of the immunoglobulin heavy (IgH) or light chain loci resulting in the deregulation of c-myc expression. However, the enhancer elements mediating c-myc deregulation in vivo remain largely unidentified. To investigate the role of the IgH 3’-enhancers in c-myc deregulation, we used gene targeting to generate knock-in mice in which four DNase I hypersensitive regions from the murine IgH 3’-region were integrated into the 5’-region of the c-myc locus. The IgH 3’-enhancers induced the up-regulation of c-myc expression specifically in B cells of IgH-3’-E-myc mice. After ~10 months, the mice developed a Burkitt-like B cell lymphoma with the phenotype of B220+, IgM+, and IgDlow. Analysis of immunoglobulin gene rearrangements indicated that the lymphoma cells were of clonal origin. The presence of a rapidly expanding population of B cells in the spleen and bone marrow of young knock-in mice at 2–4 months of age was observed. Premalignant splenic B cells of knock-in mice showed higher spontaneous and induced apoptosis; however, malignant B cells were more resistant to apoptosis. The p53-ARF-Mdm2 pathway was disabled in half of the lymphomas examined, in most cases through Mdm2 overexpression. Although c-myc expression was increased in premalignant B cells, the promoter shift from P2 to P1 was observed only in malignant B cells. Our studies demonstrate that the IgH 3’-enhancers play an important role in c-myc deregulation and B cell lymphomagenesis in vivo.

Burkitt’s lymphoma and many mouse plasmacytomas are associated with chromosomal translocations that juxtapose the c-myc proto-oncogene to regulatory elements of the immunoglobulin heavy chain locus (IgH)1 or one of the light chain loci and subsequently deregulate c-myc (1–3). In Burkitt’s lymphoma, the translocated c-myc gene is activated, whereas the normal allele is silent. Myc is a key regulator of cell proliferation, differentiation, and apoptosis (4–6), and its expression is tightly regulated at both the transcriptional and post-transcriptional levels in normal cells. Myc enhances cell proliferation by promoting cell cycle progression, inhibiting differentiation, and enhancing cellular metabolism. In addition, c-Myc overexpression triggers the apoptotic program (7, 8) and sensitizes cells to a range of apoptotic stimuli such as withdrawal of survival factors, death receptor signals, and DNA damage (9). It is thought that apoptosis acts as an intrinsic limit to the oncopgenic potential of c-Myc (10). Inhibition of apoptosis through additional mutations favors continued growth and malignant transformation of the affected cells.

Although it is believed that deregulated c-myc plays a critical role in the pathogenesis of Burkitt’s lymphoma, the identity of the regulatory elements of the IgH locus that result in c-myc activation remains unclear. Several enhancers have been identified in the murine and human IgH loci. The IgH intronic enhancer (Eμ) was the first enhancer discovered in the IgH locus and has been shown to be involved in VDJ rearrangement and gene expression in early B-lineage cells (11, 12). The Eμ enhancer is not linked to the translocated c-myc allele in most sporadic Burkitt’s lymphomas. The IgH 3’-enhancers are located ~16 kb downstream of the Ca gene in the mouse and 25 kb downstream of the human Ca gene. The 3’-enhancers consist of four DNase I-hypersensitive sites (HS1234), which have been shown to function as a locus control region in B cells (13), and they activate c-myc expression in Burkitt’s lymphoma cell lines (13, 14). The IgH 3’-enhancers also mediate a shift in promoter usage of c-myc from P2 to P1, one of the mechanisms of c-myc deregulation in Burkitt’s lymphoma. The IgH 3’-enhancers are invariably linked to the translocated c-myc gene in all Burkitt’s lymphomas with the IgH translocation and are therefore the best candidate elements to cause deregulation of c-myc expression in Burkitt’s lymphoma.

Several mouse models that link the c-myc oncogene to sequences from the immunoglobulin genes have been developed. However, most of these do not provide completely accurate models for Burkitt’s lymphoma. Transgenic mice bearing c-myc driven by the IgH Eμ enhancer produced primarily precursor-B cell malignancies (15). Mice carrying a yeast artificial chromosome with c-myc linked to a portion of IgH sequence also developed B cell tumors (16), but similar malignancies were observed in the yeast artificial chromosome-based mice with or without the Eμ enhancer (17). A transgenic mouse with a construct based on the IgA translocation produced a more appropriate model for Burkitt’s lymphoma (18).

To study the mechanism of c-myc deregulation in Burkitt’s lymphoma and to test the hypothesis that the IgH 3’-enhancers play a major role in c-myc deregulation in vivo, we established a mouse model (IgH-3’-E-myc) where the IgH 3’-enhancers were
IgH Enhancers Deregulate c-myc

EXPERIMENTAL PROCEDURES

Targeting Vector—A genomic c-myc BAC clone was isolated from a 129/J library (Incyte Genomics). A 4.0-kb fragment containing exons 2 and 3 was subcloned by digestion of the c-myc BAC clone. Two fragments of 2.6 kb (5′-flanking region) and 1.5 kb (exon 1) were amplified by PCR using primers: 5′-AGAAGTGGTACCTTTATCTCC-3′, 5′-AGCGG-TACCCTCTCTCAGGAGGAAACCAAG-3′, 5′-AGTGAGTGGATTAGACG-GAATTCCCGAGG-3′, and 5′-TGCTCTACTCCAGGACTGAT-3′.

To generate the targeting vector, the 1.5-kb PCR fragment was digested with HindIII and XhoI and inserted into the pNTloxP vector 3′ of the neomycin cassette. Then the 4.0-kb fragment was cloned adjacent to the 1.5-kb PCR fragment at the XhoI site. To make the 5′-arm, the 2.6-kb PCR fragment was cloned at the KpnI site 5′ of the neomycin cassette. A 4.2-kb fragment containing the four DNAse I hypersensitive sites of the murine IgH 3′-enhancers (IgH-3′E) was inserted between the 5′-arm and the 1.5-kb PCR fragment.

Generation of IgH-3′E-myc Knock-in Mice—R1 ES (129/J) cells were electroporated with the linearized targeting vector, selected with G418 and ganciclovir, and screened by PCR using a neomycin primer and a primer with the sequence 5′-gccggtccgctccagcagcatgatcggctcattcctggagaccggctc-3′.

To determine the targeting vector, the 1.5-kb PCR fragment was digested with HpaI and XhoI and inserted into the pNTloxP vector 3′ of the neomycin cassette. Then the 4.0-kb fragment was cloned adjacent to the 1.5-kb PCR fragment at the XhoI site. To make the 5′-arm, the 2.6-kb PCR fragment was cloned at the KpnI site 5′ of the neomycin cassette. A 4.2-kb fragment containing the four DNAse I hypersensitive sites of the murine IgH 3′-enhancers (IgH-3′E) was inserted between the 5′-arm and the 1.5-kb PCR fragment.

Clonality Assay—Genomic DNA was digested with EcoRI and examined by Southern blot analysis using a 2F2-labeled JH probe, representing the Jα4 region of the IgH (20). A DNA fragment for the probe was amplified by PCR using primers 5′-TGU-TGGTACCTTTATCTCC-3′ and 5′-CAAGATGCTCTGGACTACAGG-3′. The probe was prepared using the megaprime DNA-labeling system (Amersham Biosciences). Premalignant polyclonal B cells were obtained from mice less than 3 months of age. These cells were indistinguishable from wild-type B cells except that c-myc expression was increased, and they showed increased rates of proliferation and apoptosis.

Histology—Murine tissues were fixed in 10% neutral buffered formalin and paraffin-embedded. Four-micrometer sections were stained with hematoxilin and eosin.

Transfection of sRNA—Lymphoma cells with increased expression of Bcl-xL were grown in culture and transfected with siRNA against Bcl-xL (PharMaco) using nucleofector buffer R and program O-17 (Amaxa Biosystems). After 12 h in culture non-viable cells were removed with the Dead Cell Removal Kit (Miltenyi Biotec). The remaining cells were divided into two aliquots. One was incubated in cell culture for 24 h, the other was harvested and washed. The PCR was performed to determine the level of Bcl-xL mRNA. The other aliquot of cells was incubated with etoposide for 24 h, and the number of apoptotic cells was determined.

Promoter Usage Analysis—Transcripts from the c-myc P1 promoter and total transcripts (from both P1 and P2 promoters) were measured by real-time RT-PCR. Total RNA was isolated from purified B cells with the RNeasy mini kit (Qiagen), and cDNA was prepared using the RETROscript kit. The primers and probe for the P1 promoter were: 5′-GGGAGCTGTTAGGCGATAT-3′, 5′-TCCTGGCAACAGGGCTTTCC-3′, and 5′-6-FAM-AACCGTCGACCTCTTCTGCTTCT-3′. The mm-myc-primer-probe set was used to detect the total level of c-myc. Normalization was performed by establishing standard curves with a plasmid containing the entire murine c-myc gene. The quantity of P1 transcripts and total transcripts was then normalized to GAPDH.

RESULTS

Generation of IgH-3′E-myc Knock-in Mice—The murine IgH 3′-enhancers HS1234 were integrated 5′ of the c-myc locus by homologous recombination in murine embryonic stem (ES) cells using a targeting construct with two homologous arms, the 5′-flanking region of c-myc and the entire c-myc coding region (Fig. 1A). A neomycin cassette located between the arms was used for drug selection of ES cells. The ES cell clones were screened by PCR and Southern blot analysis of genomic DNA digested with EcoRI using a probe outside of the region of homology (Fig. 1B). Two independent ES cell clones were injected to produce chimeric mice (Fig. 1B). The neomycin cassette flanked by loxP sites in the neomycin-IgH-3′E-myc knock-in mice was removed by breeding with transgenic mice expressing the Cre recombinase under the control of the β-actin promoter (Fig. 1, C and D). The mice with neomycin deleted are designated as IgH-3′E-myc mice.

Expression of c-myc in IgH-3′E-myc Mice—Real time RT-PCR was used to analyze the expression level of c-myc driven by the IgH 3′-enhancers in different tissues of the IgH-3′E-myc mice. As shown in Fig. 1E, except for spleen, which had elevated c-myc expression in the IgH-3′E-myc mice, the level of c-myc in brain, liver, kidney, lung, and thymus from the IgH-3′E-myc mice was not different from that of wild-type mice. To further investigate the tissue-specific expression of c-myc, splenic B lymphocytes and non-B lymphocytes (mainly T cells) from IgH-3′E-myc and wild-type mice were purified and subjected to real time RT-PCR analysis (Fig. 1F). We found that elevated c-myc expression was detected only in the B cell fraction and not in non-B lymphocytes. Increased c-myc expression was detected in the spleens of both heterozygous and homozygous IgH-3′E-myc mice compared with the level of expression from...
spleens of wild-type littermates (Fig. 1G). Protein immunoblotting also demonstrated that splenic lymphocytes from knock-in mice showed higher levels of c-Myc protein (Fig. 1H). Taken together, the IgH 3’-enhancers HS1234 appear to be B cellspecific and to increase c-Myc expression only in B cells of the IgH-3’E-myc knock-in mice.

Abnormal B Cell Development in Young IgH-3’E-myc Mice—We examined the development of B and T cells in young (6 weeks–3 months) IgH-3’E-myc mice. T cell development in the thymus appeared to be normal as judged by CD4 and CD8 staining. The percentage of T cells was decreased in the spleens of IgH-3’E-myc mice, but the ratio of CD4 and CD8 cells was similar to that of wild-type mice. An analysis of the spleen revealed that the percentage of B cells (CD19+ and B220+) was increased in both heterozygous and homozygous IgH-3’E-myc mice as compared with age-matched wild-type controls (Fig. 2). There was a significant increase in a B220+ IgM+ IgDlow B cell population from the spleens of IgH-3’E-myc mice. Homozygous mice showed a more pronounced increase (7–10-fold) of this population. An analysis of bone marrow revealed that the IgH-3’E-myc mice had a slight increase in the B220+ IgM+ population (Fig. 2), and the percentage of the CD43+ B220+ pro-B population was not significantly different between the wild-type and IgH-3’E-myc mice.2

2 J. Wang and L. M. Boxer, unpublished data.
Increased Proliferation of B Cells from IgH-3 E-myc Mice in Response to Activation Signals—It is known that c-Myc plays an important role in cell proliferation and cell cycle progression. We therefore evaluated B cell proliferation from young IgH-3 E-myc mice and age-matched wild-type mice in response to B cell antigen receptor engagement signals and to bacterial lipopolysaccharide, PMA, and ionomycin. Proliferation of splenic B cells from IgH-3 E-myc mice was 2-fold higher compared with wild-type B cells treated with lipopolysaccharide, PMA, PMA plus ionomycin, anti-IgM, and anti-CD40 (Fig. 3A).

Further studies were performed to examine changes in the cell cycle. As shown in Fig. 3B, 24% of B cells from wild-type mice were in the S and G2/M phases after treatment with anti-CD40 for 24 h. The number of B cells in the S and G2/M phases from IgH-3 E-myc mice was increased to 47% with the same treatment. Similar results were obtained with treatment with PMA plus ionomycin (Fig. 3B). These results demonstrate that the B cells from IgH-3 E-myc mice have increased c-Myc expression and higher rates of cell proliferation and cell cycle progression.

Development of B Cell Lymphomas in IgH-3 E-myc Mice—Profound enlargement of the spleen and lymph nodes was observed in heterozygous mice at 12 ± 2 months and in homozygous mice at 10 ± 2 months of age. Heterozygous and homozygous mice that exhibited obvious tumors or became visibly ill were necropsied. These mice displayed moderate to severe splenomegaly with a 5–12-fold increase in the weight of the spleen over wild-type littermates (Fig. 4A). Sections of tumor tissue were fixed in formalin, paraffin-embedded, and stained with hematoxylin and eosin. Histology showed diffuse infiltration in the spleens and lymph nodes with effacement of the normal architecture by a monomorphic population of round cells with one or few nucleoli (Fig. 4, B–D). The lymphomas from IgH-3 E-myc mice displayed a “starry sky” appearance, one of the histologic hallmarks of Burkitt’s lymphoma (Fig. 4). Various degrees of infiltration of lung (Fig. 4E), kidney (Fig. 4F), and intestine were also observed.

The majority of lymphoma cells from spleen and lymph node-derived tumors displayed a phenotype of mature B cell origin.
Eosin staining of spleen from an IgH-3 E-myc mice were treated with medium alone (Uns), lipopolysaccharide (LPS, 10 μg/ml), PMA (20 ng/ml), PMA (20 ng/ml) plus ionomycin (0.5 μg/ml) (P + I), anti-IgM (15 μg/ml), and anti-CD40 (5 μg/ml) for 24 h. B, cell cycle analysis of B cells from IgH-3 E-myc and wild-type (wt) mice. Freshly purified splenic B cells were activated with anti-CD40 (5 μg/ml) or PMA (20 ng/ml) plus ionomycin (Iono) (0.5 μg/ml) for 24 h and then stained with propidium iodide. Cell cycle progression was analyzed by flow cytometry. Prior to stimulation ~9% of the lymphocytes from wild-type mice, and ~12% of the lymphocytes from the IgH-3 E mice were in S+G2/M phases. Three independent experiments were conducted, and a representative experiment is shown.

FIG. 3. Increased proliferation in response to activation signals in B cells of IgH-3 E-myc mice. A, XTT assay of B cells from premalignant (6–10-weeks) IgH-3 E-myc or wild-type (wt) mice. B cells were treated with medium alone (Uns), lipopolysaccharide (LPS, 10 μg/ml), PMA (20 ng/ml), PMA (20 ng/ml) plus ionomycin (0.5 μg/ml) (P + I), anti-IgM (15 μg/ml), and anti-CD40 (5 μg/ml) for 24 h. B, cell cycle analysis of B cells from IgH-3 E-myc and wild-type (wt) mice. Freshly purified splenic B cells were activated with anti-CD40 (5 μg/ml) or PMA (20 ng/ml) plus ionomycin (Iono) (0.5 μg/ml) for 24 h and then stained with propidium iodide. Cell cycle progression was analyzed by flow cytometry. Prior to stimulation ~9% of the lymphocytes from wild-type mice, and ~12% of the lymphocytes from the IgH-3 E mice were in S+G2/M phases. Three independent experiments were conducted, and a representative experiment is shown.

FIG. 4. Histopathological analysis of the lymphomas in IgH-3 E-myc mice. A, enlarged lymph nodes (top) and spleen (bottom) from an IgH-3 E-myc mouse with lymphoma. B, hematoxylin and eosin staining of wild-type spleen (4X magnification). C, hematoxylin and eosin staining of spleen from an IgH-3 E-myc mouse with lymphoma (4X magnification). D, higher magnification (40X) of IgH-3 E-myc spleen. Infiltration of lymphoma into lung (E) and kidney (F) (40X magnification).

with expression of CD19+, B220+, CD22, CD79a, IgM+, and low levels of IgD. Further studies revealed that they were also positive for CD24, CD16/CD32, and CD38, but negative for CD23, CD34, CD3, CD4, and CD8.3

Thirty-six heterozygous IgH-3 E-myc, 30 homozygous IgH-3 E-myc, and 20 wild-type littermates were followed to record their lifespan. The IgH-3 E-myc mice showed significantly increased mortality compared with wild-type controls with a mean age of death ~379 days for heterozygous IgH-3 E-myc mice and 314 days for the homozygous IgH-3 E-myc mice (Fig. 5A). At necropsy, all the IgH-3 E-myc mice had enlarged spleens and lymph nodes.

To determine whether the lymphomas that developed in IgH-3 E-myc mice were monoclonal, we analyzed the IgH gene configuration by Southern blot analysis. DNA from spleens of age-matched wild-type control mice showed the germ line IgH band, whereas DNA from lymphomas displayed one or more discrete, rearranged bands in addition to the germ line band, indicating a clonal origin (Fig. 5B). No clonality was observed in B cells of young IgH-3 E-myc mice despite the presence of an increased population of B cells.

Premalignant B Cells from IgH-3 E-myc Mice Prior to Lymphoma Development Show Increased Rates of Apoptosis—In addition to the role of c-Myc in promoting proliferation, elevated c-Myc expression in normal cells sensitizes them to apoptosis in response to many apoptotic stimuli such as deprivation of survival factors and treatment with chemotherapeutic agents (21). We examined the rate of spontaneous apoptosis of B cells in culture without activation stimuli. As shown in Fig. 6A, there was a significant decrease in the number of viable B cells from young IgH-3 E-myc mice (premalignant B cells) compared with that of viable B cells from age-matched wild-type mice. In contrast, B cells from IgH-3 E-myc mice with lympho-
mas remained viable in culture for longer periods of time (Fig. 6A).

To examine chemotherapy-induced apoptosis, the B cells were treated with etoposide at a range of concentrations. Again, we observed that the premalignant cells from young IgH-3'E-myc mice were more sensitive to induction of cell death compared with B cells from wild-type mice (Fig. 6B). Although the lymphoma cells were more resistant to etoposide-induced cell death than the premalignant B cells at 16 h, there was no significant difference compared with B cells from wild-type mice. Longer treatment times with etoposide revealed that the lymphoma cells were somewhat more resistant to the induction of cell death compared with wild-type cells (Fig. 6C). These results demonstrate that non-transformed B cells from IgH-3'E-myc mice display increased apoptosis, presumably because of high c-Myc levels, whereas this is no longer observed in the lymphoma cells, suggesting that changes in apoptotic pathways have occurred in the lymphoma cells of IgH-3'E-myc mice.

IgH-3'E-myc Lymphomas Show Increased Expression of Bcl-xL or Bcl-2 and Mdm2—To begin to investigate the differences in apoptosis rates in lymphoma cells compared with the premalignant cells, the levels of expression of p53, Mdm2, and several Bcl-2 family members were examined in 12 lymphoma samples by Western blot analysis. We found that all 12 lymphomas showed low levels of p53 compared with wild-type B cells and premalignant B cells (Fig. 7A). Sequence analysis of p53 cDNA reverse-transcribed from RNA of the 12 lymphomas revealed that p53 was wild-type in these lymphomas. In addition, there was no loss of the p53 gene in these cells. Seven of the lymphoma samples had increased Mdm2 expression relative to wild-type and premalignant B cells (Fig. 7A). Levels of ARF were increased in the premalignant B cells compared with wild-type and malignant cells. Bax expression in the lymphomas was slightly decreased compared with wild-type cells (Fig. 7A). Five of the lymphoma samples had elevated expression of Bcl-xL, and three had increased levels of Bcl-2 (Fig. 7A). These data are summarized in Fig. 7B.

Further investigation of the p53 response to chemotherapy was performed. As shown in Fig. 7C, there was increased p53 expression in both wild-type and premalignant B cells in response to etoposide treatment. The lymphoma cells expressed lower levels of p53 at baseline, and there was no increase with etoposide treatment.

Increased Levels of Bcl-xL Protect the IgH-3'E-myc Lymphoma Cells from Apoptosis—To further evaluate the role of increased expression of Bcl-xL in the IgH-3'E-myc lymphoma cells, siRNA was used to target Bcl-xL. The lymphoma cells could be adapted to growth in tissue culture, and the levels of the anti-apoptotic proteins Bcl-xL or Bcl-2 remained high. A lymphoma sample that expressed high levels of Bcl-xL was transfected with siRNA against Bcl-xL. As shown in Fig. 8A, Bcl-xL levels were markedly decreased. Treatment of the cells expressing the Bcl-xL siRNA with etoposide resulted in increased apoptosis compared with the cells expressing a scrambled siRNA (Fig. 8B).

The c-myc Promoter Shift from P2 to P1 Is Observed in Lymphoma Cells—In addition to increased expression of c-myc, a promoter shift from the normal P2 promoter to the P1 promoter is observed in Burkitt’s lymphoma. We used real-time RT-PCR to determine whether the P1 promoter was activated by the IgH enhancers. As expected, wild-type B cells showed predominant usage of the P2 promoter (Fig. 9). Interestingly, the premalignant B cells, which showed increased expression of c-myc, did not display activation of the P1 promoter (Fig. 9). The lymphoma cells all showed increased activity of the P1 promoter consistent with that observed in human Burkitt’s lymphoma cells. We have previously identified a binding site in the c-myc promoter for a MAZ-related factor (22). This site is required for the full activation of the P1 promoter in Burkitt’s lymphoma cell lines. We examined wild-type, premalignant, and lymphoma B cells for the presence of the MAZ-related factor by electrophoretic mobility shift assay, but no differences in specific DNA binding activity were observed.
**Discussion**

Burkitt's lymphomas invariably contain translocations of c-myc into one of the immunoglobulin loci, and the IgH 3' enhancers are linked to the translocated c-myc in all t(8; 14) translocations. We have used the IgH 3'/H11032 enhancers HS1234 to deregulate c-myc expression in murine B cells. The use of a gene-targeting approach avoided positional and copy number effects in our mouse model. We were able to study the function of the isolated 3'/H11032 enhancers without interference from other regulatory elements of the IgH locus. Our results demonstrate that the IgH 3' enhancers are sufficient to deregulate c-myc expression and cause a malignancy that resembles human Burkitt's lymphoma.

The morphology of the IgH-3'E-myc and Burkitt's lympho-
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IgH locus, such as with E\textasciimacron3 suffices to cause the c-myc expression in cell lines had suggested that the 3'-enhancers are required for the activation of the P1 promoter by the IgH 3'-region c-Myc expression. The mechanisms involved in the induction of c-myc expression and cause lymphomagenesis in B cells. This lymphoma has a number of similarities to human Burkitt's lymphoma and will allow investigations into the mechanisms involved in c-myc deregulation and provide a preclinical model for testing therapeutics.

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