Published in final edited form as:

Genes Chromosomes Cancer. 2007 October ; 46(10): 950–959. doi:10.1002/gcc.20480.

In a Model of Immunoglobulin Heavy-Chain (*IGH*)/*MYC* Translocation, the *Igh* 3' Regulatory Region Induces *MYC* Expression at the Immature Stage of B Cell Development

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Abstract

Reciprocal translocations involving the immunoglobulin loci and the cellular oncogene *MYC* are hallmark mutations of the human postgerminal center B cell neoplasm, Burkitt's lymphoma. They are occasionally found in other B cell lymphomas, as well. Translocations involving the heavy chain locus (*IGH*) place the *MYC* gene either in cis with both the intronic enhancer E μ and the *IGH* 3' regulatory region (3'RR) or in cis with only the 3'RR. The result is deregulated *MYC* expression. Recent studies have led to some controversy as to when, during B lymphocyte development, *IGH*/*MYC* chromosome translocations take place. A related issue, relevant not only to lymphoma development but also to normal controls on *IGH* gene expression, is the stage, during B lymphocyte development, at which the 3'RR is capable of activating *MYC* expression. We have developed mice transgenic for a human *MYC* (h*MYC*) gene under control of the four core enhancers from the mouse *Igh* 3'RR. Unlike other transgenic mouse models where premature and inappropriate *MYC* expression disrupts normal B cell development, the h*MYC* transgene in these studies carries a mutation that prohibits *MYC* protein synthesis. As a result, h*MYC* expression can be analyzed in all of the normal B cell compartments. Our data show that h*MYC* is expressed almost exclusively in B-lineage cells and is induced to high levels as soon as bone marrow cells reach the immature B cell stage.

INTRODUCTION

The immunoglobulin heavy and light chain (*IGH* and *IGL/IGK*) loci undergo multiple rounds of DNA recombination and somatic hypermutation as B lymphocytes form and then perfect their receptors for antigen. DNA recombination events that result in deletion of large segments of the genome are required for assembly of the variable region-encoding gene segments (V-D-J and V-J joining) and for the switch from one class of antibody to another (Fig. 1). The latter, class-switch recombination (CSR), follows antigen stimulation of the B cell and allows for the development of daughter clones that can produce antigen-reactive antibody with the

appropriate properties to combat the pathogen in question (e.g., ability to traffic to appropriate sites in the body; to destroy directly the organism through complement-mediated lysis; etc.). Somatic hypermutation (SHM) of the assembled Ig variable region genes is another consequence of antigen-stimulation and is the process by which responding B lymphocytes develop a pool of daughter cells producing antibodies with higher affinity for the invading pathogen.

The many unusual events that take place in the *IGH* locus, therefore, provide this locus with an unparalleled flexibility in gene structure that is fundamental to the role that antibodies must play in combating infection. At the same time, these events come at considerable risk. The tissue-specific enzymes initiating these events (AID and the RAGs) have potential substrates elsewhere in the genome, but cis-acting regulatory regions within the *IGH*, *IGK*, and *IGL* loci largely succeed in focusing these enzymes' efforts on the *Ig* loci. These regulatory regions include the intronic enhancer E μ and the 3' regulatory region (3'RR) of the *IGH* locus.

E μ plays an essential role in the efficient assembly of V_H genes, and one or more elements of the 3'RR is essential for normal CSR (Chen et al., 1993; Serwe and Sablitzky 1993; Cogne et al., 1994; Manis et al., 1998; Seidl et al., 1999; Pinaud et al., 2001; Dunnick et al., 2005). Both play a role in driving appropriate levels of *IGH* gene expression during different stages in B cell development, and it is probable that these two regulatory regions influence SHM, although how they do so remains unclear (Arulampalam et al., 1997; Stevens et al., 2000; Shi and Eckhardt, 2001; Terauchi et al., 2001; Morvan et al., 2003; Perlot et al., 2005; Ronai et al., 2005). What is clear is that errors take place in restricting these gene-damaging processes to the *IGH* locus.

IGH translocations with oncogene-containing chromosomes participate in the development of several types of B lymphoid and plasma cell (PC) tumors (Bergsagel and Kuehl, 2001). It has been postulated that most of these translocations represent errors in the usually *IGH* locus-restricted processes of V_H gene assembly, CSR, and/or SHM (Mills et al., 2003). Moreover, the result of these translocations is juxtaposition of the oncogenes with the regulatory regions of the *IGH* locus and subsequent oncogene deregulation. The type of B cell tumor that arises as a result of *IGH* translocations, therefore, must be influenced by at least two factors: the developmental stage at which the translocation occurs (dependent on mechanism) and the developmental stages during which the regulatory element(s) newly-associated with the oncogene is active.

In the present study, we focus on the juxtaposition of *MYC* with the 3'RR of the *IGH* locus. Our goal has been to determine when, during B cell development, the 3'RR becomes competent to influence *MYC* expression, thereby rendering cells susceptible to transformation. To this end, we created a transgene that placed the murine 3'RR in cis with the natural human *MYC* gene. Because ectopic expression of MYC protein has been shown previously to perturb normal B cell development and behavior, well before the development of tumors (Cheung et al., 2004; Wang and Boxer, 2005), we used a human *MYC* gene (h*MYC*) that carries a nonsense mutation, precluding expression of human MYC protein.

Analyses of two independent mouse lines showed that the 3'RR becomes competent to drive h*MYC* expression as soon as developing B cells in the bone marrow reach the surface Ig⁺ stage, and it maintains this competence as cells exit the bone marrow to enter the peripheral B cell pool. Although it is possible that the molecular events driving juxtaposition of *MYC* and the *IGH* 3'RR through chromosome translocation occur only after peripheral B cell activation, the present studies demonstrate that the 3'RR's ability to influence *MYC* activity is acquired much earlier in B cell development.

MATERIALS AND METHODS

Generation of 3'RR/hMYC Transgenic Mice

An 8 kb *HindIII*-*SalI* fragment encompassing human *MYC* was obtained from a human Burkitt lymphoma line (BL60) (Polack et al., 1993). This fragment includes the noncoding first exon with the natural P1 and P2 promoters and ~2.3 kb 5' flanking sequence encompassing the regulatory Far UpStream Element (FUSE). There is a frame shift in this *MYC* gene at the start of exon 2, resulting in termination at the codon for amino acid 103 (Polack et al., 1991). This frame shift was caused by a 5 bp insertion (at nucleotide 4823, Genbank Accession No. X00364). A vector carrying this fragment was kindly provided by Dr. Georg Bornkamm (GSF-National Research Center for Environment and Health, Munich, Germany). A 7.8 kb *HindIII* fragment containing the four enhancers (hs3a, hs1,2, hs3b, and hs4) of the 3'RR was obtained from plasmid γ 2bhs1-4loxphs123a (Shi and Eckhardt, 2001). The two fragments were fused to create the 3'RR/hMYC transgene (Fig. 2). Transgenic mice were developed in the C57BL/6 mouse strain in the National Cancer Institute Transgenic Core Facility under the direction of Dr. Lionel Feigenbaum. The two transgenic lines D1 and D5 were maintained as heterozygotes for the transgene. Data shown were obtained from heterozygous mice after at least six backcross generations (backcrosses to C57BL/6).

Genomic Southern Blots

Restriction enzyme-digested DNAs were loaded into individual lanes of 0.8% agarose gels (20–30 μ g DNA per sample). DNA was then transferred to nylon membranes (Cat no. 10416296, Whatman, Inc., Sanford, ME). Blots were hybridized using Quickhyb (Cat no. 201220, Stratagene, CA). Probes were labeled by the random primer method (Mega Prime™, Cat no. RPN 1605, Amersham, Arlington Heights, IL). Hybridization intensity was quantified by PhosphorImager™ (Molecular Dynamics, Sunnyvale, CA) and ImageQuant™ Software (GE Bio-Sciences Corp., Piscataway, NJ). In genomic Southern blots like those shown in Figure 3, the *Bam*HI band derived from the transgene (1 kb) was normalized to that (4.9 kb) derived from the two endogenous *Igh* loci (hs4 region of *Igh* locus). The experiment was repeated multiple times with DNA from several mice of each transgenic line (D1 and D5). Probes used were hs4 (1.4 kb *Pst*I/*Hind*III fragment), hs3a/b (1.2 kb *Xba*I fragment containing hs3b; hybridizes to both hs3a and hs3b), hs1,2 (570 bp *Kpn*I/*Xba*I fragment from pBS3'αE; Lieberson et al., 1991), and *MYC* (510 bp PCR product, nucleotides 129–638, Genbank Accession no. M13930).

Cell Culture

Cells from spleen were depleted of erythrocytes by ACK buffer lysis (PBS with 0.15 M NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA) and resting B cells enriched by negative selection with the MACS™ B-cell isolation kit (Miltenyi Biotech, Inc., Bergisch Gladbach, Germany, Cat no. 130-090-862). This isolation kit eliminates both non-B cells and activated B cells (Podojil and Sanders, 2003). In LPS (lipopolysaccharide) stimulation experiments, resting, splenic B cells were cultured at 5×10^5 cells/ml in serum-containing medium (RPMI-1640 with 20% fetal calf serum, 4 mM L-glutamine, 4 mM penicillin-streptomycin, 50 μ M β -mercaptoethanol) supplemented with 25 μ g/ml LPS (Sigma, St. Louis, MO, Cat no. L6511). Cultures were maintained for 5 days, and cell samples removed ($\sim 10^7$ cells) at 2 hr, 24 hr, 3 days, 4 days, and 5 days (experiment done twice with pooled spleens from D5 mice and once with spleens from D1 mice).

Flow Cytometry

For most antibody stains, cells were incubated for 20 min with the relevant antibody in ice-cold staining buffer (Phosphate-Buffered Saline with 0.1% bovine serum albumin, 5.6 mM

dextrose, and 0.1% sodium azide). After washing, the cells were resuspended in staining buffer and analyzed by FACScan™ or sorted by FACS Vantage™ (Becton Dickinson, San Jose, CA). Five to ten thousand events were collected and analyzed by Cell-Quest™ (Becton Dickinson) and/or Flow Jo™ (Tree Star, Inc., Ashland, OR) software.

Bone marrow B cells were enriched by the MACS™ B220-microBeads kit (Miltenyi Biotech, Germany, Cat no. 130-049-501), and then were stained with PE-anti-B220 (BD Pharmingen, Cat no. 553089) and FITC-anti-IgM (BD Pharmingen, Cat no. 553408) to sort B220 + IgM⁺ pro/pre B cells and B220 + IgM⁺ immature/mature B cells. Viable lymphocytes were identified by forward and side scatter gates. Bone marrow cells ($5-10 \times 10^8$) from 4 to 15 animals were pooled for these sorting experiments (three independent experiments done). Sorted fractions were analyzed postsort and were >95% pure. Immature and mature B cells were separated, using PE-anti-B220, FITC-anti-IgD (BD Pharmingen, Cat no. 02214D), and biotinanti-IgM (BD Pharmingen, Cat no. 553436) plus streptavidin-PE-Cy7 (BD Pharmingen, Cat No. 557598) to distinguish B220 + IgM⁺IgD⁻ immature B cells from B220 + IgM⁺IgD⁺ mature B cells (~17 and 19% of bone marrow B220⁺ cells, respectively). Bone marrow cells from 9 or 16 animals were pooled for these experiments (two independent experiments).

Real-Time RT-PCR

RNAs from heart, lung, liver, kidney, thymus, and muscle were purified directly from the tissues by using the RNeasy™ mini-prep kit (Qiagen, Valencia, CA, Cat no. 74106). Splenic B lymphocytes were purified with the MACS™ B-cell isolation kit (Miltenyi Biotech, Germany) and the non-B portion by sorting (FACS Vantage™, Becton Dickinson) for B220-negative cells. RNAs from splenic B cells, non-B cells, and the bone marrow B cell subsets were also purified with the RNeasy™ mini-prep kit. Real-time RT-PCR was performed using the ABI Prism 7500 and 7900 systems (Applied Biosystems, Branchburg, NJ). Human *MYC*, mouse *Myc*, and mouse *Gapdh* mRNA levels were determined by specific primer-probe sets from assay-on-demand™ (Applied Biosystems, Branchburg, NJ). RT-PCR reagents were from the TaqMan One-step RT-PCR kit (Applied Biosystems, Cat no. 4309169). mRNA levels were normalized to that of *Gapdh*. The program used to analyze data was supplied by Applied Biosystems (User bulletin no. 2; <http://docs.appliedbiosystems.com/pebi/docs/04303859.pdf>).

RESULTS

Generation of 3'RR/hMYC Transgenic Mice

A transgene was generated by fusing an 8 kb region encompassing human *MYC* with a 7.8 kb fragment containing the four enhancers of the murine 3' regulatory region (3'RR). A diagram of the construct is shown in Figure 2. The human *MYC* in this transgene is derived from the Burkitt lymphoma line BL60 and has been used previously in transgenic mice to generate a mouse model of Burkitt's lymphoma (Kovalchuk et al., 2000). The gene in the present study, however, carries a frame-shift mutation that leads to early termination so that MYC protein is not produced.

Two founder mice were isolated and mated with C57BL/6 mice to generate two independent transgenic lines. Liver DNA from each line was digested with *HindIII* and hybridized to 3'RR elements to confirm that the 7.8 kb *HindIII* fragment containing all of these elements remained intact in the animals. As shown in Figure 3A, this was confirmed in both mouse lines. *HindIII*/*BglIII* digests confirmed that the human *MYC* promoter region and first two exons were present and intact (Fig. 3A, and refer to map in Fig. 2). In all of these analyses, the D1 line showed stronger signals for the transgene fragments than did the D5 line. To determine copy number and for routine screens of transgenic progeny, *BamHI* digests of tail DNA were hybridized to

an *hs4* probe. The endogenous *hs4* region is found within a 4.9 kb *Bam*HI fragment, while *hs4* in the transgene lies within an ~1 kb *Bam*HI fragment (see map in Fig. 2). As shown in Figure 3B, the D5 line carried one or two copies of the transgene while D1 had ~10 copies.

B-Lymphocyte-Specific Expression of 3'RR/hMYC Transgene

Real-time PCR was used to analyze mRNA from both mouse lines. RNA was isolated from several nonlymphoid tissues, from resting and activated splenic B cells, from non-B spleen cells, and from B220+ bone marrow cells. Species-specific primers were used to distinguish between mRNA from the normal (murine) *Myc* loci (*mMyc*) in the animal and the human *MYC* transgene (*hMYC*) juxtaposed with the 3'RR. Results were qualitatively the same for both transgenic lines, regardless of the difference in copy number and integration site.

As shown in Figure 4, there was minimal *Myc/MYC* expression (endogenous or transgenic) in the heart (set to 1 in the bar graphs shown), liver, kidney, and muscle. There was elevated *hMYC* transgene expression in the lung, but this was dwarfed by the high level of expression seen in splenic B lymphocytes. Spleen cells were fractionated into B lymphocytes and non-B cells, and while *mMyc* was expressed equally in the two fractions (171 ± 35 ; 216 ± 45 , respectively, relative to heart), *hMYC* transgene expression was an average of 18-fold more abundant in the B cell fraction of spleens isolated from D5 mice (Fig. 4B) and over 80-fold more abundant in this fraction in spleens from D1 mice (Fig. 4A). We conclude that the *hMYC* transgene, when under control of the 3'RR, is expressed in a B lymphocyte-specific manner.

3'RR Modifies hMYC Response to LPS-Activation of Splenic B Lymphocytes

The method used to isolate splenic B cells was an indirect one that avoids activation of the B cells. To determine how activation of these cells might influence 3'RR/hMYC expression, we treated splenic B cells with the polyclonal activator LPS. Endogenous *mMyc* mRNA rose transiently, followed by a decline such that *mMyc* mRNA remained lower (1/10) than starting levels as many as 5 days later (Fig. 5; the transient rise in *mMyc* is early and quite brief and was not fully captured in the analysis of D1 mice). This profile for (nontranslocated) *mMyc* expression has been reported before and is consistent with the induction of *mMyc* at the onset of cycling, followed by its down-regulation as the MYC protein is produced and represses, directly and indirectly, *mMyc* transcription (Facchini et al., 1997; Chung and Levens, 2005).

The *hMYC* expression profile in LPS-treated splenic B cells differed substantially from that of *mMyc*. *hMYC* showed no early peak in transcription (in either D1 or D5 mice) but did decline from starting levels within 24 h from the start of LPS stimulation. The transgene recovered from repression much more readily than the endogenous gene, reaching prestimulation levels when endogenous *mMyc* mRNA levels remained low (Fig. 5). Importantly, all the promoter elements known to be involved in *MYC* transcriptional activation and repression are present in the *hMYC* transgene (Avigan et al., 1990; Meulia et al., 1992; Liu et al., 2000; Kim et al., 2003). The different behavior of the *mMyc* and *hMYC* genes after LPS stimulation suggests, therefore, that the 3'RR supercedes mechanisms that normally regulate *MYC* transcription.

As B-Lineage Bone Marrow Cells Reach the Surface IgM+ Stage, 3'RR/hMYC Expression Rises to High Levels

Having confirmed expression of the 3'RR-driven *hMYC* transgene in mature splenic B lymphocytes, we isolated bone marrow cells from transgenic animals to determine when, during B lymphocyte development, this transgene first becomes operational. An antibody to B220 (B lineage cell marker) was used to identify B lineage cells in the bone marrow, and an antibody to IgM further distinguished immature and mature B cells (IgM+) from pro- and pre-B cells (IgM-). Bone marrow B-lineage cells were sorted, by flow-cytometry, on the basis

of IgM expression, and the resulting subsets analyzed for purity (Fig. 6A). As shown in Figure 6B, B-lineage bone marrow cells (BM B220⁺), prior to their fractionation into IgM⁺ and IgM⁻ subsets, expressed hMYC, but at lower levels than splenic B cells. Quantitative RT-PCR assays of RNA extracted from the pro-/pre-B and immature/mature B cell fractions revealed that the bulk of the hMYC mRNA in bone marrow B-lineage cells derived from the immature/mature B cell fraction (Fig. 6B and Fig. 6C). IgM⁻, B220⁺ cells (pro and pre-B cells) expressed the 3'RR/hMYC gene but at 10-fold lower levels than either immature/mature bone marrow B cells or splenic B cells (Fig. 6B and Fig. 6C). Note that mMyC did not show the dramatic variation in levels seen for hMYC among these bone marrow subsets, supporting the notion that these differences were due to changes in 3'RR activity (Fig. 6B). We conclude from these results that transcription of the 3'RR-associated hMYC transgene is first induced to high levels at the immature/mature B cell stage.

Antibodies to B220, IgM, and IgD were used to distinguish newly-formed, immature B cells (B220⁺, IgM⁺, IgD⁻) from mature B cells (B220⁺, IgM⁺, IgD⁺) (Fig. 7A). The latter cells consist both of newly matured B cells that have not yet exited the bone marrow as well as mature B cells from the periphery that have recirculated to the bone marrow. To quantify hMYC mRNA levels in the immature B cell population as compared to this mixed population of mature B cells, cells were again sorted by flow cytometry (purity indicated in Fig. 7A). RNA was isolated from each subset and hMYC mRNA quantified by qRT-PCR. As shown in Figure 7B and Figure 7C, while hMYC mRNA was somewhat more abundant in the mature B cells, there was substantial expression in the newly-emerging, immature B cells, suggesting that this 3'RR-driven transgene becomes fully active as soon as B cells reach the surface Ig-positive stage.

DISCUSSION

Translocations between *MYC* and the *IGH*, *IGK*, and *IGL* loci are a hallmark of Burkitt's lymphoma and are also found in some other B lymphoid and plasma cell tumors (Bergsagel and Kuehl, 2001). In many cases, these translocations position *MYC* in cis with the 3' end of the *IGH* locus. It has been postulated that the regulatory sequences found at this end of the *IGH* locus (3'RR) are responsible for the consequent deregulation of *MYC* expression, resulting in the first step toward cellular transformation. Consistent with this hypothesis, animals carrying transgenes that mimic this translocation are predisposed to the development of B lymphoid tumors.

The 3'RR was first identified in rat and mouse *Igh* loci, but analogs were subsequently identified downstream of both of the *IGH* gene clusters in humans (Pettersson et al., 1990; Dariavach et al., 1991; Lieberson et al., 1991; Chen and Birshtein, 1997; Mills et al., 1997). Clearly, the 3'RR can drive transcription of assembled *IGH* genes and plays a role in promoting class-switch recombination in the *IGH* locus, but its mechanism of action and its functional roles early in B cell development are yet to be determined. Studies of the murine *Igh* locus suggest that the 3'RR undergoes step-wise changes in chromatin structure and that not all (if any) of the elements (hs3a, hs1,2, hs3b, and hs4) within the 3'RR are active throughout B cell development (Garrett et al., 2005). An understanding of how the 3'RR disrupts expression of adjacent oncogenes, when such juxtaposition takes place by chromosome translocation, awaits, therefore, a more thorough study of 3'RR function.

In a transgene mimicking *IGH/MYC* translocation, we have found that the 3'RR begins to drive high levels of *MYC* expression when cells in the B lineage reach the stage of export from the bone marrow as surface Ig-positive cells. The 3'RR/hMYC transgene was expressed in lung tissue at higher levels than in any of the other tested, nonlymphoid tissues, but its expression in lymphoid tissue far exceeded that in lung (Fig. 4). In lymphoid tissue, expression in B

lymphocytes dwarfed expression by T lymphocytes (non-B splenic cells). As shown in Figure 4, the wild-type, endogenous *mMyc* locus behaved quite differently. Its expression did not vary significantly among nonlymphoid tissues, and, while expression was higher in lymphoid than in nonlymphoid cells, it did not differ significantly between B and non-B (T lymphocytes) cells of the spleen. The 3'RR, derived from the tissue-specific *Igh* locus, therefore, largely transforms a locus that is expressed ubiquitously at basal levels (*MYC*) into one that is expressed at high levels (deregulated) almost exclusively in B-lineage cells. B cell-restricted transgene activity was also seen in a previous study in which the 3'RR was linked to an *Igh*, rather than the *MYC*, promoter (Chauveau et al., 1999).

In transgenic animal studies in which only one element of the 3'RR (hs1,2) was linked to a reporter gene (human β -globin gene and murine *Igu* gene, respectively), reporter gene expression was detected only after activation of splenic B cells (Arulampalam et al., 1994; Grant et al., 1996; Andersson et al., 1999). The high levels of hMYC transgene expression in resting splenic B cells in the present study showed that this was not the case when the four known elements (hs3a; hs1,2; hs3b; hs4) of the 3'RR were present and juxtaposed with *MYC*. Moreover, there was significant hMYC expression in immature B lymphocytes, even before these cells had exited the bone marrow and prior, therefore, to their exposure to antigen. Both sets of data identify an earlier onset of 3'RR activity than was noted for the single 3'RR sub-element hsl,2 (Arulampalam et al., 1994). This difference is consistent with work from our laboratory and others that reveal a complex interplay among the elements within this region (Ong et al., 1998).

In two earlier studies, elevated expression of a 3'RR-regulated *MYC* gene was restricted to B line-age cells and was detected in the spleen (Cheung et al., 2004; Wang and Boxer, 2005). In neither of these studies, however, was it clear whether expression both pre-dated activation of the cells and was 3'RR-dependent. In one of the studies, *mMyc* was inserted into the *Igh* locus in a fashion that made it subject both to $E\mu$ and 3'RR influences. $E\mu$ is active throughout B cell development, so it was to be expected that the inserted *mMyc* gene would be expressed in resting splenic B cells (Cheung et al., 2004). In the other study, the 3'RR was inserted into the murine *Myc* locus (Wang and Boxer, 2005). In animals carrying this insertion, B lymphocyte development was substantially perturbed. There was an appreciable increase in splenic B cell numbers, even in young animals (7- to 10-fold in homozygotes), and these B cells were both hyper-responsive to proliferative signals and prone to high levels of apoptosis. These effects were clearly due to the abnormal levels of MYC protein being produced, consistent with the notion that the modified *Myc* locus was active in B cells. However, given the abnormal phenotype of B lymphocytes in these animals, it was not possible to determine whether the 3'RR-mediated increase in *Myc* expression pre-dated or coincided with signals promoting B cell activation.

The difference in the behavior of hMYC and *mMyc* in both resting and LPS-stimulated splenic B cells supports the notion that juxtaposition of the 3'RR with *MYC* substantially modifies *MYC* promoter activity both before and after B-cell stimulation. Under normal circumstances and in normal cells, when *MYC* is activated, MYC protein is produced and, in addition to its initiating a sequence of events that promote cell division, MYC acts in a feed-back loop to turn off *MYC* transcription (Facchini et al., 1997; Chung and Levens, 2005). *mMyc* behaved in accordance with this paradigm in splenic B cells stimulated by a mitogen (LPS) to divide (Fig. 5). While the hMYC gene had both the FUSE region and more proximal promoter elements known to mediate both activation and repression of *MYC* transcription, its induction was much less pronounced than that of *mMyc*, and it recovered from repression much more readily. This suggests that the 3'RR shields the associated hMYC gene from normal controls on its expression, consistent with attributes that would render a cell prone to transformation (Liu and Levens, 2006).

In summary, the present study is the first reported analysis of 3'RR-regulated transgene expression in B lineage cells undergoing normal development. Two transgenic mouse lines carrying the transgene at two different integration sites and in different copy numbers yielded the same overall results. These results suggest that the 3'RR is competent to deregulate *MYC* expression as soon as B-lineage cells begin to express surface IgM. As shown in Figure 6, there may be some 3'RR activity in pro- and/or pre-B cells as well, since activity of the h*MYC* transgene is appreciably higher in these cells than in heart, for example. It is difficult to know whether this is due to differences in 3'RR activity or in activity of the *MYC* promoter itself, however, since endogenous m*Myc* also shows higher expression in the pro/pre-B cell fraction than in heart.

Translocations between *MYC* and the 3'RR are usually detected in tumors that appear to have arisen from postgerminal center B cells (i.e., after antigen-activation). Given the much earlier onset of 3'RR/h*MYC* activity, as demonstrated in the present study, it would appear that the cell stage at which tumors develop is a function of the timing of events that can lead to these aberrant translocations (e.g., somatic hypermutation and class-switch recombination) rather than a function of the developmental stage at which the *MYC* promoter is subject to deregulation by the *IGH* 3'RR. 3'RR translocations in B lymphoid tumors are not limited to those fusing the *IGH* and *MYC* loci. In follicular lymphoma, the *IGH* 3'RR is juxtaposed with the gene encoding *BCL2*, an antiapoptotic factor. The result is deregulated expression of *BCL2*, resulting in increased tumor resistance to programmed cell death (Heckman et al., 2003). Primary chromosomal translocations associated with the development of multiple myeloma also frequently involve the *IGH* locus (30–50%) and result in juxtaposition of the 3'RR with oncogenes such as fibroblast growth factor receptor 3 (*FGFR3*) and the cyclins D1 and D3 (Bergsagel and Kuehl, 2001). Further analyses of the 3'RR and the means by which elements within it are activated and influence oncogene expression will inform strategies aimed at treating and/or preventing these neoplasms.

ACKNOWLEDGMENTS

The authors thank Dr. Georg Bornkamm for providing a vector carrying the h*MYC* gene and Dr. Lionel Feigenbaum and his staff (National Cancer Institute Transgenic Core Facility) for generating the D1 and D5 transgenic lines. Steven Williams and Pavel Munerman are gratefully acknowledged for their expert technical assistance. The authors also thank Dr. Jill Bargonetti-Chavarria, Dr. Adrienne Alaie-Petrillo, and Dr. Michael Young for critical readings of the manuscript.

Supported by: NIH; Grant numbers: AI30653, RR-0307.

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Figure 1.

Diagram of the murine *IgH* locus. Representative Variable (V_H) and Diversity (D_H) gene segments are shown, separated by long distances (hatched lines) from the Joining (J_H) gene segments, the intronic enhancer E_μ and the tandem array of *IgH* constant region genes (μ , δ , etc). Vertical ovals upstream of each constant region gene represent Switch sequences (sites of class-switch recombination, CSR). The DNaseI hypersensitive sites (3a, 1, 2, 3b, 4) are shown within the $3'IgH$ Regulatory Region ($3'IgH$ RR). The processes of V_H gene assembly and class-switch recombination are diagrammed. Not drawn to scale.

Figure 2.

Diagram of the 3'RR/hMYC transgene. The four core enhancers of the 3'RR (hs3a; hs1,2; hs3b; hs4) are shown as hatched rectangles and lie within a 7.8 kb *HindIII* (H) fragment. Closed circles depict the three exons of hMYC, arrows indicate its two promoters (P1 and P2), and a black bar indicates the position of FUSE (far-upstream element). This MYC gene does not encode protein, due to a 5 bp insertion that leads to a frame-shift and early termination. The four probes used in Southern blots shown in Figure 3 are designated A (hs3a/b), B (hs1,2), C (hs4), and D (MYC). B = *Bom*HI, H = *Hind*III, S = *Sal*I, Bg = *Bgl*II.

Figure 3.

3'RR/hMYC transgene in two mouse lines. (A) Genomic Southern blots of *HindIII*-digested liver DNA from C57BL/6 (C57), mouse line D1, and mouse line D5 are shown in the middle three panels. DNA from the same three sources was digested with both *HindIII* and *BglII* in the right-most panel. 1 kb ladder (KB) is shown as molecular weight marker (left-most panel). Probes are shown below each blot. In the *HindIII* blots shown, the same digests were loaded in triplicate on the same gel, blotted, and the blot cut before hybridization to the three 3'RR probes. Endogenous fragments for each of the 3'RR probes (hs3a/b, hs1,2, hs4) are seen in the C57 lanes. For both hs3a/b and hs4, the endogenous and transgene fragments are close in size (8 kb vs. 7.8 kb) so that they comigrate in the transgenic lines. (B) *BamHI*-digested tail DNA

from a transgene-negative littermate (C57), a D5 animal and a D1 animal, hybridized with probe hs4 (probe C in Fig. 2). Quantification of this and similar blots (using the endogenous band = 2 copies, to normalize) indicated that the D5 line carried one or two copies of the transgene, while D1 carried ~10 copies.

A. D1 line

B. D5 line

Figure 4.

Real-time (quantitative) PCR of mRNA from transgenic mouse tissues and from splenic B and non-B cells. *hMYC/mMyc* mRNA was quantified, using species-specific primers and probes for murine *Myc* (black bars) and human *MYC* (white bars), respectively. *hMYC/mMyc* mRNA levels in each RNA preparation were normalized with *Gapdh* mRNA. *hMYC* and *mMyc* mRNA levels are expressed relative to those in heart, respectively (set to a value of 1). Tissues were pooled from three transgene-positive animals. Each RNA sample was run in duplicate and at two dilutions to confirm linearity (four determinations used to calculate standard deviation). (A) qPCR data for tissues from the D1 mouse line (multi-copy transgenic). (B) qPCR data for tissues from the D5 mouse line (one/two-copy transgenic).

A. D1 line

B. D5 line

Figure 5.

MYC mRNA levels from endogenous (*mMyc*) and transgenic (*hMYC*) loci in LPS-stimulated spleen cells. Representative realtime PCR data, normalized to *Gapdh* mRNA. Purified resting B cells from the spleens of three transgene-positive mice (for both D1 and D5) were pooled and stimulated with LPS. Cells were removed at indicated time points after addition of LPS and RNA extracted. Each RNA sample was run in duplicate and at two dilutions to confirm linearity. Note log scale for mRNA levels. (A) qPCR data for LPS-stimulated B cells from the D1 mouse line. (B) qPCR data for LPS-stimulated B cells from the D5 mouse line.

B. D1 line

C. D5 line

Figure 6.

hMYC levels in IgM^+ and IgM^- B-lineage cells of the bone marrow. (A) FACSTM profiles for viable, B220+ bone marrow cells pooled from four D1 mice. Gates used to sort pro/pre-B cells from immature/mature B cells are indicated and postsort analyses (and % purity) are shown. (B) qPCR data for RNA isolated from the subsets shown in Figure 6A. White bars = *hMYC* mRNA levels; black bars = *mMyc* mRNA levels. Unsorted splenic B (B220+) cells, and unsorted bone marrow B-lineage cells (BM B220+) were isolated from the same animals and analyzed at the same time. *mMyc* and *hMYC* levels in each cell type (or subfraction) are shown relative to those in spleen. This experiment was done twice. (C) Similar analyses of *hMYC* expression in pro/pre B cells and immature/mature B cells in bone marrow pooled from fourteen

D5 mice. *hMYC* levels in both B-lineage subfractions are shown relative to those in spleen of the same animals.

Figure 7.

hMYC mRNA levels in immature and mature, IgM⁺ bone marrow B cells. (A) FACSTM profiles of bone marrow cells pooled from sixteen D1 mice. Bone marrow samples were incubated with antibodies to B220, IgM, and IgD. Viable cells (gated by forward and side scatter) were first gated on B220 and IgM, and the B220⁺, IgM⁺ cells further subdivided by IgD expression. The indicated fractions were sorted and analyzed postsort (% purity indicated), before RNA isolation. (B) qRT-PCR data for RNA isolated from the immature and mature B cell subsets shown in Figure 7A. Unsorted splenic B (B220⁺) cells, and unsorted bone marrow B-lineage cells (BM B220⁺) were isolated from the same animals and analyzed at the same time. Data

are presented relative to splenic B cell *hMYC* expression. (C) Similar analyses of bone marrow cells pooled from nine D5 mice. Data are presented relative to splenic B cell *hMYC* expression.