Characterization of the Myc collaborating oncogenes Bmi1 and Gfi1

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Chapter 3

Retroviral insertions in the *bla1* locus result in long-range activation of the *bmi1* proto-oncogene in B cell lymphomas

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Submitted

SHORT REPORT

Retroviral insertions in the *bla1* locus result in long-range activation of the *bmi1* proto-oncogene in B cell lymphomas

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The *bmi1* proto-oncogene was identified as a common target for proviral integration in Moloney murine leukemia virus (MoMLV)-induced lymphomas of Eμ-*myc* transgenic mice. Bmi1 collaborates with c-*myc* in lymphomagenesis and suppresses c-*myc*-induced apoptosis by repressing the *INK4a/ARF* locus. This report demonstrates that all lymphomas with proviral insertion in the *bla1* locus display transcriptionally activation of the *bmi1* gene. Similar to *bmi1*, *bla1* integrations are only detected in B cell but not T cell lymphomas. Retroviral insertions in the *bla1* locus are absolute mutually exclusive with MoMLV integrations near the *bmi1* gene. The *bla1* locus maps to the same region on mouse chromosome 2 as *bmi1*, but different lambda phage clones covering 45kb of genomic *bmi1* sequence contain no overlap with 23kb cloned genomic DNA of the *bla1* locus. Additional mapping demonstrates that *bla1* is proximal of *bmi1* within a distance of less than 170kb, which is confirmed by comparative human genome analysis. Our data strongly suggest that proviral insertions at the *bla1* locus activate the *bmi1* gene over a large distance.

[*Key Words*: B cell lymphoma; *bmi1*; c-*myc*; enhancer activation; Moloney murine leukemia virus]

The development of T cell lymphomas in mice and rats following Moloney murine leukemia virus (MoMLV) infection is a multistep process. Several different pathogenic mechanisms are involved during the process of lymphomagenesis, including generation of mink cell focus-forming (MCF) recombinants (van der Putten et al., 1981;Chattopadhyay et al. 1982), hematopoietic hyperplasia and splenomegaly (Storch et al., 1985; Davis et al., 1987), activation of NF-κB (Pak and Faller 1996) and insertional activation of cellular proto-oncogenes (Jonkers and Berns 1996). The loci targeted by provirus insertion are identified either as known proto-oncogenes or as new regions of integration, which are common among the tumors analyzed. Proviral activation of the proto-oncogenes c-*myc* (Corcoran et al. 1984; Selten et al. 1984) or N-*myc* (van Lohuizen et al. 1989), *pim1* (Selten et al. 1985) or *pim2* (van der Lugt et al. 1995), and *gfi1* (Zörnig et al. 1996; Scheijen et al., 1997) occur in a large fraction of murine T cell tumors and are important collaborating oncogenes in T cell lymphomagenesis.

The mechanisms that are employed by proviruses to affect cellular host genes comprise of promoter- or enhancer-activation, mRNA destabilization or protein truncation by transcription termination (Jonkers and Berns 1996). Promoter insertions occur in close proximity and are unidirectional with respect to the cellular target gene. Enhancer activation is however orientation independent and the regulatory elements present in the long terminal repeats (LTR) are able to operate over longer distances of genomic DNA by cis-

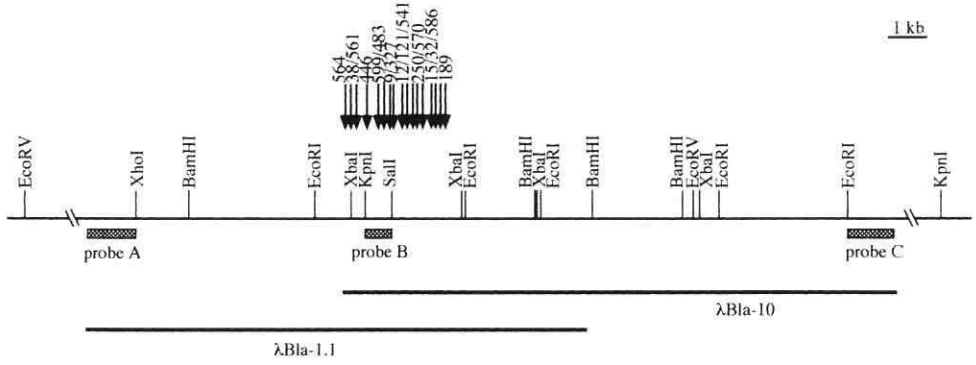

Figure 1. Restriction map of the *bla1* locus. The different proviral insertions of MoMLV, which map in this region are indicated together with the number of the tumor sample. Two overlapping SVJ129 genomic phage lambda clones were isolated (λBla-1.1 and λBla-10). Three different DNA probes were isolated from these clones. Probe A is a *Sal*I-*Xho*I end fragment of λBla-1.1, probe B is an internal unique *Kpn*I-*Sal*I fragment used to detect proviral insertions in MoMLV-induced lymphomas, and probe C is an *Eco*RI-*Sal*I end fragment of λBla-10. The relative positions of the various proviral insertions were mapped using *Kpn*I and *Eco*RV-digested genomic tumor DNA.

acting mechanisms. The ability of retroviral insertions to affect gene expression over large distances has been shown for the *evi1* and c-*myc* gene. Induction of *evi1* expression in IL-3 dependent myeloid leukemia cell lines is the result of proviral insertions in *cbl/fim3* locus (Bartholomew et al. 1989), 90 kilobases proximal of the mouse *evi1* gene (Bartholomew and Ihle 1991). Long-range activation of the c-*myc* proto-oncogene occurs after insertion of MoMLV in the common insertion sites *mlvi1/mis1/pvt1* (Villeneuve et al. 1986; Tsichlis et al. 1989) and *mlvi4* (Tsichlis et al. 1990), which map 270 and 30 kilobases distal from the c-*myc* gene (Lazo et al. 1990).

The proto-oncogene *bmi1* was identified as a common target of proviral insertion in MoMLV-induced Eμ-*myc* B cell lymphomas (Haupt et al. 1991; van Lohuizen et al. 1991b). Subsequently it was shown that mice transgenic for *bmi1* develop spontaneous B- and T cell tumors, and demonstrate clear acceleration in lymphomagenesis upon intercrossing the Eμ-*myc* transgene (Haupt et al. 1993; Alkema et al. 1997). *Bmi1* and *Mel18* are both murine and human homologues of the *Drosophila Posterior sex combs* (*Psc*) gene (Brunk et al. 1991); (van Lohuizen et al. 1991a), which is a member of the *Polycomb* group (*Pc*-G) gene family. The *Pc*-G genes are essential for maintaining the proper spatial restricted expression pattern of homeotic loci during *Drosophila* and mammalian development. Besides regulating the expression boundaries of specific homeobox gene clusters (Alkema et al. 1995; van der Lugt et al. 1996), Bmi1 also controls the expression levels of the *INK4a/ARF* locus (Jacobs et al. 1999a). *Bmi1*$^{-/-}$ primary mouse embryonic fibroblasts show elevated levels of the tumor suppressors p16^{INK4A} and p19^{ARF} and display premature replicative senescence. Overexpression of *bmi1* on the other hand reduces *INK4a/ARF* levels and inhibits c-*myc*-induced apoptosis (Jacobs et al. 1999b).

Previously, *bla1* was also identified as a common insertion site in MoMLV-induced lymphomas of Eμ-*myc* transgenic mice (van Lohuizen et al. 1991b). An inverse PCR (IPCR) based assay had been used to obtain a distinct fragment flanking the 5' end of a proviral integration in the *bla1* locus and 4 out of 25 independent lymphomas of Eμ-*myc* mice showed a proviral insertion in the *bla1* region. To obtain further information about this locus, several strategies were employed. First, independent *bla1* genomic clones were obtained by screening a mouse 129/SVJ genomic lambda phage library

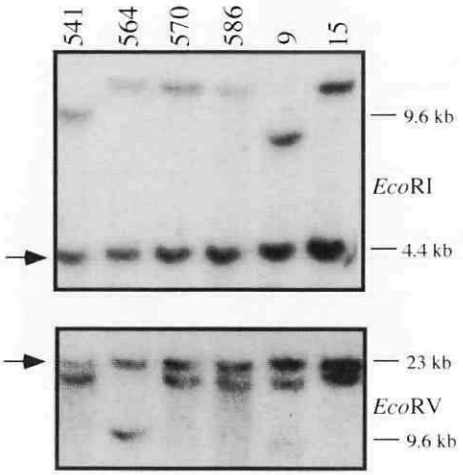

Figure 2. Rearrangements in the *bla1* locus due to Moloney murine leukemia proviral insertions. Genomic tumor DNA was digested with either *Eco*RI or *Eco*RV, separated on an 0.7%-agarose gel, transferred to nitrocellulose membrane and hybridized with a ^{32}P-random priming- labeled *bla1* probe B. The position of the endogenous allele is indicated with an arrow. Due to the absence of endogenous *Eco*RI restriction sites in the MoMLV provirus, a larger fragment will be recognized with a probe flanking the proviral insertion. The size of the full-length provirus is 8.5kb, whereas recombined proviruses will generate smaller sized fragments. In contrast, there are several internal *Eco*RV restriction sites in MoMLV, the first one being localized in the LTR. Therefore, fragments smaller than the endogenous allele will be detected on a Southern blot. Notice that tumor 9 contains a proviral insertion at the position of probe B, where both flanking fragments can be detected.

with the IPCR probe. Two overlapping lambda phage clones were isolated, λBla-1.1 and λBla-10, and characterized using a panel of restriction endonucleases (Fig. 1). Together they covered a region of 23kb genomic DNA sequence. Subsequently, different probes were generated from the *bla1* locus for DNA hybridization. These consisted of a proximal 1.4kb *Sal*I-*Xho*I fragment (probe A), a central 0.7kb *Kpn*I-*Sal*I fragment (probe B), and a distal 1.3kb *Eco*RI-*Not*I fragment (probe C).

Secondly, we decided to screen additional tumor panels to obtain more information about the frequency of insertions and the locations of proviral integrations in the *bla1* locus. Therefore Southern blots with *Eco*RI- and *Eco*RV-digested tumor DNA's isolated from MoMLV-induced lymphomas of wild type mice (T cell lymphomas), Eμ-*myc*, Eμ-*myc/pim1*$^{-/-}$*;pim2*$^{-/-}$ and Eμ-*myc/bmi1*$^{+/-}$ compound mice (pre-B and T cell lymphomas), and H2K-*myc* transgenic mice (T cell lymphomas) were analyzed with probe B (Fig. 2). We found that the *bla1* locus was a target for proviral insertions only in B cell and not T cell lymphomas of Eμ-*myc* single and compound transgenic mice (ranging from 8 to 15%) (Table 1). No integrations were detected in a large panel of T cell lymphomas derived from wild type (n=104) or H2K-*myc* transgenic mice (n=69). Similar lineage specificity is seen for *bmi1* integrations. Furthermore, it became evident that all retroviral integrations mapped in a region of 4kb, which roughly coincided with the endogenous *Eco*RI fragment (Fig.1).

Thirdly, we wanted to assess in which oncogenic complementation group *bla1* could be categorized. Our previous analysis led to the identification of three distinct complementation groups in primary MoMLV-induced lymphomas, consisting of *myc*, *pim* and *bmi1/gfi1* (Scheijen et al. 1997; Berns et al. 1999). Each group harbors either homologous genes, like c-*myc* and N-*myc*, or gene products assumed to function in a similar signaling pathway, like for *pim1*, c-*kit* and *tpl2*. The nuclear proteins Bmi1 and Gfi1 have been assigned to the same oncogenic complementation group based on the following findings. MoMLV-induced T cell lymphomas in Eμ-pp-*bmi1* transgenic mice contain in only a very small fraction insertions near *gfi1* (8% versus 44% in wild type mice) (Alkema et al. 1997). In addition, crosses between Eμ-pp-*bmi1* and Eμ-pp-*gfi1* mice reveal no significant synergy in lymphomagenesis (Scheijen and Berns, unpublished results).

However, proviral insertions near *gfi1*, within the *eis1/gfi1/pal1/evi5* locus (Scheijen et al. 1997), and *bmi1* in Eμ-*myc* pre-B cell lymphomas are not absolute mutually exclusive. As much as 25% (10/40) of the lymphomas with proviral insertions near *bmi1* also contain MoMLV integration in the *eis1/gfi/pal1/evi5* lo-

Table 1. Frequency of different common integration loci in B- and T cell lymphomas

		Common proviral insertion sites		
Genotype	Tumor phenotype	*bmi1*	*bla1*	*gfi1/pal1/evi5*
Wild type	T cell	0/104 (0%)	0/104 (0%)	14/32 (44%)
Eμ-*myc*	B and T cell[a]	26/49 (49%)	8/53 (15%)	8/34 (24%)
Eμ-*myc*/*pim1*$^{-/-}$;*pim2*$^{-/-}$	B and T cell	9/36 (25%)	4/36 (11%)	13/36 (36%)
Eμ-*myc*/*bmi1*$^{+/-}$	B and T cell	12/37 (32%)	4/52 (8%)	11/37 (30%)
H-2K-*myc*	T cell	0/62 (0%)	0/62 (0%)	44/59 (75%)

[a]Eμ-*myc* mice (transgenic line 186) develop after MoMLV infection B cell (75-90%) and T cell lymphomas (10-25%). Proviral integrations in *bmi1* and *bla1* loci are only detected in B cell and not in T cell tumors.

cus, arguing that maybe other genes in the vicinity of *gfi1* are implicated in the onset MoMLV-induced B cell lymphomas. Alternatively, activation of *bmi1* in the context of Eμ-*myc* transgene expression, provides additional cooperative function(s), which are not provided by Gfi1 action, like inhibition of c-*myc* induced apoptosis in pre-B cells (Jacobs et al. 1999b; Scheijen et al. submitted). For comparison, the overlap between *bmi1* and *gfi1* is still far less than observed for co-integrations near *pim* and *bmi1*, which is 50% (20/40). The analysis performed on *bla1* indicated that pre-B cell lymphomas with proviral integration in the *bla1* locus, showed no insertions near *bmi1*, whereas 20% (2/10) had retroviral integrations in the *eis1/gfi1/pal1/evi5* locus. This fraction was significant lower than the observed overlap of 75% (6/8) between *pim* and *bla1* (data not shown). Therefore we concluded that *bla1* most likely belongs to the *bmi1/gfi1* complementation group.

Positional mapping using an interspecific mouse backcross analysis with progeny derived from matings of (C57BL/6J x *Mus spretus*)F_1 mice with C57BL/6J, indicated that *bla1* was located at the same position as *bmi1* on mouse chromosome 2 (N. Copeland, personal communication). Since *bla1* integrations were mutually

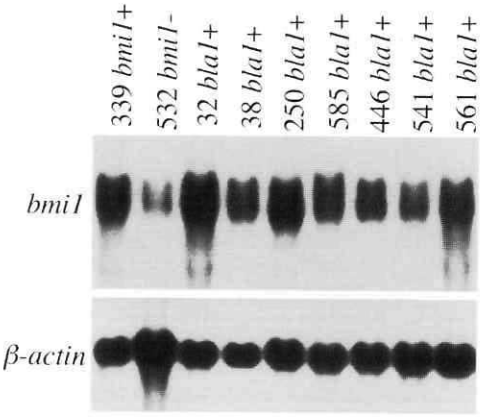

Figure 3. B cell lymphomas with proviral integration in the *bla1* locus display increased *bmi1* expression. Total RNA isolated from various B cell lymphomas containing proviral insertions in the *bla1* locus, was analyzed by Northern blotting, to detect expression of *bmi1*. To confirm upregulation of *bmi1* mRNA levels, two control samples were included, derived from a B cell lymphoma without any activation of *bmi1* expression (tumor 532), and a tumor with an insertion near the *bmi1* gene (tumor 339). For each sample 15μg total RNA was loaded on 1%-agarose/paraformaldehyde-containing gel. After electrophoresis the RNA was blotted onto nitrocellulose filter, and hybridized to *bmi1* cDNA probe, followed by *β-actin* probe using standard conditions as described before (Scheijen et al., 1997).

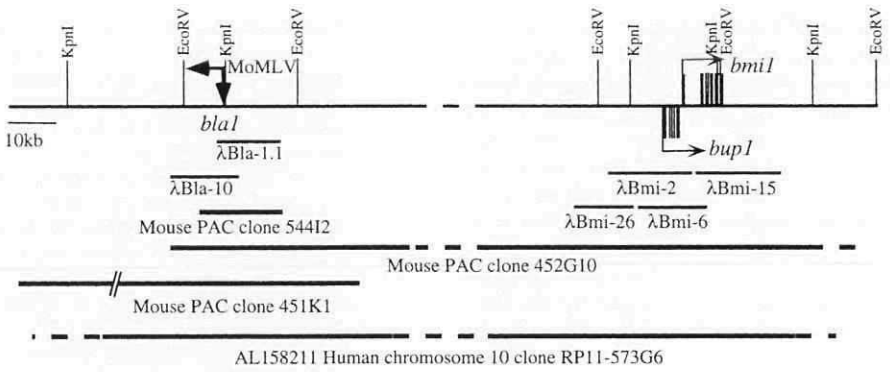

Figure 4. Schematic representation of the genomic localization of *bla1* and *bmi1*. Four overlapping genomic phage lambda clones covering a region of 45kb, harbor the murine *bmi1* and *bup1* genes on mouse chromosome 2. The transcriptional orientation of the two genes is indicated. Three different mouse PAC clones were isolated from C57BL6 genomic PAC library (from Rosewell Park Cancer Institute) using *bla1* probe B, of which one clone (452G10) ±180 kb in size, showed cross hybridization with *bmi1* cDNA probe. Mapping indicated that *bla1* is between 60 and 170kb proximal to *bmi1*. The orientation of the integrated MoMLV proviruses at the *bla1* locus is opposite with respect to the transcriptional orientation of *bmi1*. A similar genomic organization was deduced from the partial sequenced human genome database, where accession clone AL158211 on human chromosome 10 (192 kb in size) contains both human *BMI1* and a region homologous in sequence with mouse genomic *bla1*.

exclusive with *bmi1*, we tested the hypothesis that proviral insertions in the *bla1* locus might activate the *bmi1* gene. For this purpose, total RNA isolated from *bla1*$^+$ B cell lymphomas was checked for *bmi1* expression. It was evident that all tumors analyzed showed at least 4-fold higher mRNA levels than tumors with no *bmi1* activation, and was comparable to the induction seen in tumors with integrations in the near vicinity of *bmi1* (Fig. 3).

To establish whether the *bla1* locus was in close proximity to *bmi1*, we checked if individual lambda phage clones covering 45kb of genomic *bmi1* sequence, which also included the closely located *bup1* gene (Haupt et al. 1991), showed overlap with the *bla1* lambda phage clones. This was not the case. The *bup1* gene is apparently not a target for proviral activation in B cell lymphomas with MoMLV integration near *bmi1* (M. Alkema and M. van Lohuizen, personal communication). Subsequently, a mouse B6 genomic PAC library was screened with probe B derived from the *bla1* locus and *bmi1* cDNA, to obtain genomic fragments that allowed coverage of the gap between the two loci. One PAC clone of ±180kb (452G10) was positive for both probes (Fig. 4). Control hybridizations revealed no indications that this PAC clone was rearranged. Additional mapping pinpointed *bla1* proximal to the *bmi1* gene. The majority of the proviral insertions in the *bla1* locus had the opposite orientation with respect to the transcriptional start site of *bmi1* (Fig. 4). To confirm the localization of mouse *bla1* with respect to *bmi1*, the sequence database of the human genome project was consulted. Reasonable genomic sequence homology between different stretches of mouse and human *bla1*, allowed the identification of human chromosome 10 clone RP11-573G6, which is 192kb in size and also harbored the human *BMI1* gene (Fig. 4) (Alkema et al. 1993).

All together our results strongly suggest that proviral insertions in the *bla1* locus activate *bmi1* over a large distance. The estimated distance between the retroviral insertions in the *bla1* locus and the transcription start site of *bmi1* is at least 60 kb and maximal 170kb, based on the insert size of the mouse and human genomic PAC clones. The exact mechanism employed by the provirus to achieve long-range activation of *bmi1* transcription in case of retroviral insertion into the *bla1* locus is not clear at this moment. The in-

crease in *bmi1* expression could most likely be explained by direct enhancer activation in cis, like has been shown for *evi1* and c-*myc*. Short-range (<15kb) and long-range enhancers act mechanistically identical, where by some target specificity enhancer-bound transactivators are juxtaposed to proteins interacting with the proximal promoter, looping out or bending the intervening DNA (Blackwood and Kadonaga 1998). However, alternative models have been proposed to explain long-range action, including the scanning/tracking and linking model (Bulger and Groudine 1999).

Alternatively, proviral integrations within the *bla1* locus cause indirect upregulation of *bmi1* transcription, by affecting the activity of an intermediate gene. Preliminary results indicate that a potential new gene might be located at the 4kb *bla1* integration region, since a few human ESTs map to this location based on the human sequence. Whether proviral insertions in the *bla1* locus alter the transcript size or level of the mouse homologue remains to be established. Since it is known that proviral insertions will occur more preferentially in transcriptionally active regions within the genome, the mere presence of open chromatin structure might have favored this 4kb *bla1* region for retroviral insertions.

Acknowledgments

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