Chapter 16

C-MYB AND LEUKAEMOGENESIS

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Abstract: The c-*myb* proto-oncogene has repeatedly been a target of retroviral insertional mutagenesis in murine and avian haemopoietic neoplasms. The most common mechanism by which avian and murine retroviruses activate c-*myb*'s oncogenic potential is promoter insertional mutagenesis where the viral LTR function replaces the endogenous transcriptional control resulting in constitutive expression of the *myb* mRNA. Another mechanism of activation of c-Myb is achieved through the integration of retroviruses into the 3' region of the c-*myb* locus. The 3' untranslated region is replaced with viral polyA causing an increased stability of the c-*myb* mRNA. In addition, this type of activation results in the carboxyl-terminal truncation of the c-Myb protein providing increased proteolytic stability and transactivation capacity. Several virus integration sites were also mapped within the genomic region surrounding the c-*myb* locus suggesting that retrovirus integrations outside of the coding region can also impose activation via the long-range effect of retroviral regulatory elements.

1. INTRODUCTION

The proto-oncogene c-*myb* encodes a transcription factor that is expressed in cells of the haemopoietic lineage and regulates the transcription of genes involved in proliferation, differentiation and apoptosis. Interest in this gene has been stimulated by the numerous examples of its involvement in haemopoietic neoplasias of the myeloid and lymphoid lineages in animals.

In this review, we will summarise the different modes of oncogenic activation of c-*myb* by retroviral insertional mutagenesis in chickens and mice and their potential role in Myb-induced leukaemogenesis with respect to known functions and modes of regulation of the c-Myb transcription factor. Our emphasis will be primarily on murine model systems. Because

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several other review articles about v-Myb proteins have been published (Introna and Golay, 1999; Lipsick and Wang, 1999), aspects of AMV and E-26 v-Myb induced transformation will be mentioned only briefly, to underscore some unifying mechanistic concepts. A more comprehensive description of its protein structure and molecular properties can be found in several excellent reviews published recently (Ness, 1999; Oh and Reddy, 1999). At the end we also discuss the possible role of the c-Myb oncoprotein in human leukaemia.

2. TRANSFORMATION OF HAEMOPOIETIC CELLS

c-Myb was identified more than 30 years ago through the discovery of the transforming v-Myb protein encoded by two avian retroviruses that induce leukaemia in chickens. The viruses, AMV and E-26, which encode different versions of the c-Myb oncoprotein, transform cells of the myeloid lineage. *In vitro* assays, using cells from tissues rich in haemopoietic progenitor cells, confirmed the role of v-Myb in transformation of myeloid lineage cells (Lipsick and Wang, 1999). Sequence analysis showed that both v-Myb proteins suffered severe truncations at the amino and carboxy-termini. Although there are 10 amino acid substitutions in AMV v-Myb compared to c-Myb, none of them are required for transformation. Some of these substitutions affect the phenotype of the transformed cells (Lipsick and Wang, 1999).

c-*myb* can be activated by retroviral insertional mutagenesis following inoculation of animals with replication competent retroviruses. For a review of insertional mutagenesis see Jonkers and Berns, 1996. In the animals, viral DNA integrates randomly into the genomic DNA during its life cycle and the mutagenic effects of the integrated provirus on c-*myb* are selected due to a growth advantage conferred by the virus. These experimentally induced leukaemias in chickens and mice have facilitated our identification of the alterations in c-*myb* gene and its protein product that lead to transformation of haemopoietic cells.

In chickens, insertional mutagenesis at the c-*myb* locus has been associated with lymphoid neoplasms. Inoculation of embryos with either RAV-1 or EU-8 results in rapid induction of B-cell lymphomas that have activated c-*myb* (Kanter et al., 1988; Piser and Humphries, 1989; Piser et al., 1992; Press et al., 1995). In addition, activation of c-*myb* by a RAV-1 provirus was discovered in an avian T-cell lymphoma cell line derived from chickens inoculated with Marek's disease virus (Le Rouzic and Perbal, 1996).

In mice, c-*myb* is activated with high frequency in a model for promonocytic leukaemia due to integration of Moloney murine leukaemia virus (M-MuLV), Friend MuLV or amphotropic virus 4070A (Wolff, 1996). These leukaemias are often referred to as MML for murine myeloid leukaemias. In this model, leukaemia develops in virus-inoculated susceptible strains of mice undergoing a pristane-induced chronic inflammation in the peritoneal cavity (Wolff et al., 1988; Wolff et al., 1991). There is a rather long latency for disease development in this animal model that suggests there is a multi-step process in the transformation of myeloid cells. Activated c-*myb,* therefore, must collaborate with other oncogenic events. Intriguingly, leukaemias with a mutagenised c-*myb* locus develop only in mice that have the inflammatory granuloma induced by pristane. In its absence, virus-inoculated mice do not develop leukaemia, despite the fact that, in haemopoietic tissues, 100% of animals have detectable viral *gag-myb* transcripts, shown to be a consequence of proviral integration in the c-*myb* locus (Nason-Burchenal and Wolff, 1992; Nason-Burchenal and Wolff, 1993). These results also suggest that, in this model, c-*myb* activation is probably one of the first oncogenic events in disease progression, because cells expressing aberrant *myb* messages are detected within the first 2-3 weeks following virus inoculation and can be found in the bone marrow and spleen (Nason-Burchenal and Wolff, 1993). Interestingly, expression of the aberrant fusion message is also detected in the thymus, but lymphomas never develop (Belli et al., 1995); it is believed this is due to a lack of a sufficient number of cooperating events. Thus, putative mutagenic events involved in progression of the promonocytic leukaemia seem to be strictly tissue-specific. Promonocytic leukaemias, induced by retroviruses in mice with activated c-*myb* expression can be adapted to growth *in vitro* in the absence of growth factors (Wolff et al., 1988) although initial growth is facilitated with the addition of granulocyte-macrophage colony stimulating factor (GM-CSF).

In other experimental murine models involving retroviruses, the c-*myb* region has been identified as a target of insertional mutagenesis. These include myeloid leukaemias induced by Cas-Br-M (Shen-Ong et al., 1986; Joosten et al., 2002) and myeloid leukaemias in BXH2 mice (Blaydes et al., 2001).

Similar to the avian system, a transformation assay for murine Myb has been developed (Gonda et al., 1993; MacMillan and Gonda, 1994; Ferrao et al., 1995). Murine retroviruses expressing wild-type c-Myb or carboxy-terminally truncated c-Myb can transform foetal liver cells, but only in the presence of required growth factors. Transformed cells have morphological characteristics of myeloid progenitors and respond to growth factors such as GM-CSF and interleukin-3 (IL-3). Interestingly, these experiments also

showed that under specific experimental conditions including growth at high density, enforced expression of full-length c-Myb has transforming potential (Ferrao et al.1995).

Although most of the *myb* related leukaemias studied in mice have been myeloid, a T-cell lymphoma model involving Myb was reported by Kathy Weston and colleagues. In v-Myb transgenic mice, with T-cell-specific expression, high-grade T-cell lymphomas develop in older animals (Badiani et al., 1996).

3. MECHANISMS THAT ACTIVATE MYB'S ONCOGENIC POTENTIAL

Experiments from many laboratories have shown that truncation of either end of the c-Myb protein contributes to increased transforming potential (Lipsick and Wang, 1999). During activation of c-*myb* by retroviral insertional mutagenesis, for example, the protein is truncated on or other end depending on the location of the provirus (Figure 1). In addition, regulatory LTR sequences of the provirus enhance or promote transcription or cause termination of transcription. In the following sections, we will discuss in more detail the alterations caused by integrated proviruses in the c-*myb* gene as well as their impact on regulation of the transcription factor.

3.1 Activation of c-Myb by Retroviral Integration into the 5' End of the Gene

Proviruses are found at the 5' end of c-*myb* in at least 95% of murine promonocytic leukaemias, which are induced by the combination of replication competent virus and pristane, and in an equally high percentage of avian retrovirus-induced B-cell lymphomas. The integration sites are found in the first, second, or third introns. In these leukaemias transcription of aberrant c-*myb* mRNA is initiated in the retroviral 5' LTR. Read-through c-*myb* transcripts, in both the murine and avian neoplasms, are spliced. In the mouse promonocytic leukaemias, the splice sites utilised a cryptic *gag* donor splice site and one of the normal splice acceptors of c-*myb* at the next available exon (Shen-Ong and Wolff, 1987). In the chicken lymphomas, splicing is similar except a normal donor splice site in *gag* is utilised instead of a cryptic donor site (Kanter et al., 1988).

Interestingly, integrated proviruses at the 5' end of c-*myb* are positioned in the genome in a manner that allows a transcriptional pause site to be bypassed (Mukhopadhyaya and Wolff, 1992; Piser et al., 1992; Jiang et al., 1997). It should be emphasised that the normal down-regulation of c-*myb*

expression observed during maturation of haemopoietic cells does not occur at the level of transcriptional initiation, but rather at the level of transcriptional elongation. The endogenous c-*myb* promoter does not have a TATA motif, and transcription appears to initiate at multiple sites within a CpG island located in the promoter region (Bender and Kuehl, 1986; Dvorak et al., 1989). This type of TATA-less promoter is usually characterised by low-level, constitutive activity and is not subject to rapid transcription factor-induced regulation arising from extracellular signals. Therefore, the transcriptional pause site within the first intron is an important and major mechanism of regulation of c-*myb* expression (Watson, 1988; Reddy and Reddy, 1989; Wang et al., 1994). The bypassing of the c-*myb* elongation block is likely to be the most critical event in the transformation process, because it permits escape from maturation-associated down-regulation of c-*myb* expression. In addition, the viral LTR provides strong promoter/enhancer elements that contribute to the constitutive transcription. As shown in *in vitro* studies, this constitutive expression of c-Myb provides continued growth of cells even during the G-CSF induced 32D cells differentiation to the granulocyte lineage and IL-6-induced macrophage maturation (Bies et al., 1995; Bies et al., 1996). Interestingly, there was no difference observed between truncated and full-length protein in these experiments, emphasising the role of inappropriate expression in transformation (Selvakumaran et al., 1992; Bies et al., 1995). Similarly, others found that under specific conditions, enforced expression of full-length c-Myb transforms foetal liver cells (Ferrao et al., 1995).

The observations that proviruses cause constitutive expression of c-Myb in leukaemic cells that have undergone insertional mutagenesis and that full length c-Myb can block differentiation and induce transformation *in vitro*, might suggest that mutation of c-*myb* alleles is not required for Myb-specific transformation. However, leukaemia-associated c-*myb* alleles with proviral integrations at the 5' end, always undergone some alteration in sequences affecting the amino-terminal coding region of c-Myb (Mukhopadhyaya and Wolff, 1992, Piser et al., 1992; Jiang et al., 1997), and it is, therefore, possible that these alterations do facilitate the transformation of haemopoietic cells. In murine promonocytic leukaemias, the most common sites of integration are located in introns 2 and 3 with removal of 47 or 71 amino acids from amino-terminus of c-Myb, respectively (Figure 1). More rarely integrations have been found in intron 1 and result in truncation of 20 amino acids (Shen-Ong and Wolff, 1987; Mukhopadhyaya and Wolff, 1992). This is in contrast to chicken B-cell tumours, where the most common integration site is intron 1 (Piser et al., 1992). All of these deletions remove the conserved acidic region at the amino-terminus including phosphorylation sites Ser 11 and 12 (Figure 1).

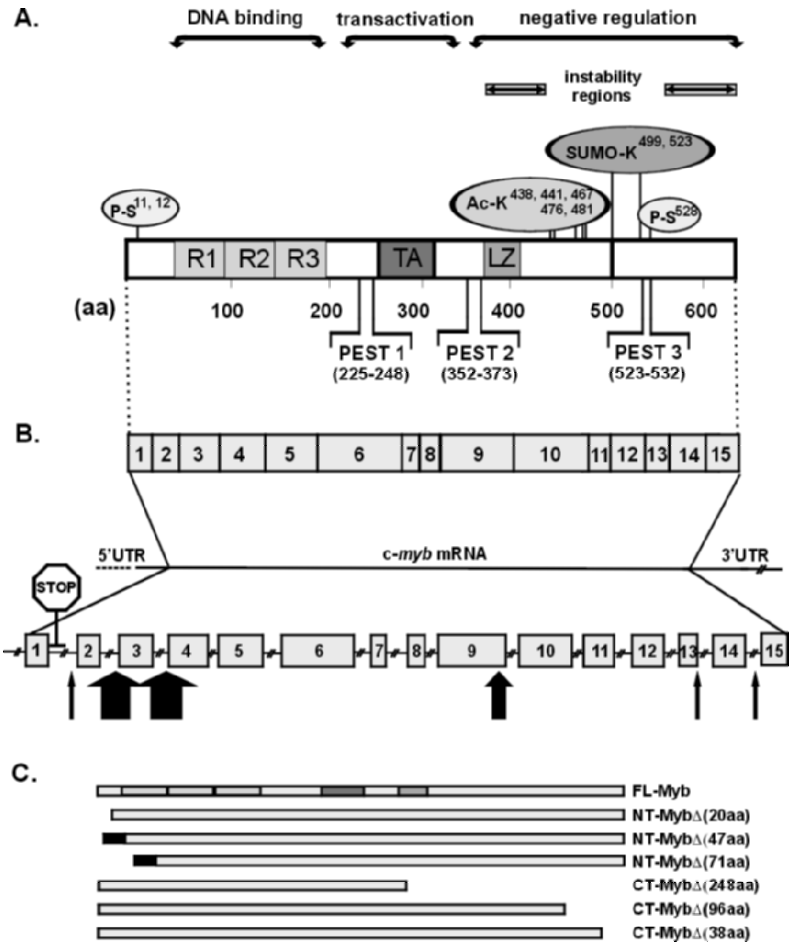

Figure 1

(A) Structure of the c-Myb protein: R1, R2, R3, imperfect tandem repeats composing the DNA-binding domain; TA, acidic and highly hydrophilic domain that is part of the transactivation region; LZ, putative leucine zipper structure, PEST1, 2, and 3 regions identified by PEST-FIND program. Posttranslational modification of c-Myb: SUMO-K, SUMO-1-conjugated lysines; Ac-K, acetylated lysines; and P-S, phosphorylated serines. (B) Activation of the c-Myb by retroviral integration into c-myb locus. Schematic diagram of the genomic structure of c-myb with transcription pause site (STOP sign), mRNA, 5' and 3' untranslated regions (UTRs), and exon structure. Black arrows represent locations of proviruses identified in murine myeloid leukaemias (MML); thickness of arrows reflects frequency of integration sites in MML. (C) Wild type and truncated forms of the protein detected in MML. Black regions in NT-MybΔ(47aa) and NT-MybΔ(71aa) represent viral Gag protein sequences.

Phosphorylation of both serines by casein kinaseII (CKII) was implicated in the negative regulation of DNA-binding affinity of c-Myb (Lüscher et al.,

1990; Oelgeschlager et al., 1995). Truncation of c-Myb by 47 or 71 amino acids removes part of the R1 repeat of the DNA binding domain. While the R1 repeat is not involved in direct contact of c-Myb with DNA, it was suggested that this repeat can either affect DNA-binding affinity of c-Myb (Dini and Lipsick, 1993) or it can facilitate intramolecular interaction with the carboxy-terminus (Dash et al., 1996). More recent studies with murine proteins with similar size amino-terminal truncations revealed that the most extreme truncation of the amino-terminus disrupted the activation of chromatin embedded target genes in collaboration with the transcription factor C/EBPβ (Oelgeschlager et al., 2001). This result suggests a new role for the R1 repeat domain in cooperation with other transcription factors in the transcriptional regulation of resident genes in intact chromatin. In addition, Oelgeschlager and coauthors also suggested an inverse correlation between activation of chromatin embedded genes and leukaemogenic potential of the amino-terminally truncated Myb proteins (Oelgeschlager et al., 2001). Since the products of studied genes are associated with the differentiation process rather than with proliferation, the inability of some oncogenic forms of c-Myb to activate these types of genes may to some extent enhance the transforming capability of the c-Myb proteins.

At present, the only evidence that an amino-terminal truncation is in itself oncogenic was provided by experiments in chickens where embryos were infected with retroviral vectors expressing wild type c-Myb or c-Myb truncated by 20 amino acids. This truncated protein produced a high incidence of rapid onset tumours while the wild-type c-Myb was only weakly oncogenic (Jiang et al., 1997).

3.2 Activation of c-Myb by Retroviral Integration into the Middle or 3' End of the Gene

Integration of retroviruses into the middle or at the 3' end of the c-*myb* gene has been found less frequently than integration at the 5' end (Wolff, 1996) and only in murine myeloid leukaemias. The most frequent site of virus integration in this category is within exon 9. The virus LTR at this site causes premature termination of transcription and translation of truncated c-*myb* RNA is terminated at stop codon within the LTR sequence. This produces a c-Myb protein that is severely truncated at the carboxy-terminus by 240 to 248 amino acids. Examples of myeloid leukaemia cell lines with these truncations are a myelomonocytic leukaemia cell line NFS60 (Shen-Ong et al., 1986) and a promonocytic leukaemic cell line R1-4-11 (Mukhopadhyaya and Wolff, 1992). A very similar truncation was also detected in an IL-3-dependent cell line, VFLJ2, generated *in vitro* by retroviral infection (Weinstein et al., 1987). Additional integration sites in c-

myb were also reported and include sites in exon 13 and the intron 14. These integrations result in carboxy-terminal truncations by 96 and 38 amino acids, respectively (Nazarov and Wolff, 1995; Bies et al., 1999).

3.2.1 mRNA stabilisation

Integration of viral promoter/enhancer sequences into the 3' end of the c-*myb* gene could potentially affect the endogenous enhancer elements and transcriptional pause site, thus mimicking the effects of proviruses integrated at the 5'end of the gene (see above). However, we were unable to show that integration of retroviruses in the down-stream region could prevent down-regulation of c-myb expression in cells treated with differentiation-inducing agents (Haviernik et al., 2002). Therefore, viruses must employ a different strategy to keep sufficient levels of c-Myb to prevent growth arrest in these cells when they are exposed to differentiation-inducing cytokines *in vivo*. Changes in the stability of mRNA or protein might provide sufficient steady-state levels of c-Myb to keep cells in a proliferative state until additional mutagenic events occur that promote progression of leukaemia.

c-*myb* mRNA in myeloid cells has a short half-life of around 45 minutes (Figure 2) and its turnover can be rapidly accelerated during the initial stages of differentiation (Watson, 1988). Decay of many unstable mRNAs is controlled via *cis*-acting structural elements, located in the 3' untranslated region (3'-UTR). These elements can bind *trans*-acting factors and accelerate mRNA decay. The best characterised *cis*-acting destabilising elements in 3'-UTR are AU-rich elements (ARE) found in many short-lived mRNAs (Guhaniyogi and Brewer, 2001). The most common AREs found in unstable mRNAs consist of multiple pentamers of AUUUA, or AU- or U-rich elements (Mitchell and Tollervey, 2000). c-*myb* mRNA contains five copies of the AUUUA sequence in its 3'UTR (Figure 2).

Interestingly, integration of a retrovirus into the 3' end of the c-*myb* locus causes aberrant termination of c-*myb* transcription in 5'LTR and replaces the endogenous c-*myb* 3'-UTR containing AREs with viral LTR sequences. This leads to the dramatic increase in the stability of c-*myb* RNAs. The half-life of c-*myb* mRNA in M1 cells is around 45 minutes, while the half-lives of aberrant c-myb mRNAs in cell lines RI-4-11 (provirus integration in exon 9) and 45-16 (integration in exon 13) is substantially longer. As shown in Figure 2, we detected only 20% of full-length c-*myb* mRNA in M1 cells treated with actinomycin D for 120 minutes, while we measured 90% of the two oncogenic mRNAs forms in RI-4-11 and 45-16 cells. Importantly, the half-life of c-*myc* mRNA in all three cell lines was very similar, suggesting that the dramatic difference of c-*myb* mRNA stability is indeed an intrinsic feature and is not cell line-specific.

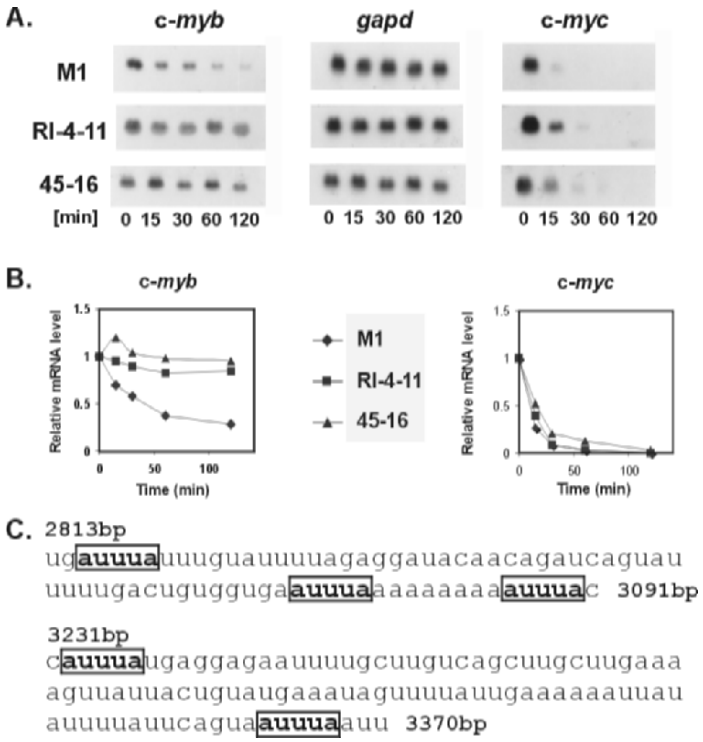

Figure 2

Stabilisation of c-*myb* mRNA as a consequence of proviral integration into 3' end region. (A) Cell lines expressing wild type c-*myb* (M1) as well as *myb* mRNAs truncated at the 3' end due to retrovirus integration (RI-4-11 - provirus integrated into exon 9; 45-16 - provirus integrated into exon 13) were cultivated in the presence of the RNA synthesis inhibitor actinomycin D (5µg/ml) for the indicated times. Isolated RNA samples were analyzed by northern blotting using radioactively labelled cDNAs for murine c-*myb*, c-*myc* and visualised by radiography. Hybridisation with a GAPDH probe was used as a control for loading and the integrity of RNA samples. (B) Quantitative analysis was performed on a PhosphoImager 425 using IMAGEQUANT software. Levels of c-*myb* and c-*myc* at each time point were normalised to GAPDH level and plotted on the graph. Each point represents the relative amount of either c-*myb* or c-*myc* mRNA at different times. RNA levels were assigned to 100% at the beginning of actinomycin D treatment. (C) c-*myb* 3' UTR removed from oncogenic *myb* RNAs due to integration of proviruses into the 3' end of c-*myb* gene. Destabilising heptamers sequences "auuua" are boxed.

3.2.2 Protein stabilisation

Removal of the 3'end of the c-*myb* gene causes not only loss of sequences important for targetting mRNA for decay, but it also removes protein sequences important for targetting the protein for degradation. We

have shown that c-Myb is a very unstable protein rapidly degraded by the ubiquitin/26S proteasome pathway (Bies and Wolff, 1997). Attachment of polyubiquitin chains serves as a recognition signal for 26S proteasome machinery ultimately leading to proteolysis. Recently we found that the targetting of the protein to the proteasome depends on Ser/Thr phosphorylation (Bies et al., 2000; Bies et al., 2001). The previously identified phosphorylation sites, Ser 11, 12 and 528, however, are not involved. We located two independent instability determinants, one in the extreme carboxy-terminus and one overlapping the putative leucine zipper (Bies et al., 1999). The ubiquitin/26S proteasome pathway is the only proteolytic system described for tightly controlling the amount of c-Myb in cells. The half-life does not change during proliferation and differentiation of myeloid cells (Feikova et al., 2000) and we have not detected a situation where degradation of c-Myb is induced. This suggests that its turnover is a constitutive process. However, the fact that inhibition of Ser/Thr protein phosphatases rapidly causes hyperphosphorylation-induced conformational changes in the carboxy-terminus and accelerated proteolytic breakdown of c-Myb, suggests that there may be a signal transduction pathway that regulates proteolysis of this protein.

As mentioned above, deletion of the carboxy-terminal negative regulatory domain of c-Myb results in protein stabilisation. Evidence suggests that carboxy-terminally truncated proteins are less efficiently ubiquitinated and, therefore, have increased resistance to degradation (Bies and Wolff, 1997). c-Myb is not the only protein found to be activated to become oncogencic by stabilisation. Examples of other transcription factors where stablisation occurs in conjunction with oncogenic activation are c-Myc, c-Fos and c-Jun (Hershko and Ciechanover, 1998). Therefore, stabilisation of proteins with oncogenic potential may be a common mechanism for increasing transforming potential.

3.2.3 Increased transactivation

Carboxy-terminal truncation of c-Myb increases transforming activity (Gonda et al., 1989; Grasser et al., 1991; Ferrao et al., 1995) and this is related, in part, to the fact that the carboxy-terminal portion of the protein, beyond the transactivation domain, negatively regulates c-Myb activity as a transcription factor. Early studies with carboxy-terminal deletion mutants suggested that carboxy-terminal negative regulatory domain (NRD) decreases the transactivation capacity (Sakura et al., 1989; Kalkbrenner et al.1990; Hu et al., 1991), as well as the DNA-binding affinity of c-Myb (Ramsay et al., 1991; Tanaka et al., 1997). Although removal of protein instability determinants in the NRD alone may account in part for the

observed increases in transactivation upon removal of the NRD, removal of other regulatory elements are likely to account for these increases in activity as well.

The NRD of c-Myb is subjected to post-translational modifications. Phosphorylation of serine 528 by MAPK kinase or other cellular proline-directed kinase has been implicated in the negative regulation of c-Myb transactivation on some promoters without affecting DNA-binding affinity of Myb (Aziz et al., 1995; Miglarese et al., 1996).

Recently, we described a post-translational modification of c-Myb by a ubiquitin-like protein SUMO-1. We showed that sumolation negatively regulates c-Myb transactivation and increases its stability (Bies et al., 2002). SUMO-1 is covalently attached to the NRD of c-Myb on lysine residues 499 and 523 and another lysine residue that has not been specifically identified. Sumolation of Lys523 is necessary for subsequent covalent attachment of a second molecule of SUMO-1 to Lys499, and modification of both Lys523 and Lys499 is required for conjugation of the third molecule of SUMO-1 to the unidentified lysine (Bies et al., 2002). It is assumed that sumolation of the first Lys523 in c-Myb can affect conformation of the carboxy-terminus so that it allows covalent attachment of SUMO-1 to other lysines. We provided evidence for the involvement of SUMO-1 in negative regulation of transactivation, using the K523R mutant that is completely deficient in sumolation. This mutant was shown to have an increased transactivation capacity on a Myb-responsive promoter. One proposed mechanism by which SUMO-1 could inhibit transactivation is by steric hindrance of acetylation at the carboxy-terminus of c-Myb (Figure 1), as it was reported that acetylation of several lysine residues by p300/CBP in carboxy-terminus can positively regulate its transactivation capacity (Tomita et al., 2000; Sano and Ishii, 2001).

Several other components of the NRD with a specific role in negative regulation of c-Myb have been identified. First, a leucine zipper-like (LZ) sequence, located in a region spanning amino acids 375-403, has negative regulatory activity. Deletion or point mutation of the LZ increased transactivation and transforming activity of c-Myb (Kanei-Ishii et al., 1992). It was proposed that the negative activity of the leucine zipper is due to the binding of specific inhibitor proteins. The first inhibitor that was suggested was c-Myb itself. It was shown that the leucine zipper-like sequence of c-Myb is capable of forming homodimers *in vitro* (Nomura et al., 1993). However, there is no evidence so far for the presence of full-length c-Myb homodimers in cells. Use of a GST-LZ-Myb pull-down assay led to the identification and molecular characterisation of a c-Myb binding protein p67/p160. It was shown that p67 (the amino-terminal portion of p160 protein) represses transactivation of c-Myb (Tavner et al., 1998). The LZ

can be interrupted by alternative splicing and the alternatively spliced form identified in many normal and tumour cell lines encodes a protein with increased transactivation activity (Shen-Ong, 1987; Woo et al., 1998). Interestingly, the LZ structure also overlaps with one of the two instability regions identified in the negative regulatory domain suggesting its role in proteolytic processing (Bies at al., 1999).

Two regions within the c-Myb NRD have been shown to be important in the negative regulation of DNA-binding affinity and this effect would be predicted to influence transactivation (Tanaka et al., 1997). A region containing the PEST3/EVES motif within the NRD was shown to be capable of interacting with the DNA-binding domain (Dash et al., 1996). The p100 protein contains an identical EVES motif through which it can also interact with the DNA binding domain of c-Myb (Dash et al., 1996), and compete with the EVES region binding (Ness, 1999). Therefore, transactivation may be inhibited by the intramolecular interaction and activated by binding of the p100. More recently, an adenovirus E1A-associated protein BS69, was shown to interact with carboxy-terminus of c-Myb and inhibited its transactivation capacity (Ladendorff et al., 2001).

Thus, deletion of the negative regulatory domain, which is observed frequently in activated forms of c-Myb, increases stability of truncated proteins as well as facilitates an escape from multiple negative regulations imposed by post-translational modifications and protein-protein interactions.

4. RETROVIRUSES INTEGRATED 30-100 KB UPSTREAM AND DOWNSTREAM OF THE C-MYB TRANSCRIPTIONAL UNIT

In murine and feline leukaemias, sites of integrated proviruses have been mapped as far as 25-100 kbp upstream or downstream of the c-*myb* gene transcriptional unit on chromosome 10. *Mml1*, *Mml2, and Mml3* are sites that have been identified in our laboratory in myeloid promonocytic leukaemias and map 25-70 kbp upstream of the c-*myb* locus (Koller et al., 1996, Haviernik et al., 2002) (Figure 3). Mapping even further upstream, by approximately 30 kbp, is another feline leukaemia virus-common integration site in thymic lymphomas *Fit-1* (*Fti1*) (Tsujimoto et al., 1993; Hanlon et al., 2003). Analysis of the region encompassing these proviruses did not reveal the presence of any gene, but led to the identification of two potential scaffold (matrix) attachment regions (SARs/MARs) (Haviernik et al., 2002). SARs/MARs are sites where chromatin attaches to the nuclear matrix. These sequences play an important role in organising and regulating nuclear processes including transcription (Deppert, 2000). The important regulatory

function of the nuclear matrix makes it a likely target for structural alteration during neoplastic transformation.

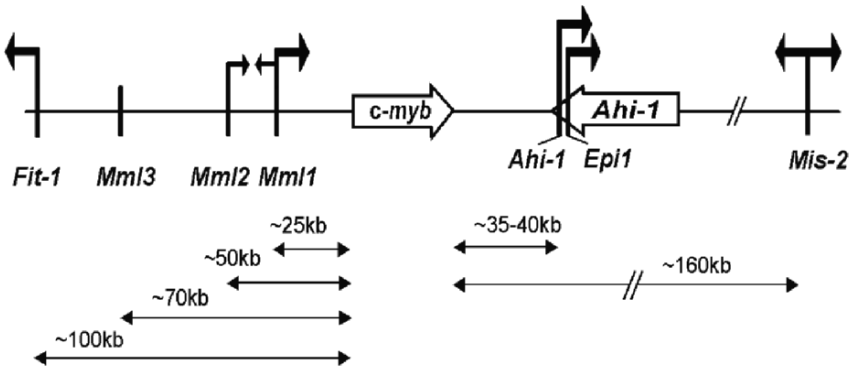

Figure 3

Common integration sites upstream and downstream of c-myb transcription unit. Positions of proviruses in genomic DNA on mouse chromosome 10 surrounding c-myb and Ahi-1 genes are marked by vertical black lines. Open arrows show the orientation of the two genes. The orientations of the proviruses at different sites are showed by black arrows above the integration sites, and the approximate distance of common integration sites from c-myb gene are marked under the diagram.

There is evidence to suggest that alterations in chromatin may be important in cancer. For example, the transforming potential of simian virus 40 (SV40) large T antigen is closely associated with perturbation of chromatin structure and changes in nuclear architecture (Malyapa et al., 1996). Also, oncoproteins with an A/T hook domain can bind directly to A/T rich sequences in SARs. The mixed lineage leukaemia (MLL) protein is an example of a protein with oncogenic potential and it contains an A/T hook domain. Competitive binding of proteins like MLL and nuclear matrix for binding to MARs could modulate chromatin structure and ultimately lead to altered regulation of gene expression (Caslini et al., 2000). Interestingly, the gene that encodes MLL is frequently involved in translocations in human acute myeloid and lymphoid leukaemias and the translocations encode fusion proteins between the SAR binding portion of MLL and other protein domains.

It is conceivable that integration of the proviral genome into regions that bind nuclear matrix could also perturb normal regulation of gene expression and ultimately lead to cancer. Because no new transcription units were found in the upstream genomic region, the most probable candidate affected by these proviruses remains to be c-*myb* itself (Haviernik et al., 2002). Although, promonocytic leukaemias with provirus in *Mml1* do not always express c-*myb*, it is possible that c-Myb expression was crucial for

leukaemia progression, but only during earlier stages of development. Development of promonocytic leukaemia may involve an early immature stage in which regulation of c-*myb* transcription requires the upstream region. A recent paper by Hanlon and colleagues strongly indicates that c-*myb* is a key target gene affected by long-range transcriptional activation (Hanlon et al., 2003). They suggest that c-*myb* expression may become dispensable during cultivation of cells *in vitro* or *in vivo* during the progression of virus-induced leukaemias in mice.

Two other common murine leukaemia virus insertion loci, *Ahi-1* (Jiang et al., 1994) and *Mis-2* (Villeneuve et al., 1993), were mapped approximately 35 and 160 kbp downstream of c-*myb* in murine lymphomas. Recently, a somatically acquired common retroviral integration site, *Epi1,* located just 30-40 kbp downstream of c-*myb* was described in murine myeloid leukaemias in BXH2 mice (Blaydes et al., 2001) and may overlap *Ahi-1* sites. Although these proviruses are integrated at the end of a recently described gene *Ahi-1* (Jiang et al., 2002), this gene does not seem to be over-expressed in association with retrovirus integration. Therefore, it remains possible that downstream proviruses affect c-*myb* expression as well. Interestingly, proviruses found in *Epi1* and *Ahi-1* were integrated in the same transcriptional orientation as the c-*myb* gene, and this observation is in complete agreement with the theory of viral enhancement, where the enhancer in 5' LTR activates a gene located upstream of provirus integration site (Jonkers and Berns, 1996). Northern analyses did not reveal increased c-*myb* expression in tumours with viruses integrated in *Epi1*. However, it is possible c-*myb* plays a role in leukaemogenesis in these tumours during an earlier stage of development as suggested by Hanlon and colleagues (Hanlon et al., 2003).

That c-*myb* is a target of retrovirus activation in leukaemia has been further emphasised in high throughput retroviral tagging of genes (Joosten et al., 2002; Lund et al., 2002; Mikkers et al., 2002; Suzuki et al., 2002). The c-*myb* locus was among the most frequently targetted genes identified in genome-wide screenings involving murine myeloid and lymphoid leukaemias. Whether these sites of integrated proviruses are located within or outside the immediate c-*myb* transcriptional unit has not been reported. However, this further implicates for c-Myb as having a crucial role in the induction of these diseases.

5. C-MYB TARGET GENES IMPLICATED IN MYELOID CELL TRANSFORMATION

The ability of c-Myb to both promote transcription and transform cells (Lane et al., 1990; Kanei-Ishii et al., 1992) indicates that it induces myeloid and lymphoid leukaemia through activation of specific target genes. An increasing amount of evidence from the past two decades has confirmed a direct role of c-Myb in regulation of cellular processes such as proliferation, differentiation and programmed cell death (Wolff, 1996; Oh and Reddy, 1999). Several Myb-target genes with "leukaemogenic" potential have been identified and will be briefly discussed.

A gene regulated by c-Myb that is critical to its transforming capacity and involved in the regulation of the cell cycle is c-*myc*. Initially, it was shown that the c-*myc* promoter is responsive to c-Myb (Cogswell et al., 1993; Evans et al., 1990; Zobel et al., 1992) with its highest level of activity in myeloid cells. However, until recently there was a lack of evidence that expression of the endogenous c-*myc* promoter is controlled by this transcription factor. Studies employing conditional expression of Myb and dominant negative forms of Myb demonstrated that the resident chromosomal c-*myc* gene is regulated directly by c-Myb in myeloid leukaemic cells (Schmidt et al., 2000, Wolff et al., 2001, Chen et al., 2002).

Other genes, proposed to be targets of c-Myb and essential for proliferation of haemopoietic cells, include p34*cdc2* (Ku et al., 1993), DNA topoisomerase IIα (Brandt et al., 1997), c-*kit* (Hogg et al., 1997; Ratajczak et al., 1998 Vandenbark et al., 1996) and *cyclinA1* (Muller et al., 1999).

More recently, it was reported that c-Myb inhibits the expression of the proposed tumour suppressor gene $p15^{INK4b}$, which encodes an inhibitor of cyclin dependent kinases cdk4/6 (Schmidt et al., 2001). Ectopic expression either full-length c-Myb or its truncated forms prevented the induction of $p15^{INK4b}$ mRNA in M1 cells during interleukin-6-induced monocytic differentiation. The effect of c-Myb on $p15^{INK4b}$ expression appears to be indirect and not due to the action of c-Myb on the Ink4b promoter (our own unpublished data). However, this function of c-Myb is probably an important mechanism in the transformation process, because the majority of c-Myb-induced tumours (in contrast to Myc-induced monocytic tumours) do not express this gene (Schmidt et al., 2001). Therefore, the inhibition of a growth arrest pathway involved in monocyte differentiation is another mechanism by which c-Myb promotes proliferation.

Modulation of programmed cell death by c-Myb represents another important mechanism involved in transformation of haemopoietic cells by Myb. Several groups identified *bcl-2* gene, which encodes an anti-apoptotic protein as a direct target for the c-Myb transcription factor in lymphoid and

myeloid cells (Taylor et al., 1996; Frampton et al., 1996; Schmidt et al., 2001), and placed c-Myb oncoprotein into a family of transcription factors with "survival" function. It is important to note, however, that under some conditions c-Myb can actually promote apoptosis rather than prevent it (Selvakumaran et al., 1994, Sala et al., 1996).

Based on the evidence mentioned above, transformation of haemopoietic cells by c-Myb seems to be achieved through modulation of at least two distinct pathways, one involving proliferation, and one related to programmed cell death. Therefore, deregulation of c-Myb via oncogenic activation, which increases its stability or its ability to transactivate genes, plays an important role maintaining cell cycle progression and preventing programmed cell death. Since c-Myb induces c-*myc* expression and this oncogene is known to activate the p53 pathway leading to apoptosis, it is to c-Myb's advantage as an oncogene to counteract c-Myc's anti-tumour effects by preventing c-Myc induction of apoptosis.

6. C-MYB AND HUMAN LEUKAEMIA

The human c-*myb* oncogene is located on chromosome 6q22-24 (Harper et al., 1983). Abnormalities at this locus, such as amplification or deletion, have been observed in leukaemic cells with over-expression of unaltered c-*myb* (Pelicci et al., 1984; Barletta et al., 1987; Okada et al., 1990). The only activated form of c-Myb in human leukaemia detected to date was a carboxy-terminally truncated c-Myb (Tomita et al., 1998). It was observed in the TK-6 cell line, which was established from a patient with chronic myelogenous leukaemia and resulted from a large deletion in the chromosome. This abnormality was associated with late progression, because it was acquired after T-cell blast crisis. A recent screening for activating mutations in the negative regulatory domain of c-Myb in patients with myeloid leukaemia did not reveal any abnormalities (Lutwyche et al., 2001). Therefore, so far it is not clear whether full length c-Myb over-expression or timing of expression, due to altered regulation, plays a role in human leukaemia.

Although little evidence of c-Myb's involvement in leukaemia has been reported so far there are ways one may envision that the transcription factor could positively affect transformation of human haemopoietic cells. The ideas are based on what we have learned from animal model systems. It is clear that c-Myb is required for proliferation of haemopoietic cells and the only examples of proliferating haemopoietic cells that lack c-Myb expression, as far as we know, are those with deregulated c-Myc. The first mechanism to consider would be a disturbance in the abundance of the

protein. Alterations in the abundance of critical proteins are frequently observed defects in cancer cells. Among the primary mechanisms used by cells to adjust protein concentrations are gene dosage, mRNA abundance, and protein stability. We have shown that increased RNA and protein stability significantly contribute to increased levels of c-Myb in murine myeloid leukaemia. Interestingly, increased stability of c-*myb* RNA was observed in patients with acute myeloid leukaemia (Baer et al., 1992). In addition, microsatellite deletions in the c-*myb* transcriptional attenuator located in the first intron was associated with overexpression of normal c-Myb in colon carcinomas providing more evidence for an oncogenic potential of deregulated full-length c-Myb (Thompson et al., 1997). In regard to mRNA regulation in animal models, several retroviral integrations at a distance from the c-*myb* locus have been hypothesised to positively affect transcription (Koller et al., 1996, Haviernik et al., 2002, Hanlon et al., 2003). This leaves open the possibility that alterations that disturb these distal chromosomal regulatory mechanisms, could affect transformation of human cells as well. Alterations that cause changes in abundance may be subtle and difficult to detect, however, at present, they cannot be ruled out. The second mechanism to consider, based on evidence in animal models, is an alteration that would increase directly or indirectly c-Myb's transactivation potential. Several post-translational modifications such as phosphorylation, acetylation and sumolation are involved in the control of c-Myb activity and/or proteolytic processing. Altered regulation of these pathways in leukaemic cells could theoretically result in potentiating c-Myb's oncogenic ability to drive proliferation and prevent apoptosis.

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