The c-myb proto-oncogene product (c-Myb) regulates proliferation of hematopoietic cells by inducing the transcription of a group of target genes. Removal or mutations of the negative regulatory domain (NRD) in the C-terminal half of c-Myb leads to increased trans-activating capacity and oncogenic activation. Here we report that TIF1β directly binds to the NRD and negatively regulates the c-Myb-dependent trans-activation. In addition, three corepressors (Ski, N-CoR, and mSin3A) bind to the DNA-binding domain of c-Myb together with TIF1β and recruit the histone deacetylase complex to c-Myb. Furthermore, the Drosophila TIF1β homolog, Bonus, negatively regulates Drosophila Myb activity. The Ski corepressor competes with the coactivator CBP for binding to c-Myb, indicating that the selection of coactivators and corepressors is a key event for c-Myb-dependent transcription. Mutations or deletion of the NRD of c-Myb and the mutations found in the DNA-binding domain (DBD) in the N-terminal region of c-Myb lead to increased interaction with these corepressors and weaken the corepressor-induced negative regulation of Myb activity. These observations have conceptual implications for understanding how the nuclear oncogene is activated.

The c-myb proto-oncogene is the cellular progenitor of the v-myb oncogenes carried by the chicken retroviruses avian myeloblastosis virus (AMV) and E26, which cause acute myeloblastic leukemia or erythroblastosis (1, 2). The level of c-myb expression is high in immature hematopoietic cells, and its expression is turned off during terminal differentiation (3). c-myb-deficient mice show a defect in definitive hematopoiesis in the fetal liver due to a severe reduction in the number of progenitor cells, indicating that c-myb is essential for the proliferation of immature hematopoietic cells (4). Analysis of homozygous null c-myb/Rag1 chimeric mice indicates that c-myb is also essential for early T-cell development (5). The myb gene is well conserved not only in vertebrates but also in other species. Drosophila melanogaster has one myb gene (dmyb), which is required in diverse cellular lineages throughout the course of development (6).

The c-myb gene product (c-Myb) is a transcriptional activator that recognizes the specific DNA sequence 5'-AACNG-3' (7–10). Some of the c-Myb target genes, including c-myc, are required for the G2/S transition in the cell cycle (11, 12). In contrast, dmyb is required for the G2/M transition (6), and cyclin B expression is directly regulated by Dmyb (13). Several other target genes, including mim-1, GBX2, and bcl-2, are involved in lineage commitment in differentiation and blockage of apoptosis (10, 14–16). c-Myb has three functional domains that are responsible for DNA binding, transcriptional activation, and negative regulation (8). The DNA-binding domain (DBD) in the N-terminal region of c-Myb consists of three imperfect tandem repeats of 51–52 amino acids, each containing a helix-turn-helix variation motif. Repeats 2 and 3 (R2 and R3) are sufficient for binding to the target DNA sequence (17). The transcriptional activation domain is adjacent to the DBD, to which the transcriptional coactivator CBP binds (18).

Analysis of various oncogenically activated myb genes suggests that truncation of either the N or C terminus of c-Myb can cause oncogenic activation. For example, the v-Myb protein encoded by AMV is N- and C-terminally truncated versions of c-Myb (1). Deletion of the negative regulatory domain (NRD) located in the C-terminal portion of the molecule increases both the trans-activation and transformation capacity of c-Myb, implying that the NRD normally represses c-Myb activity (8, 19–21). The v-Myb encoded by AMV lacks the C-proximal region of the NRD. In addition, the mutations of only the leucine-rich region in the NRD result in oncogenic activation of c-mycb (22). Thus, the NRD appears to contain multiple subdomains, and the deletion of any of these may result in the oncogenic activation of c-mycb. However, the mechanism by which c-Myb is regulated by NRD still unclear.

The ski gene is also a nuclear oncogene. The products of the c-ski proto-oncogene and its related gene sno (ski-related novel) (c-Ski and Sno) directly bind to two other corepressors, N-CoR/SMRT and mSin3A, and act as transcriptional corepressors (23). mSin3A and N-CoR/SMRT also interact with each other (24–26) and form macromolecular complexes with class I and II histone deacetylase (HDAC), respectively (27–29). All three corepressors (Ski/Sno, mSin3A, and N-CoR/SMRT) are required for transcriptional repression by Mad and non-ligated thyroid hormone receptor β (30, 23–26), suggesting that different corepressor-HDAC complexes interact with each other and mediate transcriptional repression together. c-Ski also directly binds to other multiple transcription factors, including Smads.
and Gli3, and mediates transcriptional repression or inhibits transcriptional activation (31–33).

Here, we demonstrate that four corepressors, including c-Ski, directly bind to c-Myb via multiple domains in the c-Myb molecule to negatively regulate c-Myb activity. Deletions or mutations of the NRD or the point mutations found in v-Myb reduces the affinity with these corepressors, leading to increased c-Myb activity. Thus, our results suggest that selection of coactivators or corepressors is a key event for oncogenic activation of c-Myb.

MATERIALS AND METHODS

Yeast Two-hybrid Screening and in Vitro Binding Assays—The yeast two-hybrid screening was performed using the mouse embryonic cDNA library as described previously (23). The protein containing the C-terminal 312 amino acids of mouse c-Myb was used as bait. GST pull-down assays were performed as described previously (23). To increase the solubility of GST fusion proteins expressed in bacteria, the thioredoxin coexpression system (34) was used. The binding buffer used for most of the experiments consists of 20 mM Hepes, pH 7.5, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 100 mM KCl (for interactions between Myb and mSin3A or N-CoR) or 150 mM KCl (for interactions between Myb and c-Ski). The binding buffer used for the experiments with the R23 fragment consists of 50 mM phosphate buffer, pH 6.8, 20 mM dithiothreitol, and 100 mM KCl.

Coimmunoprecipitation and HDAC Assay—For coimmunoprecipitation of endogenous proteins, lysates were prepared from Molt-4 cells by mild sonication in NET buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Nonidet P-40, protease inhibitor mixture) containing 150 mM NaCl. Anti-c-Myb monoclonal antibody 1-1, the rabbit anti-N-CoR antibody (23), the anti-mSin3A antibody (Santa Cruz Biotechnology, AK-11), or normal rabbit IgG were used for immunoprecipitation. The immunocomplexes were used in Western blotting with rabbit anti-TIF1β polyclonal antibodies raised against GST-TIF1βC, anti-c-Ski monoclonal antibody, rabbit anti-mSin3A antibody (Santa Cruz Biotechnology, AK-11), or anti-c-Myb monoclonal antibody 1-1. To study the interaction between c-Ski and various forms of c-Myb, 293 cells were cotransfected via the CaPO4 method with the c-Ski expression plasmid and treated with antibody against each corepressor. The immunocomplexes were used in Western blotting with rabbit anti-TIF1β monoclonal antibody. Assays for HDAC activity were performed essentially as described (23) using lysates prepared from 293 cells that were transfected with 10 μg of the c-Myb expression plasmid.

Analysis of Repressor Domains in c-Myb—The cytomegalovirus promoter was used to express the Gal4-c-Myb fusion proteins consisting of the Gal4 DNA-binding domain fused to various portions of c-Myb. CV-1 cells were transfected with a mixture of 3 μg of the luciferase reporter containing the TK promoter and six Gal4-binding sites, 0.33 μg of the Gal4-c-Myb or Gal4 expression plasmids, and 1 μg of the internal control plasmid pRL-TK (Promega). The luciferase assays were performed using the dual-luciferase assay system (Promega).

Subcellular Localization of c-Myb and Corepressors—CV-1 cells were transfected with a mixture of 1.5 μg of the FLAG-c-Myb expression plasmid and 1.5 μg of the plasmids that express c-Ski, mSin3A, or N-CoR. Forty hours after transfection, cells were fixed and stained as described (23) with anti-c-Myb, anti-c-Ski, and anti-FLAG antibodies. The proteins of different immunoreactive profiles were visualized by rhodamine- and fluorescein isothiocyanate-conjugated secondary antibodies and analyzed by confocal microscopy.

Chromatin Immunoprecipitation Assays—The retroviral expression plasmids for wild-type c-Myb or CT3 were constructed using the MSCV (murine stem cell virus)-based retroviral vector, and viruses were prepared (37). To generate TIF1β M1 cell clones that express c-Myb together with the neomycin resistance gene, M1 cells were infected with viruses and then grown in the presence of G418 (400 μg/ml). ChIP assay was carried out essentially by using the method of Weinmann and Farnham (36). In brief, 1.5 × 10⁶ M1 cells were fixed with 1% formaldehyde for 10 min at room temperature. Nuclei were isolated and suspended in nuclear shearing buffer (50 mM HEPES, pH 8.1, 10 mM EDTA, 1% SDS, protease inhibitors) and sonicated. After the centrifugation, the supernatant was diluted with IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HEC, pH 8.1, 167 mM NaCl) and treated with antibody against each corepressor. The immunocomplexes were collected and was incubated at 65 °C with IP elution buffer (50 mM NaHCO₃, 1% SDS) to release the proteins and DNA complex. DNA was extracted and used for PCR. PCR reaction (94 °C for 45 s, 55 °C for 30 s, and 72 °C for 3 min) was carried out with [3²P]dCTP for 30 cycles. PCR products were analyzed by electrophoresis on an 8% polyacrylamide gel. The primers used for the amplification of myc promoter were as follows: 786 GTGCGGACTCAATAACTGTA 205 and 1106 GGCAGTGAGCCGACGAG 1424

Luciferase Reporter Assays—In the experiments using the reporter containing multiple Myb-binding sites, CV-1 cells (2 × 10⁶ cells per 60-mm dish) were cotransfected using LipofectAMINE Plus (Invitrogen) with the 6MBS-I-SV40-luc reporter (0.2 μg), the c-Myb expression plasmid (0.03 μg), the corepressor expression plasmid (0.5 or 1 μg), and the internal control plasmid pRL-TK (0.05 μg), followed by luciferase assays. The chicken β-actin promoter was used to express c-Myb and various corepressors. In the case of assays using the 6MBS-I-TK-luc reporter (1 μg), the plasmid to express c-Myb (0.1 μg), or the v-Myb (0.03 μg) was used together with the same amounts of other plasmids as described above. The dominant negative form of the TIF1β expression plasmid was constructed by inserting the DNA fragment encoding the RBCC motifs and the artificial nuclear localization signal into the β-actin promoter-based vector. In the experiments using the reporter containing the c-myc promoter, CV-1 cells (4 × 10⁶ cells per 100-mm dish) were cotransfected with the CaPO₄ method with the myc-CAT reporter (4 μg) (11), the c-Myb expression plasmid (4 μg), the corepressor expression plasmid (0.5 or 2.5 μg), and the internal control plasmid pact-β-gal (0.5 μg), followed by luciferase assays. The total amount of plasmid DNA was adjusted to 12 μg by adding the control plasmid DNA lacking the cDNA.

Genetic Interaction between Bon and dMyb—Two alleles of dmyb mutants were described previously (13). The bon21β and bon487β mutations (37) were provided by H. J. Bellon. Imaginal discs were dissected from late third-stage larvae, fixed, and stained with the anti-Cy3B antibody (gift from C. Lehner) as described (13). For analysis of the lethal stages, dmybβ1057 (hypomorph) and dmybβ hypomorph) were balanced by FM7cY and bon21β (amorph) (57) was balanced by TM6B Tb, dmybβ1057/FM7cY and dmybβ/FM7cY females were mated with males of dmybβ1057/Tb, dmybβ1057/bon21B, and dmybβ/Tb. Egg laying was grown at 25 °C, and the males of third instar larvae hemizygous for dmyb and FM7c Ybac were selected by the size of the gonads embedded in the opaque fat body. Furthermore, the male larvae were separated by the presence or the absence of the y+ cuticular phenotypes. The bon21β chromosome was isolated by the absence of the Tb phenotype. The viability was calculated as a standard value from the number of male larvae hemizygous for FM7cY or FM7cYbon21β-1. Results

TIF1β Binds to the NRD of c-Myb—To identify putative inhibitors that bind to the NRD of c-Myb, we performed yeast two-hybrid screening using the NRD of mouse c-Myb as bait. This resulted in the identification of clones encoding TIF1β (also called as KAP-1). TIF1β, which contains a RING finger, B boxes, a coiled-coil region, and a plant homeodomain (PHD) finger, was originally identified by two groups as a protein that binds to the heterochromatin protein-1 (HP-1) (38) or to the KRAB repression domain (39). TIF1β was subsequently found to act as a corepressor that associates with the HDAC complex and HP-1 (40, 41). The TIF1β clones isolated in our screening encoded the 92-amino acid region that includes the B1 box (Fig. 1A). In vitro translated c-Myb efficiently bound to the GST-TIF1βN protein containing the N-terminal half of TIF1β, but not to the GST-TIF1βC protein that contains the C-terminal half of TIF1β (Fig. 1A, lower middle panel). The bacterially expressed recombinant NRD also bound to the GST-TIF1βN protein (Fig. 1A, lower right panel).

We then performed GST pull-down assays using in vitro translated TIF1β and GST-NRD fusion proteins that contain the NRD of c-Myb. TIF1β efficiently bound to the GST-NRD, but mutation of the leucine-rich region (L34P) dramatically decreased but did not completely abrogate the affinity with TIF1β (Fig. 1B, lower left panel, see also Supplementary Fig. 1B for GST-NRD proteins). We also performed GST pull-down assays using GST-TIF1βN and a series of in vitro translated...
C-terminally truncated forms of c-Myb bearing the mutation L34P in the leucine-rich region. The c-Myb protein truncated up to amino acid 500 still retained the capacity to interact with TIF1β, but truncation up to amino acid 444 completely abrogated binding, indicating that TIF1β binds to the region between amino acids 444 and 500 (Fig. 1B, lower right panel). Thus, TIF1β interacts with c-Myb at both the leucine-rich region and a C-terminal region in the NRD. Supporting this is that TIF1β binds to AMV-v-Myb, which lacks the C-terminal region of the NRD, with lower affinity than to c-Myb (Fig. 1B).

**Binding of c-Myb with Multiple Corepressors**—A recent study on corepressors demonstrates that multiple corepressors bind to the same transcriptional factor. This raises the possibility that several other corepressors may bind to c-Myb together with TIF1β. To investigate this, we analyzed the interaction between c-Myb and three other known corepressors, c-Ski, N-CoR, and mSin3A. All three in vitro translated proteins bound to GST-c-Myb (Fig. 2A). In vitro translated mSin3A and c-Ski also bound to GST-TIF1βN (Fig. 2A). We previously demonstrated that c-Ski binds to both N-CoR and mSin3A (23). Thus, N-CoR, mSin3A, and TIF1β can all bind to c-Ski. Given that the four corepressors can interact with each other, these results suggest that these corepressors bind to c-Myb simultaneously or sequentially (see "Discussion").

To investigate the in vivo interaction between c-Myb and the corepressors, we performed the coimmunoprecipitation experiments using Molt-4 cell lysates (Fig. 2B). The anti-Myb antibody precipitated TIF1β, c-Ski, and mSin3A. Furthermore, c-Myb and TIF1β were coprecipitated using the anti-N-CoR antibodies. The control IgG precipitated none of the corepressors or c-Myb. We then asked whether the c-Myb complex contains HDAC activity. We used anti-Myb to generate the immunocomplexes from 293 cells that had been transfected with the c-Myb expression plasmid and assessed their HDAC
activity. A significant level of HDAC activity was observed compared with the immunocomplexes prepared with control IgG (Fig. 2C). The immunocomplexes of the c-Myb mutant containing the mutated leucine-rich region in the NRD (L34P) had slightly lower HDAC activity than those containing wild-type c-Myb (Fig. 2C). These observations are consistent with the fact that TIF1β only partly interacts with c-Myb through the leucine-rich region.

Using various forms of in vitro translated c-Myb, we determined which of the one or more regions of c-Myb is bound by c-Ski (Fig. 3A). The results obtained using GST-Ski fusion proteins indicate that c-Ski binds to the DBD of c-Myb (CT5), but not to the mutant lacking the DBD (ΔDB) (Supplementary Fig. 1, data are summarized in Fig. 3A). The region containing only R2 and R3 (R23) was sufficient for c-Ski interaction. Interestingly, removal of the NRD (CT3) or mutations in the leucine-rich region (L34A and L34P) almost completely abrogated the interaction with c-Ski, although c-Ski does not bind to NRD. It may be that the mutations or removal of NRD alters the conformation of some of the regions such as the transactivating domain, which then blocks access to c-Ski.

We then performed GST pull-down assays using various forms of in vitro translated c-Myb and GST-mSin3A-NCT. The latter protein contains the central region of mSin3A bearing the second and third putative amphipathic helical domains (PAH2 and PAH3). This is the region responsible for binding to c-Myb (Supplementary Fig. 2A). As with c-Ski, mSin3A binds to R2 and R3 of c-Myb (Supplementary Fig. 2B, and data are summarized in Fig. 3A). The mutations of leucine-rich region of the NRD (L34A and L34P) also partly decrease the affinity with mSin3A, but removal of the NRD (CT3) does not affect the affinity with mSin3A. To determine which region of c-Myb interacts with N-CoR, similar assays were performed. c-Myb binds to the N-terminal 427-amino acid region of N-CoR (Supplementary Fig. 3A), and when GST-N-CoR, which contains this region, was used in GST pull-down assays, N-CoR was found to interact with R2 and R3 of c-Myb (Supplementary Fig. 3B, and data are summarized in Fig. 3A). However, the
loss of NRD (CT3) or mutations of the leucine-rich region (L34A and L34P) did not affect the interaction of N-CoR with c-Myb.

To confirm that the three corepressors directly bind to the DBD of c-Myb, GST pull-down assays were performed using the bacterially expressed c-Myb protein containing only the three DBD repeats (R123) and the c-Ski, mSin3A, or N-CoR GST fusion proteins. The R123 protein containing only the three repeats from the c-Myb DBD was incubated with GST fusion proteins containing c-Ski, mSin3A, or N-CoR, or GST alone as a control, and mixed with glutathione-Sepharose. After washing, bound proteins were eluted and analyzed by SDS-PAGE followed by Coomassie Blue staining.

**Fig. 3.** Direct binding of corepressors to the DBD of c-Myb. A, identification of the corepressor-binding regions in c-Myb. The binding of various forms of *in vitro* translated c-Myb to GST resin bearing c-Ski, mSin3A, or N-CoR is shown (Supplementary Figs. 1–3). The various c-Myb constructs used are indicated, and the results of binding assays are summarized on the right. ND, not determined. B, c-Ski, mSin3A, and N-CoR directly bind to the DBD of c-Myb. The R123 protein containing only the three repeats from the c-Myb DBD was incubated with GST fusion proteins containing c-Ski, mSin3A, or N-CoR, or GST alone as a control, and mixed with glutathione-Sepharose. After washing, bound proteins were eluted and analyzed by SDS-PAGE followed by Coomassie Blue staining.

**Negative Regulation of c-Myb**

To confirm that the three corepressors directly bind to the DBD of c-Myb, GST pull-down assays were performed using the bacterially expressed c-Myb protein containing only the three DBD repeats (R123) and the c-Ski, mSin3A, or N-CoR GST fusion proteins. The R123 proteins bound directly to these GST-corepressor fusions (Fig. 3B). Thus, the three corepressors interact directly with the DBD.

**c-Ski Abrogates the Interaction between c-Myb and CBP**—The binding of c-Myb with the corepressor-HDAC complexes suggests that c-Myb bears a transcriptional repressor domain. We thus examined the repressor activities of fusion proteins containing the NRD (NRD) or the c-Myb mutant lacking the transcriptional activation domain (∆TA) significantly repressed the activity of the Gal4 site-containing promoter. The Gal4-NRD fusion containing mutations of the leucine-rich region had a slightly lower repressor activity than that containing the normal NRD (Fig. 4A). These results are consistent with our observations that the corepressors c-Ski, mSin3A, and N-CoR interact with c-Myb via the DBD and the corepressor TIF1β interacts via the NRD. Furthermore, because we know that the mutation of the leucine-rich region partly and completely disrupts the interaction with TIF1β and c-Ski, respectively, this may explain why L34P had lower repressor activity than the wild type NRD.

That the three corepressors c-Ski, mSin3A, and N-CoR all bind to the DBD of c-Myb suggests the corepressors could bind
to c-Myb simultaneously or that they compete in their c-Myb binding. To discriminate these possibilities, we investigated the binding of in vitro translated [35S]mSin3A to a small amount of GST-c-Myb resin in the presence of increasing amounts of in vitro translated c-Ski (Fig. 4B). The addition of c-Ski to the binding reaction did not decrease the amount of mSin3A bound to GST-c-Myb, suggesting that these corepressors do not compete in their binding to the c-Myb DBD. Given that the four corepressors can interact with each other, these results suggest that the corepressors bind to c-Myb simultaneously or sequentially (see “Discussion”).

For the c-Myb-mediated transcriptional activation, CBP must bind to the transcriptional activation domain of c-Myb. The simultaneous binding of the four corepressors to c-Myb may mask the surface of the c-Myb protein and thereby block CBP interaction with c-Myb. To examine this hypothesis, we investigated whether the corepressors compete with CBP for binding to c-Myb (Fig. 4C). Thus, the binding of in vitro translated c-Myb to the GST-CBP fusion protein, which contains the Myb-binding domain (KIX) of the CBP molecule, was measured in the presence of an increasing amount of c-Ski translated in vitro. Wild-type c-Ski inhibited the interaction between CBP and c-Myb. Binding of in vitro translated c-Myb to GST-CBP was examined in the presence of increasing amounts of either wild-type c-Ski or mutant c-Ski lacking its C-terminal region (Δ493–728).

Loss of the NRD Lowers the Affinity between c-Ski and c-Myb—c-Ski and N-CoR have been reported to colocalize to dot-like domains in the nuclei (23). To confirm that c-Myb associates with the corepressors in vivo, we investigated whether c-Myb colocalizes with the corepressors. When c-Myb was coexpressed with c-Ski, N-CoR, or mSin3A in transfected CV-1 cells, most of the c-Myb signals were colocalized with the corepressors in dot-like structures (Fig. 5A). However, when c-Ski was coexpressed with the c-Myb mutant lacking the NRD (CT3) or containing a mutation in the leucine-rich region (L34P), the nuclear punctate structures of c-Myb were disrupted, resulting in c-Ski being mainly localized at the peripheral region in the nuclei (Fig. 5B). In these cells, c-Myb formed the nuclear dot-like structures, and the c-Ski and c-Myb signals did not overlap. Thus, removal of the NRD of c-Myb disrupts an interaction with c-Ski.

To confirm the effect of deleting the NRD on the interaction of c-Myb with c-Ski, coimmunoprecipitation experiments were performed (Fig. 5C). 293 cells were transfected with the plasmids that express c-Ski and the FLAG-linked wild-type or mutant forms of c-Myb. Lysates were prepared and used for coimmunoprecipitation. Anti-FLAG antibody coprecipitated c-Ski with wild-type c-Myb, but only a small amount of c-Ski was coprecipitated with the CT3 mutant that lacks the NRD. Although a significant amount of c-Ski was coprecipitated with L34P mutant, which has a mutant leucine-rich region, the number of c-Ski molecules per L34P molecule precipitated was still less than that with wild-type c-Myb. Thus, loss of the NRD or mutations of the leucine-rich region in the NRD decrease the affinity of c-Myb with c-Ski.

We then examined the recruitment of the corepressors by c-Myb to the c-myc gene promoter by chromatin immunoprecipitation (ChIP) assays. It was shown that the transcription of c-myc gene is directly regulated by c-Myb in a myeloblastic cell line, M1 (12). In addition, we previously demonstrated that the recombinant c-Myb proteins directly bind to the multiple sites in the c-myc promoter region (11). We generated M1 cell clones that constitutively express wild-type c-Myb or the CT3 mutant as well as control clones containing the empty vector by using...
the retrovirus vector (Fig. 5D). Anti-c-Myb antibody precipitated the c-myc promoter DNA fragment of the control M1 cells, which contains multiple Myb-bindings sites (Fig. 5E, left panels), and other regions that contain no Myb-binding sites were not precipitated (data not shown). These results indicate that the c-Myb proteins bind to the c-myc promoter region in M1 cells. Overexpression of wild-type or CT3 c-Myb increased the amounts of c-myc fragment precipitated by anti-Myb antibody (Fig. 5E, left panels), suggesting that the number of c-Myb molecules that bound to the c-myc promoter increased in Myb-overexpressing M1 cells. The antibodies against Ski, mSin3A, or N-CoR precipitated a small or undetectable amount of c-myc DNA fragment of the control M1 cells (Fig. 5E, right panels). Overexpression of wild-type c-Myb induced an increase in the occupancy by Ski and N-CoR of the c-myc gene promoter (Fig. 5E, right panels). Because c-Myb binds to either the corepressors or the coactivators, some of c-Myb molecules on the c-myc promoter probably interact with the corepressors. Therefore, an increase in the c-Myb molecule on the c-myc promoter leads to an increase in the corepressor molecule on the c-myc promoter. However, the amounts of mSin3A on the c-myc promoter were not dramatically increased by overexpression of wild-type c-Myb, suggesting that enough of the mSin3A occupies the c-myc promoter via the endogenous c-Myb. Furthermore, the amounts of c-Ski, N-CoR, and mSin3A recruited by the overexpressed CT3 mutant to the c-myc promoter were less than those by overexpressed wild-type c-Myb (Fig. 5E, right panels). These results suggest that CT3 has less affinity with these corepressors than wild-type c-Myb. Thus, loss of the NRD decreases the affinity of c-Myb with the corepressors on the c-myc promoter.

**Negative Regulation of c-Myb-dependent Transcriptional Activation by Corepressors**—We then investigated the effect of the corepressors on c-Myb-mediated activation of a promoter that contains the c-myc-binding sites (Fig. 6). CV-1 cells were cotransfected with the luciferase reporter containing the SV40 promoter linked to six tandem c-Myb-binding sites (MBS-I). c-Myb stimulated luciferase expression 6.2-fold. This c-Myb-mediated activation was inhibited by mSin3A and N-CoR in a
The dominant negative form of TIF1α/H9252/ or N-CoR/H11006/4.1 or 1.0/N11001/CT3, or L34P c-Myb and 0.5 (H11002/)/the plasmid expressing wild-type, (BMBS-I-SV40-luc) with (H11001/) or without (BMBS-I-SV40-luc) the SV40 promoter linked to six Myb-sites with the luciferase reporter bearing the thymidine kinase promoter. CV-1 cells were transfected on the trans-activating capacity of c-Myb. mSin3A did not inhibit or only weakly when TIF1α was overexpressed (data not shown), probably because sufficient levels of endogenous proteins were present (Supplementary Fig. 5A). We therefore examined the effect on Myb-mediated activation of the dominant negative form of TIF1α (DN-TIF1α), which contains only the N-terminal RING finger and B boxes (Fig. 6D). DN-TIF1α enhanced the wild-type c-Myb-mediated activation in a dose-dependent manner, but it did not affect the activation mediated by the CT3 mutant.

We also investigated the effect of the corepressors on c-Myb-mediated activation of the human c-myc promoter (Fig. 6E). CV-1 cells were cotransfected with the c-myc-CAT reporter and the c-Myb expression plasmid. CAT expression was enhanced 5.0-fold. Coexpression of mSin3A decreased CAT expression to 80% of the control in a dose-dependent manner. When v-Myb encoded by AMV was used instead of c-Myb, mSin3A inhibited its activity more poorly (to 78% of the control) than when wild-type c-Myb was employed.

Although the less efficient inhibition of the trans-activating capacity of v-Myb by the corepressors may be, at least partly, due to the lack of the C-proximal TIF1α-binding site in v-Myb,
there might be an additional mechanism. Thus, we examined
the effects of point mutations in the DBD of v-Myb on the
interaction with the corepressors. We performed the GST pull-
down assays using the in vitro translated c-Myb fragment
containing only the R2 and R3 of the DBD (R23) under the less
stringent condition compared with that in Fig. 3. The R23
fragment of c-Myb had a higher affinity with GST fusion pro-
teins containing c-Ski, mSin3A, or N-CoR than the R23 con-
taining the three point mutations found in v-Myb (Fig. 6
F). In
the wild-type c-Myb, these three residues are hydrophobic
amino acids on the surface of DBD (42), whereas these residues
are mutated to non-hydrophobic amino acids in v-Myb. These
results suggest that c-Ski, mSin3A, and N-CoR have the lower
affinity with v-Myb than c-Myb due to the point mutations in
R2.

The Drosophila TIF1β Homolog Inhibits Drosophila Myb
Activity—To confirm that TIF1β negatively regulates c-Myb,
we used the Drosophila member of the TIF1β family and c-
Myb, namely, Bonus (Bon) and dMyb. Like TIF1β, the Bonus
protein has RBCC motifs in its N-terminal half, whereas its
bromo domain is preceded by its PHD finger. Bon exhibits 29%
identity (50% similarity) with mouse TIF1β and is thought to
be the Drosophila homolog of TIF1β (37). In the GST pull-down
assays using the GST-dMyb fusion protein and in vitro trans-
lated Bon, Bon bound to GST-dMyb efficiently (Fig. 7
A). Re-
cently, we isolated two alleles of dmyb
mutants, namely,
el1
and
el2507
(13). These two alleles encode 514- and 305-amino
acid proteins. Bon bound to the dMyb 2507 protein but not to
dMyb1
(Fig. 7
A), indicating that, like the vertebrate homolog,
Bon interacts with dMyb via the C-terminal half of dMyb.

We then investigated the genetic interaction between Bon
and dMyb by using their mutants (Fig. 7B). Because the dmyb
gene is on the X chromosome, the dmyb mutation in males
(hemizygotes) was lethal at the first or second larval instar

Fig. 7. Bon, the Drosophila TIF1β homolog, inhibits dMyb activity. A, Bon binds to the C-terminal half of dMyb. The domain structure of Bon and dMyb, and the dMyb proteins encoded by two mutant dmyb alleles, are shown. The binding of in vitro translated Bon to GST-
dMyb was examined. B, viability of progeny at the third instar larval stage. The numbers of viable third instar larvae of the genotypes shown on the left were scored and shown as percentages by the bar graph. C, immunostaining of the eye imaginal disc with anti-cyclin B antibody. Eye discs were prepared from the flies of the genotype shown below. The widths of the cyclin B-expressing cells are indicated by white bars. Anterior is to the left; dorsal is upward.
stage. Upon reaching the third larval instar stage, the mutant larvae died in the ensuing 2–3 days without further growth and development. The mutant larvae displayed obvious morphologic abnormalities as judged by larval cuticle preparations (data not shown). Only 6.9% of the dmyb2507 hemizygotes survived until the third instar larval stage. However, the loss of one copy of bon increased the viable population to 80.8%, suggesting that Bon negatively regulates dMyb activity. In contrast, the loss of one copy of bon did not affect the lethality of dmyb1 hemizygotes. This is consistent with the observation that Bon binds to the dMyb2507 protein but not to the dMyb1 protein.

We found recently that dMyb directly regulates the expression of cycB in eye imaginal discs and the cycB expression in the posterior region of eye discs is lost in dmyb mutant clone cells (13). We observed here that cycB expression is also almost completely lost in the posterior region of the dmyb2507 eye discs (Fig. 7C) but that loss of one copy of bon appeared to recover the cycB expression. Thus, Bon acts as a negative regulator of dMyb.

**DISCUSSION**

The present study demonstrate that four complexes containing each of four corepressors, TIF1β, mSin3A, c-Ski, and N-CoR, directly bind to c-Myb via the NRD and DBD of c-Myb. These corepressor complexes block the trans-activating activity of c-Myb. Removal or mutation of the NRD and the mutations found in the DBD of v-Myb abrogate the interaction of c-Myb with these corepressor complexes, leading to increased c-Myb activity. These observations suggest that the oncogenic activation of c-Myb due to the truncation of the c-Myb C terminus is caused by a loss of the corepressor-dependent negative regulation of c-Myb.

We have demonstrated that four corepressors directly bind to c-Myb. The non-liganded thyroid hormone receptor β (TRβ) also binds to three corepressors (N-CoR/SMRT, mSin3A, and Ski), and a lack of either of these corepressors significantly decreases the repression capacity of TRβ (23, 24, 30). These three corepressors are not the components of the same complex. The purified N-CoR complex contained neither mSin3A nor Ski (28), whereas the purified mSin3A complex involved neither N-CoR nor Ski (27). Therefore, the three different complexes containing either of these three corepressors bind to TRβ. However, it remains unknown whether the binding of these complexes to TRβ occurs simultaneously or sequentially. Similarly, it is unknown whether the four complexes containing either four corepressors (TIF1β, Ski, N-CoR, and mSin3A) bind to
c-Myb simultaneously or sequentially. Because the mSin3A and N-CoR complexes contain the class I and II HDACs, respectively, the recruitment of these different types of HDACs may be needed for efficient transcriptional repression. TIF1β binds to two sites in NRD, namely, the leucine-rich region and additional C-terminal site between amino acids 444 and 500. These results support our previous study indicating the presence of a putative leucine-zipper structure in the NRD, which interacts with some inhibitors (22). It is noteworthy that this region also contains two PXXPh motifs that were recently demonstrated to be critical for interaction between nuclear hormone receptors and their coactivators (49). Other proteins have also been found to bind to the leucine-rich region, namely, p67 and p160 (44). Recently, Laddendorff et al. (45) found that BS69 also binds to the C-terminal region of c-Myb. All of these proteins inhibit the trans-activating capacity of c-Myb. BS69 was originally identified as an adenovirus E1A-associated protein and was recently demonstrated to interact with N-CoR (46). Thus, other factors may also bind to c-Myb together with the four corepressors.

Although c-Ski directly binds to the DBD, not to the NRD, the interaction between c-Ski and c-Myb is almost completely abolished by either the deletion of the NRD or mutations of the leucine-rich region in the NRD. One possible mechanism is that the deletion or mutation of the NRD alters the conformation of the c-Myb protein, thereby causing the transcriptional activation domain to block the interaction between c-Ski and the DBD. mSin3A and N-CoR also directly bind to the DBD of c-Myb. In contrast to c-Ski, however, the deletion of the NRD does not disrupt their binding to c-Myb. However, both corepressors inhibit the trans-activating capacity of the CT3 mutant that lacks NRD less efficiently than they inhibit wild-type c-Myb. This is probably because all of the four complexes containing each corepressor bind simultaneously or sequentially to c-Myb and to each other. Thus, the loss of binding of any complex might result in the decreased interaction of the other corepressor complexes with the NRD and therefore less repression (Fig. 8).

C-Ski and CBP compete with each other for binding to c-Myb (Fig. 8). These observations raise the important questions, what causes c-Myb to be bound selectively by coactivators or corepressors and when does c-Myb act as transcriptional repressor? In the wild-type c-Myb, these three residues are hydrophobic amino acids on the surface of DBD (42). In v-Myb, these residues are mutated to non-hydrophobic amino acids. It is known that these three wild type amino acids are involved not only in interacting with C/EBPβ but also with Cyp-40 (49–51). Thus, these three amino acids are involved in the interaction of c-Myb with various factors, including corepressors.

If the loss of corepressor-mediated negative regulation would result in the oncogenic activation of c-Myb, the corepressors would act as tumor suppressors. Recently, we demonstrated that Ski and its related gene product Sno indeed act as tumor suppressors in mice (52, 35). Ski and Sno are already known to negatively regulate cellular proliferation by mediating the transcriptional repression by the two tumor suppressors Mad and Rb (23, 53). Thus, the negative regulation of c-Myb could be another mechanism by which Ski and Sno act as tumor suppressors.

Acknowledgments—We are grateful to P. Chambon for the TIF1β cDNA (26) and N. Eisenman for the mSin3A cDNA. H. J. Bellen for the Drosophila mob mutants, C. Lehner for the anti-cyclin B antibody, and T. J. Gonda for helpful discussions.

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Teruaki Nomura, Jun Tanikawa, Hiroshi Akimaru, Chie Kanei-Ishii, Emi Ichikawa-Iwata, Md Matiullah Khan, Hiroki Ito and Shunsuke Ishii

J. Biol. Chem. 2004, 279:16715-16726.
doi: 10.1074/jbc.M313069200 originally published online February 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313069200

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