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Two regions in c-*myb* proto-oncogene product negatively regulating its DNA-binding activity

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Abstract The c-*myb* proto-oncogeneproduct (c-Myb) is a transcriptional regulator that binds to the specific DNA sequence. Deletion of the negative regulatory domain (NRD) in the carboxyl-proximal region of c-Myb results in both increased *trans*-activating capacity and oncogenic activation. One possible mechanism to modulate c-Myb activity is a regulation of DNA-binding activity. However, it is not known whether any region in NRD affects the in vivo DNA-binding activity of c-Myb. Using the highly transfectable cell line 293T, we developed a system to precisely measure the DNA-binding activity of Myb expressed in mammalian cells. Using this system, two regions in NRD were shown to repress DNA-binding activity. These results suggest that DNA-binding activity of c-Myb is independently regulated by multiple mechanisms through these subdomains.

Key words: myb proto-oncogene; Functional domain; DNA binding; Negative regulation; Protein–protein interaction

1. Introduction

The *myb* gene was originally identified as an oncogene carried by the chicken retroviruses, avian myeloblastosis virus (AMV) and E26 [1,2], that transform myelomonocytic hematopoietic cells [for review, see [3]]. Homozygous c-*myb* mutant mice are severely anemic and die in utero due to defective fetal hematopoiesis [4], indicating that the *myb* gene product (Myb) plays a critical role in the development of a normal hematopoietic system. c-Myb is a transcriptional regulator that binds to the specific DNA sequences, 5′-AACNG-3′ [5–8].

The c-*myb* proto-oncogene has no transforming capacity [9–11], but v-*myb* carried by AMV or E26 can transform myelomonocytic cells [2,3]. Comparison of c-Myb with the oncogenically activated forms indicated that a carboxyl (C)-terminal truncation induces oncogenic activation. c-Myb has three functional domains responsible for DNA-binding, transcriptional activation, and negative regulation (in that order) from the N-terminus [12]. The negative regulatory domain (NRD) normally represses c-Myb activity, and removal of this domain results in increased *trans*-activating capacity and oncogenic activation [10,12]. NRD contains the leucine zipper motif, and disruption of this leucine zipper increases both the *trans*-activating and transforming capacities of c-Myb, suggesting that the putative inhibitor represses the c-Myb activity by binding to c-Myb through this leucine zipper [13].

The DNA-binding domain of c-Myb is located within the N-terminal region, and consists of three imperfect tandem repeats of 51–52 amino acids (R1, 2, and 3 from the N-terminus) [12]. R3 and R2 cooperatively recognize the AACNG sequence, while R1 is not involved in the sequence recognition, but increases the stability of the Myb–DNA complex [14,15]. Structure analyses indicated that both R3 and R2 contain three helices and that the third helix in each is a recognition helix. The C-truncated form of c-Myb that lacks NRD has a higher DNA-binding activity than the full-length form [16], suggesting that NRD represses the DNA-binding activity. To identify the mechanism of oncogenic activation of c-*myb*, it is important to find any uncharacterized subdomain(s) in NRD and to discover how they repress c-Myb activity. However, it has not been found which region(s) in NRD represses DNA-binding activity. This was mainly due to the lack of an assay system to precisely measure the DNA-binding activity of various forms of c-Myb.

Electrophoretic mobility shift assay (EMSA) using nuclear extracts prepared from culture cells transfected by the c-Myb expression plasmid, is one method for examining the DNA-binding activities of various forms of c-Myb. In fact, mammalian cells such as NIH 3T3 cells transfected with the Myb expression plasmid expressed some Myb, judged by Western blotting. Using EMSA with nuclear extracts prepared from these transfected cells, however, only a very low level of DNA-binding activity of c-Myb was detected (data not shown). This could be due to the low level of expression and lability of Myb in transfected cells.

In this study, using the highly transfectable 293T cell line, we have developed a system to measure the DNA-binding activities of various forms of Myb. Using this system, two subdomains that repress the DNA-binding activity have been identified in NRD.

2. Materials and methods

2.1. Plasmid construction

All plasmids to express wild-type c-Myb and C-terminal truncated mutants CT1, CT2, and CT3 have been described [12]. The three plasmids to express mutants CTY, CTX, and CTV are truncated at amino acids 460, 448, and 435, respectively [10]. To construct the C-terminal truncated mutant CT471, termination codons were introduced at nucleotide 1450 (nucleotide numbers are as in [17]) by the polymerase chain reaction method. In the CT372 expression plasmid, the G-residue at 1155 was deleted, so a frameshift was produced and a termination codon was introduced 2 codons past this 1-bp deletion. To construct all internal deletion mutants, the oligonucleotide containing sequences on either side of the amino acids to be deleted were synthesized and used. This resulted in an in-frame deletion of the entire region of interest.

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2.2. DNA transfection and preparation of nuclear extracts

The 293T cell line was derived from the adenovirus type 5-transformed human embryonic kidney 293 cell line, and contains the simian virus 40 large tumor antigen [18]. 293T cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. On the day before transfection, 1.4×10^6 293T cells were plated onto 6-cm plastic tissue culture dishes, and left to adhere overnight. Four hours after a medium change, 10 μg of the plasmid DNA to express various forms of Myb was added to cells in the form of a calcium phosphate precipitate, and the cells were placed in an incubator for 4 h to permit the uptake of the DNA–$CaPO_4$ precipitate. The cells were then trypsinized and replated onto a 10-cm dish. Forty to 48 h after transfection, nuclear extracts were prepared as described by Dignam et al. [19].

2.3. Western blot analysis

Nuclear extracts, which were prepared from 293T cells transfected with plasmids encoding c-Myb mutants, were separated by SDS-PAGE on 10% gels and then transferred to nitrocellulose filters. The c-Myb proteins were detected by sequential binding of the anti-c-Myb monoclonal antibody 5.1 [20], horseradish peroxidase-labeled anti-mouse antibody, and enhanced chemiluminescense (ECL) Western blotting detection reagents (Amersham). To precisely measure the relative amount of c-Myb, a series of diluted protein samples were used for Western blot analyses, and the densities of protein signals were measured by a Bioimage analyzer (Milligen Biosearch).

2.4. EMSA

The nuclear extracts containing various forms of c-Myb were incubated for 15 min at 25°C with 1 ng of ^{32}P-labeled MBS-I oligonucleotide probe in 20 μl of binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 10 mM dithiothreitol), containing 2 μg poly(dI-dC), 200 μg bovine serum albumin, 1 μg calf thymus single-stranded DNA, and 0.1 μg mutant MBS-I(9C→T) oligonucleotide. The reaction mixture was then put onto a 4% polyacrylamide gel in 0.25×TBE (22.25 mM Trizma base, 22.25 mM boric acid, 0.5 mM EDTA) and electrophoresed. The amount of nuclear extract used for EMSAs was adjusted based on the result of Western blotting, and the difference in the amount of total protein was within 2-fold between each reaction.

For the competition analysis, varying amounts of cold competitor oligonucleotide were mixed with ^{32}P-labeled MBS-I probe and binding reactions were done as described above. For supershift analysis, the anti-c-Myb monoclonal antibody 1.1 or 5.1 [20] was added to the reaction mixture. The radioactivity of the Myb–DNA complex was measured using a Bioimage analyzer (Fuji).

3. Results

3.1. EMSAs by using nuclear extracts containing authentic c-Myb

Nuclear extracts prepared from 293T cells transfected with the wild-type c-Myb expression plasmid or from untransfected 293T cells as a control were used for EMSAs with the DNA probe containing the Myb-binding site, MBS-I (Fig. 1A). Two retarded bands (marked by asterisks in Fig. 1A) were observed with the control nuclear extracts, while nuclear extracts containing c-Myb generated two additional bands (marked by arrows in Fig. 1A) (Fig. 1A, lanes 1 and 2). Addition of the Myb-specific monoclonal antibody 1-1, of which the epitope is located in NRD, supershifted the upper additional band (the supershifted band is like a smear and is not visible) (Fig. 1A, lane 3). Addition of another antibody, 5-1, of which the epitope is located in the transcriptional activation domain blocked the generation of two additional bands (Fig. 1A, lane 4). Since the transcriptional activation domain is next to the DNA-binding domain, the antibody 5-1 may inhibit the DNA binding of Myb by masking the DNA-binding domain. These results indicate that the upper and lower bands are generated by full-length c-Myb and the degraded c-Myb lacking C-proximal portion, respectively.

All of the four retarded bands were competed with by the addition of a 50-fold molar excess of the MBS-I competitor

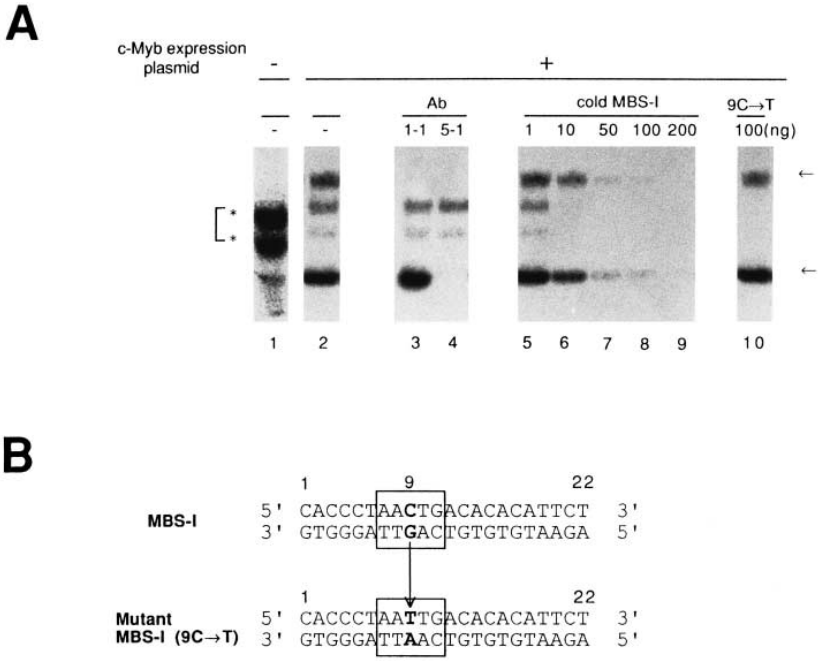

Fig. 1. Sequence-specific DNA-binding by authentic c-Myb. A: EMSAs. EMSAs were done with nuclear extracts from control 293T cells (lane 1) or 293T cells transfected with the c-Myb expression plasmid (lanes 2–10) and the ^{32}P-labeled oligonucleotide MBS-I. Lanes 3,4: Myb-specific monoclonal antibodies 1-1 and 5-1 were added, respectively. Lanes 5–9: The indicated amount of cold DNA competitor containing MBS-I site was added in the amounts shown at the top. Lane 10: 100 ng of cold DNA competitor MBS-I(9C→T) was added. The Myb–DNA complexes are indicated by arrows. The upper and lower arrows show the complexes generated by full-length and degraded c-Myb, respectively. Two bands indicated by asterisks were generated by proteins other than c-Myb. B: DNA sequence of the MBS-I probe and the MBS-I(9C→T) competitor. The consensus sequence AACNG recognized by c-Myb is boxed.

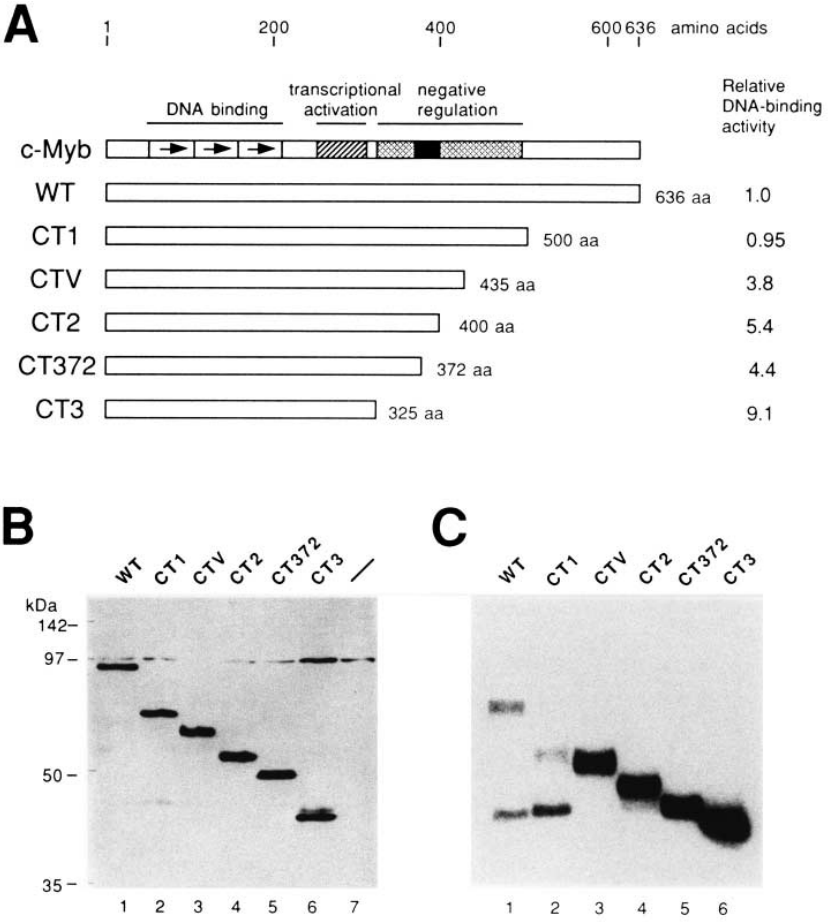

Fig. 2. Carboxyl truncation of Myb increases DNA-binding activity. A: Structures of C-truncated mutants used. The three functional domains of mouse c-Myb are shown at the top. The relative DNA-binding activity of each mutants measured by EMSAs in (C) is indicated on the right. B: Immunodetection of c-Myb mutants in transfected cells. Nuclear extracts were prepared from 293T cells transfected with plasmids encoding the c-Myb mutants shown above each lane or control 293T cells without transfection (lane 7). After separation by SDS-PAGE on a 10% gel, c-Myb proteins were detected by Western blotting. To precisely measure the relative amounts of c-Myb, a series of diluted samples were used for Western blotting. Here, typical results obtained with the appropriate amounts of protein are shown. The 97-kDa band above the full-length c-Myb band is the non-specific band generated by the 293T cell lysates. C: EMSAs. The MBS-I probe was incubated with nuclear extracts used in (B) in the presence of 100 ng of the mutants MBS-I(9C→T) oligonucleotide. Amounts of nuclear extracts used was normalized by the results of Western blotting to have an equal amounts of c-Myb proteins.

(Fig. 1A, lanes 5–9), indicating that two bands observed with 293T control extracts are also generated by specific binding of some proteins to the MBS-I probe. Since these two bands may disturb the analyses of Myb–DNA complex formed by various length of Myb, we tried to block the formation of these two bands by addition of an appropriate oligonucleotide competitor. We had used various oligonucleotides to analyze the effects on Myb binding to point mutations in MBS-I [21]. A series of oligonucleotides used in that study were added to the reactions of EMSAs, and one oligonucleotide MBS-I(9C→T) was found to block the generation of two retarded bands observed with the control 293T extracts, but not that of two other bands containing Myb (Fig. 1A, lane 10). In the oligonucleotide MBS-I(9C→T), the third C residue in the Myb-recognition sequence AACNG was changed to T (Fig. 1B), and Myb cannot bind to this oligonucleotide. These results indicate that the proteins that are involved in the two bands observed with the 293T control extract are not endogenous c-Myb of 293T cells. Thus, by addition of a specific oligonucleotide as a competitor, the EMSAs suitable to measure the DNA-binding activity of various forms of Myb have been developed.

3.2. *Presence of two subdomains*

To identify the subdomain(s) in NRD that repress the DNA-binding activity, we have examined the effects of C-terminal truncation on DNA-binding activity by using the system described above. The expression vectors for a series of C-terminal truncated mutants were constructed (Fig. 2A), and transfected into 293T cells. Nuclear extracts were prepared from transfected cells, and amounts of c-Myb in each preparation of nuclear extracts were measured by Western blotting using a series of diluted extracts (Fig. 2B). The EMSAs were done using the normalized amounts of nuclear extracts based on the results of Western blotting to have an almost equal amount of c-Myb (Fig. 2C). Deletion of the C-terminal 136-amino-acid region (CT1) did not affect the DNA-binding activity, indicating that the region downstream of NRD does not regulate the DNA-binding activity. Truncation to amino acid 435 (CTV) increased DNA-binding activity 3.8-fold. Fur-

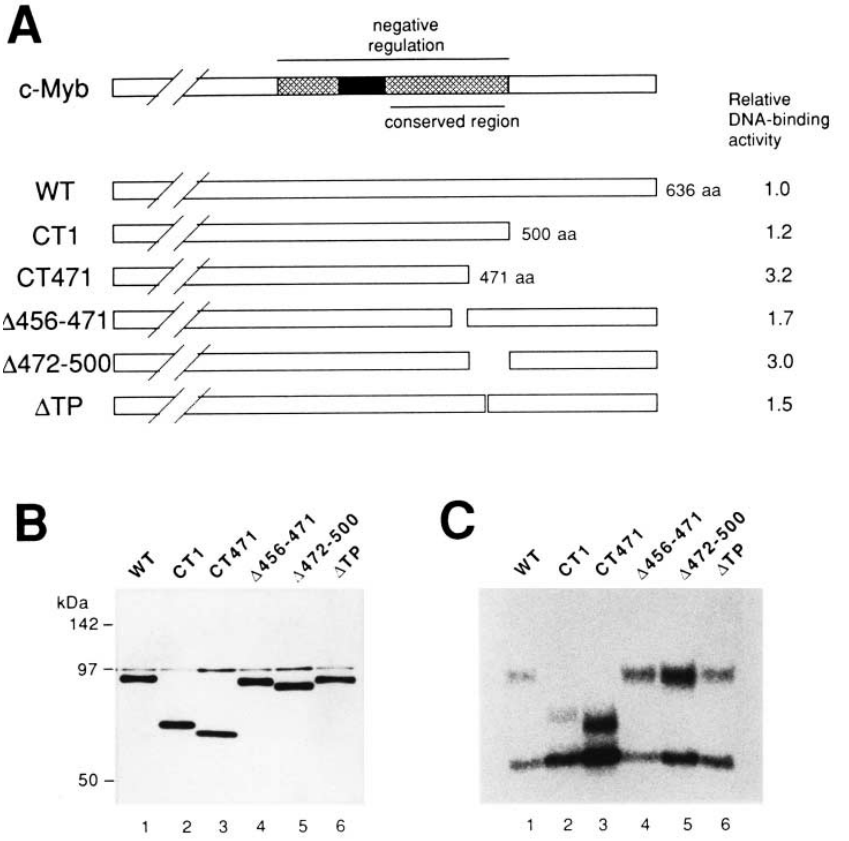

Fig. 3. Identification of the C-proximal subdomain, NRD2. A: Structures of truncated and internal deletion mutants used. The results of EMSAs shown in (C) are summarized on the right. ΔTP is a mutant lacking the putative phosphorylation site for proline-dependent kinase between amino acids 472 and 500. B: Immunodetection of c-Myb mutants in transfected cells. C: EMSAs. The experiments in (B,C) were done and the results are shown as in Fig. 2.

ther truncation to amino acid 400 (CT2) or 372 (CT372) slightly increased DNA-binding activity compared with CTV, but this increase was not significant. However, further truncation to amino acid 325 (CT3) significantly increased DNA-binding activity (9-fold) compared with CTV. Thus, two regions in NRD, amino acids 435-500 (C-proximal subdomain: NRD2) and 325-372 (N-proximal subdomain: NRD1), appear to negatively regulate the DNA-binding activity of c-Myb.

3.3. *Identification of the C-proximal subdomain, NRD2*

To further narrow down the C-proximal subdomain, we made three additional constructs to express CT471, CTY, and CTX, and examined their DNA-binding activities. DNA-binding activities of all three mutants were higher by about 3-fold than that of wild type (data not shown), suggesting the region that represses DNA-binding activity is between amino acids 471 and 500. To confirm that this region really represses DNA-binding activity, we made a expression vector for an internal deletion mutant lacking this region (Δ472-500) (Fig. 3). This mutant had 3-fold higher DNA-binding activity than wild type, indicating that the C-proximal subdomain designated as NRD2 is in this region. In addition, a small deletion of the region next to this subdomain (Δ456-471) did not affect the DNA-binding activity. Therefore, to repress DNA-binding activity, a further N-proximal region is not required, suggesting that the region between amino acids 471–500 is sufficient for negative regulation of DNA binding. In this region, the putative phosphorylation site by proline-dependent protein kinase or MAP kinase (484 PQTPSHAV 491) is located. However, deletion of this putative phosphorylation site, the TP residue at amino acids 486 and 487, (ΔTP) did not affect the DNA-binding activity (Fig. 3).

3.4. *Identification of the N-proximal subdomain, NRD1*

We next tried to narrow down the N-proximal subdomain (Fig. 4). The results in Fig. 1 indicate that the region between amino acids 325 and 372 is critical for negative regulation of DNA-binding activity. To confirm this result, we constructed an internal deletion mutant lacking this region (Δ326-372) and examined its DNA-binding activity by EMSAs. This mutant had 3.4-fold higher DNA-binding activity than wild type. Then, two mutants lacking either the N- or C-terminal half (Δ326-349 and Δ350-372) of this region were made, and their DNA-binding activities were examined. Both of them had almost the same activity as wild type. These results indicate that deletion of the whole region between amino acids 326 and 372 is necessary to increase DNA-binding activity. This N-proximal subdomain was designated as NRD1.

3.5. *Role of leucine zipper, NRD1, and NRD2 in DNA binding of c-Myb*

We have examined the effects of the leucine zipper mutation on the DNA-binding activity of authentic c-Myb using the

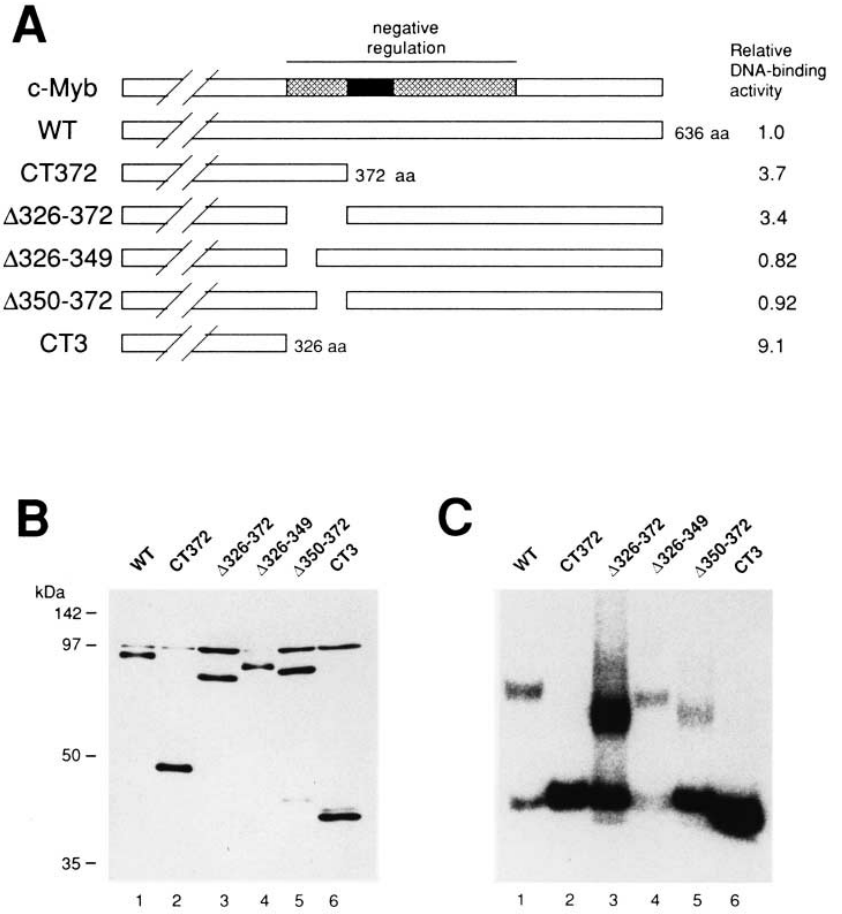

Fig. 4. Identification of the N-proximal subdomain, NRD1. A: Structures of mutants used. The results of EMSAs shown in (C) are summarized on the right. B: Immunodetection of c-Myb mutants in transfected cells. C: EMSAs. The experiments in (B,C) were done and the results are shown as in Fig. 2.

293T cell system. The results of EMSAs indicated that the DNA-binding activity of the leucine zipper mutant L34P was almost the same as that of wild type (Fig. 5). To further confirm whether NRD contains only two subdomains (NRD1 and NRD2) in addition to the leucine zipper, a mutant (ΔNRD1+2) that internally lacks both subdomains were constructed and its DNA-binding activity was examined (Fig. 5). The DNA-binding activity of ΔNRD1+2 was comparable to that of CT3 lacking the whole region of NRD. These results confirm that the critical subdomains that negatively regulate the DNA-binding activity of the authentic c-Myb are NRD1 and NRD2.

4. Discussion

Using the chicken cell line, two groups reported analyses of the DNA-binding activity of c-Myb expressed in virus-infected or plasmid-transfected cells [22,23]. However, it seemed to be difficult to analyze the DNA-binding activity of various forms of c-Myb precisely using these systems due to the presence of degraded products of c-Myb. In the present study using the 293T cell system, we have identified two subdomains in NRD, NRD1 and NRD2, that negatively regulate DNA-binding activity of c-Myb (Fig. 6A). The N-proximal subdomain, NRD1, contains two putative phosphorylation sites by proline-dependent kinase or MAP kinase, one site by glycogen synthetase kinase-3, and one site by casein kinase II (Fig. 6B). The C-proximal subdomain, NRD2, also contains one putative phosphorylation site for proline-dependent kinase (Fig. 6B). However, phosphorylation at this site in NRD2 is probably not critical for regulation of DNA-binding activity, since deletion of this putative phosphorylation site did not affect the DNA-binding activity. At present, it is not known whether the conformation of these regions, or the modification of c-Myb by the factor(s) such as phosphorylation, is important for regulation of DNA-binding activity.

Krieg et al. expressed full-length c-Myb in HeLa cells using a recombinant vaccinia virus, and found that the purified full-length c-Myb binds to DNA with almost the same affinity as the protein containing the DNA-binding domain alone [24]. This indicates that a deletion of the C-terminal region does not increase the DNA-binding activity, and is inconsistent with our results. However, the significant difference between their system and ours is that our EMSA used the crude nuclear extract, not the purified c-Myb. Since the reaction for DNA binding in our system contains many factors, some factor(s) may regulate the DNA-binding activity by binding to NRD1 and NRD2.

Using the bacterially made c-Myb that lacks the downstream region from the leucine zipper, we had demonstrated

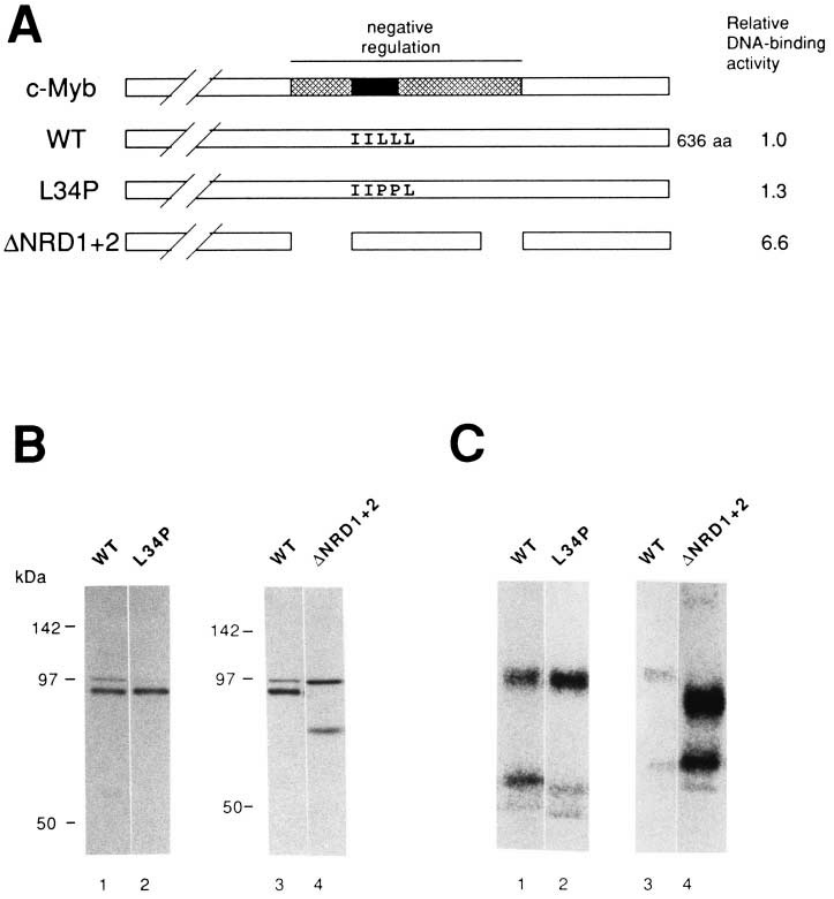

Fig. 5. Role of three subdomains in DNA binding of authentic c-Myb. A: Structures of mutants used. The results of EMSAs shown in (C) are summarized on the right. B: Immunodetection of c-Myb mutants in transfected cells. C: EMSAs. The experiments in (B,C) were done and the results are shown as in Fig. 2.

that the leucine zipper mutant had 3–4-fold higher DNA-binding activity than wild type [25]. In the 293T cell system described here, however, the DNA-binding activity of the leucine zipper mutant was almost the same as that of wild type. Since the effect of the leucine zipper mutations on the *trans*-activating capacity of c-Myb varies between the cells used [13], the effects of the leucine zipper mutation on DNA-binding activity could also depend on the cells used.

The v-Myb encoded by AMV lacks NRD2, and the v-Myb encoded by E26 lacks NRD2, the leucine zipper motif, and the half of NRD1 (Fig. 6A). So far, transforming capacities of various C-truncated forms of Myb were examined by using the mouse [9,10] and chicken [11] systems. In both systems, removal of the whole region of NRD dramatically increased transforming activity, and truncation of the region containing NRD2 identified in this study (CTY construct in the mouse system) slightly but significantly increased transforming capacity. Disruption of the leucine zipper motif also resulted in oncogenic activation in mouse system [13], but the effects of the leucine zipper disruption on transforming activity were not examined in the chicken system. It was also not examined whether deletion of NRD1 induces oncogenic activation in mouse and chicken system. Presence of multiple subdomains that negatively regulate c-Myb activity suggest that c-Myb activity is regulated multiple mechanisms and oncogenic activation is also induced by multiple mechanisms.

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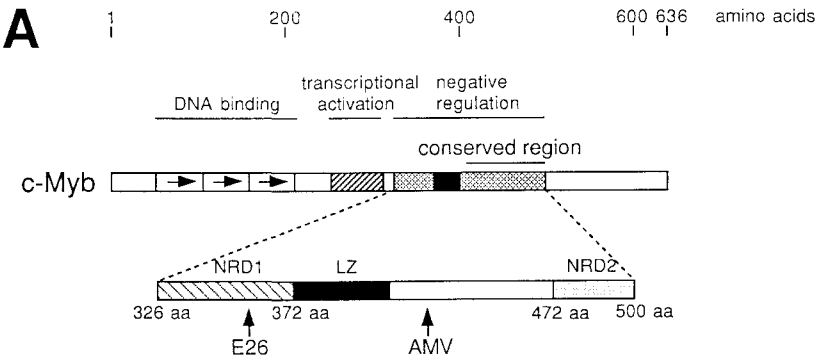

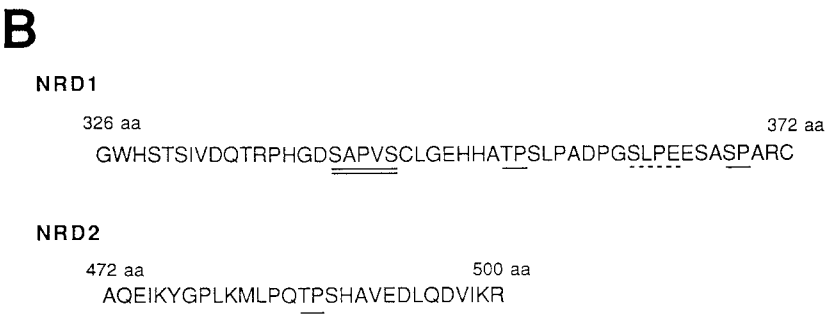

Fig. 6. Schematic representation of three subdomains in NRD. A: Relationship between the subdomains and the v-Myb. The three subdomains, NRD1, NRD2, and the leucine zipper, are indicated by hatched, stippled, and closed boxes, respectively. The conserved region shown by a overhead line is conserved between c-Myb, A-Myb, and B-Myb. The positions of C-terminal end of v-Myb encoded by AMV and E26 are indicated by arrows. B: The amino acid sequence of NRD1 and NRD2. The putative phosphorylation sites by proline-dependent kinase, glycogen synthetase kinase III, and casein kinase II are indicated by single, double, and dotted underlines, respectively.

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