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Delineation of three functional domains of the transcriptional activator encoded by the c-*myb* protooncogene

(simian virus 40 enhancer/sequence-specific DNA-binding protein/negative regulatory region/repression/nuclear localization)

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ABSTRACT **The c-*myb* protooncogene encodes a sequence-specific DNA-binding protein (c-Myb) that induces transcriptional activation or repression. We have identified three functional domains of the mouse c-Myb protein that are responsible for DNA binding, transcriptional activation, and negative regulation, respectively. In addition to the DNA-binding domain, which is located near the N terminus, an adjacent region (the transcriptional activation domain) containing about 80 amino acids was found to be essential for transcriptional activation. Deletion of a region spanning about 175 amino acids of the C-proximal portion increased transcriptional activation markedly, revealing that this domain normally represses activation. Differences between the transcriptional activation and repression functions of c-Myb and v-Myb are discussed in the light of these functional domains. Our results suggest that transcriptional activation may be involved in transformation by *myb* gene products.**

The viral oncogene v-*myb* carried by two avian leukemia viruses, avian myeloblastosis virus and E26, is a truncated form of its cellular homolog c-*myb* (1, 2) and is involved in the transformation of hematopoietic cells (3, 4). The c-*myb* gene is conserved among various species including *Drosophila melanogaster* (5–10). Both c-*myb* and v-*myb* encode nuclear proteins (c-Myb and v-Myb) that bind DNA directly (11, 12). Recently, both v-Myb and c-Myb were shown to exhibit a sequence-specific DNA-binding activity and recognize the sequence 5′-VHYAACYR-3′ (ref. 13; H.N., T.N., and S.I., unpublished results), where V = A, C, or G, and H = A, C, or T. c-Myb binds to two sites in the simian virus 40 (SV40) genome, MBS-I (positions 246–264) and MBS-II (positions 128–146 and 184–202 in the SV40) (unpublished results). When the sequences of the MBS-I and MBS-II sites were compared, 11 of 19 base pairs (bp) were identical. MBS-I is a high-affinity site and was shown to be a c-Myb-dependent enhancer element. MBS-II is a low-affinity site, and tandem repeats of the sequence containing this site induce c-Myb-dependent transcriptional repression (unpublished results). Here we report the identification of three functional domains of c-Myb: a DNA-binding domain, a transcriptional activation domain, and a negative regulatory domain. The functional differences between c-Myb and v-Myb are discussed in the light of the structures of these functional domains.

MATERIALS AND METHODS

Plasmid Construction. The effector plasmids pact-c-myb, in which the 5′ regulatory region of the chicken cytoplasmic β-actin gene is linked to the mouse c-*myb* gene, and pact1, which was constructed by deletion of the c-*myb* sequence from pact-c-myb, have been described (14). The reporter plasmids pMFcolCAT6MBS-I and pMFcolCAT6SV-II contain six tandem repeats of the MBS-I and the SV-II sequence, respectively, in the *Bam*HI site of the plasmid pMFcolCAT, in which the bacterial chloramphenicol acetyltransferase (CAT) gene is linked to the mouse α2(I)-collagen promoter. The SV-II sequence contains the MBS-II site and corresponds to positions 184–218 in the SV40 genome. All plasmids designed to express mutant c-Myb proteins in cultured cells were generated from the plasmid pact-c-myb. To make the CT1, CT5, CT6, and CT7 mutants, termination codons were introduced at nucleotides 1537, 616, 463, and 307, respectively, by site-specific mutagenesis as described by Kunkel *et al.* (15). Nucleotide numbers are as in ref. 5. To obtain the NT1, NT3, and NT4 mutants, the sequence recognized by the restriction enzyme *Nco* I was introduced at nucleotides 147, 296, and 455, respectively, by site-specific mutagenesis, and the regions between the introduced *Nco* I sites and the *Nco* I site at nucleotide 36 that overlaps the normal c-*myb* initiation codon were deleted. In-frame deletion mutants ΔDB, ΔNR, and ΔTA were constructed by deletion of the 480-bp *Rsa* I–*Bal* I fragment (nucleotides 264–743), the 714-bp *Sma* I–*Ssp* I fragment (nucleotides 1012–1725), and the 255-bp *Hinc*II–*Sma* I fragment (nucleotides 757–1011), respectively. To generate CT4 and CT3, the 280-bp *Bal* I–*Hinc*II fragment (nucleotides 668–947) and the 785-bp *Sma* I–*Rsa* I fragment (nucleotides 1012–1796) were deleted. These deletions cause a frameshift so that a termination codon is introduced 5 amino acid codons past the *Hinc*II site (nucleotide 947) and 1 codon past the *Rsa* I site (nucleotide 1796) in CT4 and CT3, respectively. The CT2 mutant was made by joining the cDNAs MM49 and MM46 (5) via their common *Sma* I site at nucleotide 1011. Because MM46 contains a 17-bp deletion (nucleotides 1240–1256), a frameshift is produced and a termination codon is introduced 2 codons past the 17-bp deletion. To make NT2, a linker containing a eukaryotic translation initiation codon was inserted at the *Rsa* I site at nucleotide 264, and the region between the generated *Nco* I site and the *Nco* I site at nucleotide 36 was deleted. The NCT mutant was constructed by joining NT2 and CT2 via their common *Sma* I site at nucleotide 1011. The v-*myb* construct was obtained by joining the v-*myb* gene of avian myeloblastosis virus and synthetic DNA corresponding to sequences derived from viral *gag* gene. To express ΔDB, ΔNR, and CT5 mutants in

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Abbreviations: CAT, chloramphenicol acetyltransferase; SV40, simian virus 40.

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Escherichia coli, the normal c-*myb* sequence in the pAR2156myb expression vector (16), in which the mouse c-*myb* cDNA sequence is under control of the phage T7 late promoter (17), was replaced by the mutant sequence via common restriction enzyme sites.

DNA Transfection and CAT Assay. A set of in-frame deletion mutants were placed under the control of the chicken cytoplasmic β-actin promoter (18) for expression in cultured cells. The activity of the protein was monitored by its ability to stimulate or repress transcription from the reporter plasmids pMFcolCAT6MBS-I and pMFcolCAT6SV-II. Mixtures of 6 μg of reporter plasmid DNA, 6 μg of effector plasmid DNA, and 2 μg of pRSV-β-gal plasmid DNA (19) were transfected into African green monkey kidney (CV-1) cells as described (14). The plasmid pRSV-β-gal, which carries the *E. coli* β-galactosidase gene linked to the Rous sarcoma virus long terminal repeat promoter, was used as an internal control for differences in transfection efficiency between precipitates. CAT assays were performed according to Gorman *et al.* (20). The amounts of cell extract used for the CAT assays were normalized with respect to β-galactosidase activity.

DNA-Binding Analysis of Bacterially Synthesized c-Myb. The normal and mutant c-Myb proteins were synthesized in bacteria, partially purified, and used for further analyses as described (16). DNase I protection ("footprint") analysis was done as described (21).

Immunofluorescence Studies. Subconfluent monolayers of murine NIH 3T3 cells, grown on glass cover slides in 35-mm tissue culture plates, were transfected with 5 μg of plasmid DNA by the calcium phosphate precipitation method (31). Forty hours later, cells were fixed for 10 min in phosphate-buffered saline (PBS) with 2% formaldehyde, permeabilized for 10 min in PBS with 0.1% Triton X-100, and incubated with anti-Myb monoclonal antibody 1.1 (ref. 16; diluted 1:100 in PBS with 2% bovine serum albumin) for 1 hr at 37°C followed by fluorescein-conjugated goat anti-mouse immunoglobulin (1:200 in PBS containing 2% albumin) for 1 hr at 37°C.

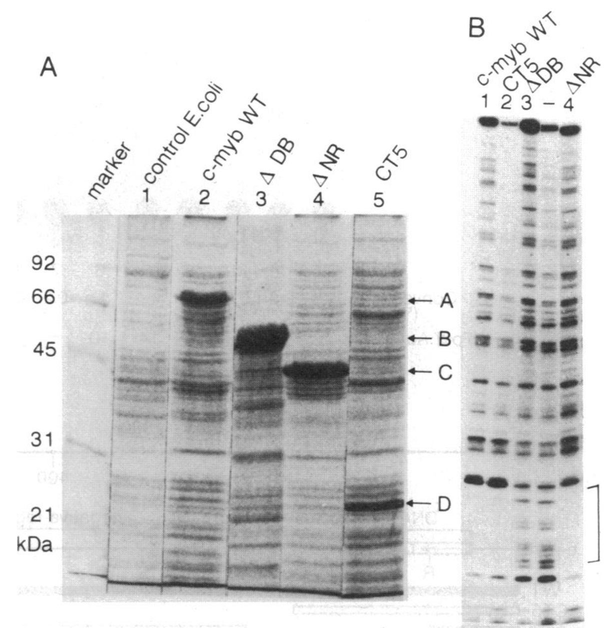

FIG. 1. Sequence-specific DNA-binding activity of c-Myb mutants. (*A*) Expression of c-Myb mutants in *E. coli*. Insoluble proteins prepared from bacteria carrying plasmids that express wild-type (WT) c-Myb (lane 2), ΔDB (lane 3), ΔNR (lane 4), or CT5 (lane 5) were solubilized by urea, electrophoresed in a SDS/12% polyacrylamide gel, and stained with Coomassie brilliant blue. In the control experiment, insoluble proteins prepared from bacteria lacking the c-*myb* expression plasmid were also analyzed (lane 1). Myb proteins are indicated by arrows A–D. Molecular size markers are shown at left. (*B*) DNase I footprint analysis. The ^{32}P-labeled DNA fragment containing the SV40 enhancer was incubated with 120 ng of WT (lane 1) or mutant (lanes 2–4) c-Myb proteins or without any protein (lane −). Bracket indicates fragments not produced in the presence of sequence-specific DNA-binding protein. Digestions were carried out with DNase I at 5 μg/ml.

RESULTS

DNA-Binding Domain. Klempnauer and Sippel (12) showed that the N-terminal region of the v-Myb protein, which lies within a threefold tandem repeat of 51–52 amino acids (5), was essential for binding to DNA-cellulose. To confirm that this region also confers sequence-specific DNA-binding activity, CT5 and ΔDB proteins were synthesized in *E. coli* (Fig. 1*A*, lanes 5 and 3) and used for DNase I footprint analysis. CT5 lacks the region downstream from the C-terminal end of the third repeat and ΔDB lacks most of the region containing the three repeats (Fig. 2*B*). CT5 bound to the high-affinity c-Myb binding site, MBS-I, in the SV40 enhancer with almost the same affinity as wild-type c-Myb (Fig. 1*B*, lanes 1 and 2), while ΔDB did not protect the binding site at all (Fig. 1*B*, lane 3). Moreover, ΔDB has no transcriptional stimulatory activity (Fig. 2*A*, lane 4), although it was shown by immunoblot analysis to be expressed at almost the same level as the normal c-Myb protein (data not shown). These results indicate that the region containing the three repeats contains the sequence-specific DNA-binding domain. Furthermore, the NT1, NT3, and NT4 proteins—which lack the region upstream from the N-terminal end of the first repeat, the first repeat itself, and both the first and second repeats, respectively—were examined for functional activity. Both NT1 and NT3 stimulated transcription, although the degree of activation was about 30% lower with NT1 and 50% lower with NT3 than with normal c-Myb (Fig. 2*A*, lanes 5 and 6). By contrast, NT4 had no transcriptional stimulatory activity (Fig. 2*A*, lane 7). These results imply that the region upstream from the first repeat and the first repeat itself are not essential for DNA binding, because sequence-specific DNA binding is presumably required for transcriptional activation.

Transcriptional Activation Domain. The CT5 mutant, which contains the N-terminal 193 amino acids, exhibits sequence-specific DNA-binding activity (Fig. 1*B*, lane 2) but cannot stimulate transcription (Fig. 2*A*, lane 3). Because the CT2 mutant retains its transcriptional activation function (Fig. 2*A*, lane 15), the region between the C-terminal ends of the CT5 and CT2 constructs (Fig. 2*B*; amino acids 194–401) should include the transcriptional activation domain. The results with mutants ΔNR, CT4, and ΔTA (Fig. 2*A*, lanes 11–13) indicate that the transcriptional activation domain is encoded in the region between the *Hin*cII and *Sma* I sites (Fig. 2*B*, nucleotides 756–1011, amino acids 241–325).

Negative Regulatory Domain. The ΔNR mutant protein bound to the c-Myb binding site MBS-I in the SV40 enhancer with almost the same affinity as did the normal c-Myb protein (Fig. 1*B*, lane 4) but transcriptional activation with ΔNR was about 10-fold higher than with normal c-Myb (Fig. 2*A*, lane 11), indicating that the region deleted in the ΔNR mutant does not repress the DNA-binding activity of normal c-Myb but regulates the transcriptional activation negatively. The results of transcriptional activation with mutants CT1, CT2, and CT3 (Fig. 2*A*, lanes 10, 15, and 14) indicated that this negative regulatory region was localized between amino acids 326 and 500. Although the level of mRNA for ΔNR mutant was almost the same as for normal c-Myb (data not shown), the monoclonal antibody 1.1 did not detect the ΔNR mutant protein due to the localization of its epitope in the deleted portion of the ΔNR mutant. Therefore, we cannot

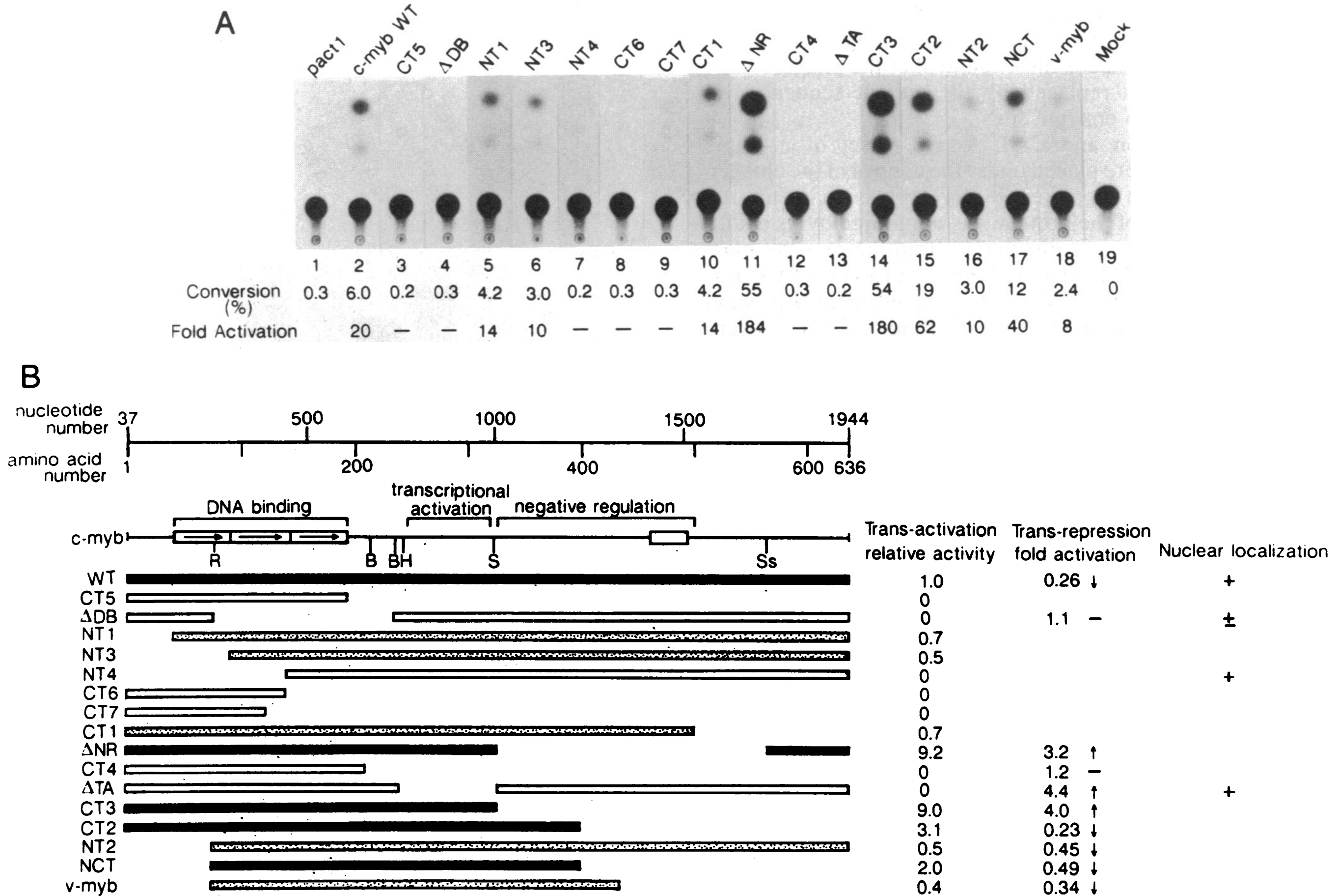

FIG. 2. Transcriptional activation by c-Myb mutants. (*A*) Transient expression of CAT activity. A mixture of reporter plasmid pMFcolCAT6MBS-I, effector plasmid that expresses wild-type (WT) or mutant Myb as shown above each lane, and pRSV-β-gal plasmid was transfected into CV-1 cells, and extracts of transfectants were assayed for CAT activity. CAT activity is shown below each lane as percent conversion of [^{14}C]chloramphenicol to acetylated derivatives. Experiments were repeated three times, and typical results are shown. The differences between each set of experiments were within 20%. (*B*) Summary of deletion analysis of c-Myb. Three functional domains inferred from our results and the restriction enzyme sites used for construction of mutants are shown. Two regions conserved among various species, including *Drosophila*, are indicated by boxes. Arrows represent the threefold tandem repeats of 51 or 52 amino acids. Mutants that activated transcription more efficiently than the WT are indicated by solid bars, those that activated less than the WT by stippled bars, and those that were inactive by open bars. On the right, activities, obtained from three independent experiments, are expressed relative to that of the WT protein. Results of transcriptional repression shown in Fig. 3 are also summarized and indicated as degree of activation. Nuclear localizations of proteins are indicated according to the results shown in Fig. 4. The mutants that have the nuclear localization signal are indicated by +, and the mutant that has partially lost the nuclear localization signal by ±.

exclude the possibility that removal of this negative regulatory domain increases the level of c-Myb protein by affecting its stability.

Converting a Transcriptional Repressor into an Activator. Deletion of the DNA-binding domain (ΔDB) eliminated the transcriptional repression function of c-Myb (Fig. 3, lane 3), while deletion of the C-terminal portion of the negative regulatory domain (CT2) did not affect the repression function (lane 9). The mutants ΔTA, ΔNR, and CT3 acted as transcriptional activators (Fig. 3, lanes 5–7), revealing that deletion of amino acids 241–401 converts a repressor into an activator. This region includes the transcriptional activation domain and the N-terminal half of the negative regulatory domain.

Nuclear Localization Signal. Because the region downstream of the transcriptional activation domain is not required for transactivation function and ΔTA, which lacks the transcriptional activation domain, is localized in the nuclei, as is normal c-Myb (Fig. 4 *a–d*), the region upstream of the transcriptional activation domain should contain the nuclear localization signal. In fact, ΔDB appears to have partially lost the nuclear localization signal, since 30% of the cells expressed the ΔDB protein in both nuclei and cytosol, while 70% of the cells expressed it only in the nuclei (Fig. 4 *g* and *h*). However, NT4, which lacks the region involved in DNA-binding (and thus lacks the transactivation function) was found in the nucleus (Fig. 4 *e* and *f*) and thus has retained the nuclear localization signal(s). These results indicate that the nuclear localization signal is dispersed over an N-terminal region of about 200 amino acids containing three repeats of 51 or 52 amino acids and that part of this region is enough to localize the protein in nuclei, as in the case of NT4.

Transcriptional Activation and Repression by v-Myb. The v-Myb protein has 40% of the transcriptional activation and 75% of the transcriptional repression activities of c-Myb (Fig. 2*A*, lane 18; Fig. 3, lane 11). In v-Myb, both the N-terminal and C-terminal portions of c-Myb are deleted (2, 22). To examine the effect of deletion of each portion, transcriptional regulation by the NT2, CT2, and NCT mutants was analyzed. Deletion of the N-terminal 76 amino acids of c-Myb decreased activation by about half (Fig. 2*A*, lane 16), whereas deletion of the C-terminal portion increased this activity about 3-fold (lane 15). These effects were partially canceled in a mutant lacking both portions (NCT), which showed twice the transcriptional activation activity of the wild-type protein (lane 17). The results with the mutant lacking both the

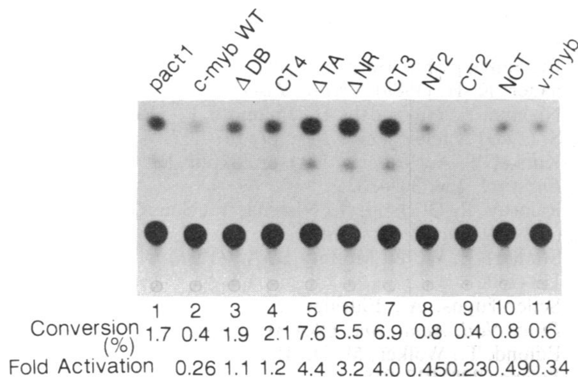

FIG. 3. Transcriptional repression function of c-Myb mutants. A mixture of reporter plasmid pMFcolCAT6SV-II, effector plasmid that expresses wild-type (WT) or mutant Myb protein as shown above each lane, and pRSV-β-gal plasmid was transfected into CV-1 cells, and CAT activity was assayed. CAT activity is shown below each lane as percent conversion. Experiments were repeated three times, and typical results are shown. The differences between each set of experiments were within 20%.

N-terminal and C-terminal portions are not consistent with the results with v-Myb, although both NCT and v-Myb lack similar (but not identical) portions of c-Myb (see *Discussion*). The transcriptional repressor activity of v-Myb was slightly lower than that of c-Myb, a result consistent with the activity of the NCT mutant (Fig. 3, lanes 10 and 11).

DISCUSSION

The transcriptional activation domains of the yeast GCN4 and GAL4 transcriptional activators are clusters of acidic amino acids (23, 24). The region identified as the transcriptional activation domain of c-Myb (amino acids 241–325) is also acidic; in particular, amino acids 286–308 (DEDPEKEKRIKELELLLMSTENE) comprise 9 acidic residues and 4 basic residues, for a net charge of −5.

The function of the negative regulatory domain of c-Myb resembles the negative regulatory function of the hormone-binding domain of the steroid hormone receptor, because deletion of the hormone-binding domain engenders a constitutively active receptor (25). The negative regulatory domain of c-Myb involves another region (amino acids 460–494 in mouse c-Myb) that is conserved in many species including *Drosophila* (10), and the amino acid sequence upstream of this conserved region (amino acids 415–459) is also conserved between the human c-*myb* and the related gene B-*myb* (26).

So far, two examples of enhancer-binding proteins, the glucocorticoid receptor (27) and the thyroid hormone receptor (28), are known to have both the transactivator and the transrepressor activities. Both can repress transcription by competing with other transactivators, such as the cAMP response element-binding factor (CREB) or the estrogen receptor; this occurs in each case because the binding sites of two trans-acting factors overlap. In the case of transcriptional repression by c-Myb, the same kind of mechanism is possible. Deletion of amino acids 241–401 converted the c-Myb transcriptional repressor into an activator. It is possible that this deletion mutant stimulates the binding of the other transactivator to the MBS-II site and activates transcription, whereas the wild-type c-Myb competes with that transactivator.

The region of v-Myb removed by N-terminal and C-terminal truncation includes a part of the DNA-binding domain and a part of the negative regulatory domain, respectively. Truncation of the N-terminal portion inhibits both the transcriptional activation and repression functions by about 30%. In contrast, C-terminal truncation alone stimulates the transcriptional activation function—about 3-fold with the CT2 construct—and has no effect on the repression function. However, it should be noted that NCT and CT2 lack a 35-amino acid region (amino acids 402–436) found at the C terminus of v-Myb. Thus, the repression functions of v-Myb and the doubly-truncated mutant NCT are slightly lower than that of normal c-Myb. However, the activation function of v-Myb is about one-third that of normal c-Myb and is lower than that of the NCT mutant. It is not clear whether this discrepancy results from (*i*) the absence in NCT of the 35-amino acid region present in v-Myb, (*ii*) point mutations reflecting the different species of origin of the *myb* genes, or (*iii*) alteration(s) other than truncation (such as addition of a small portion derived from viral *gag* and/or *env* genes or point mutations). Comparison of the biological activities of

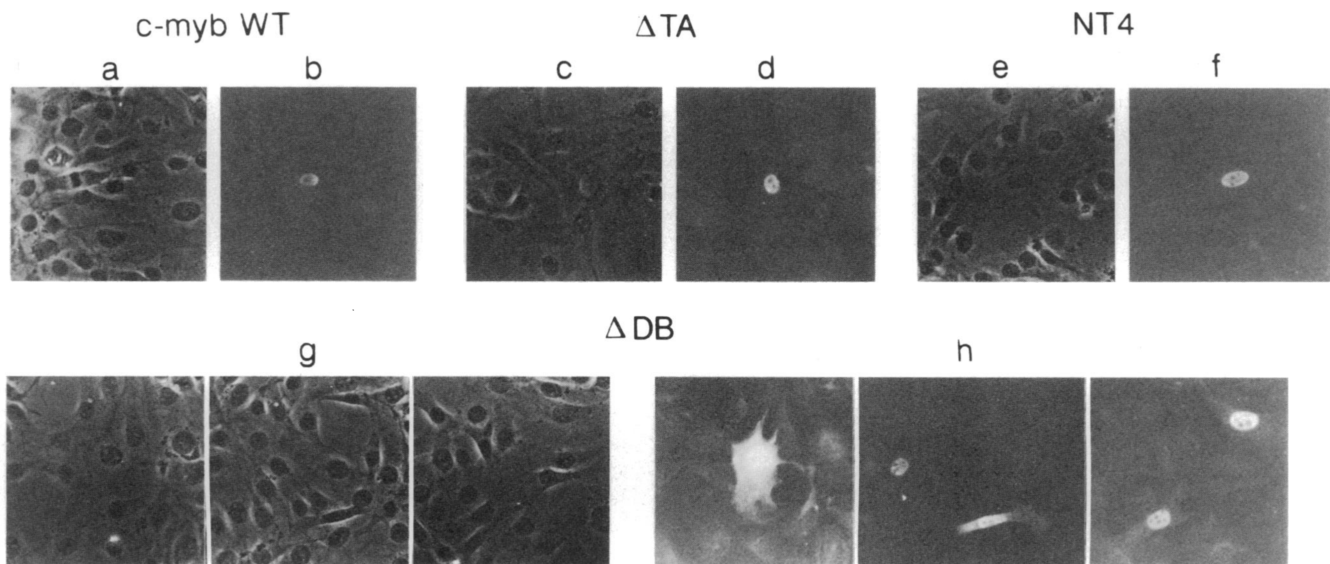

FIG. 4. Analysis of nuclear localization signal of c-Myb on the basis of subcellular distribution of c-Myb mutants. NIH 3T3 cells transfected with plasmids that express wild-type (WT) c-Myb protein (*a* and *b*) and the mutants ΔTA (*c* and *d*), CT7 (*e* and *f*), and ΔDB (*g* and *h*) were stained with anti-c-Myb monoclonal antibody 1.1 and fluorescein-labeled second antibody (*b*, *d*, *f*, and *h*). Cells were also viewed and photographed by phase-contrast microscopy (*a*, *c*, *e*, and *g*).

these mutants will be necessary to determine the significance of the functional domains identified here. Nevertheless, it is provocative that many transformation-associated Myb proteins (16, 29, 30) lack part of the negative regulatory domain and, moreover, that the CT2 mutant (which has enhanced transcription-activating capacity) is activated with respect to wild-type c-Myb in its ability to transform murine hematopoietic cells *in vitro* (T.J.G., unpublished results). This in turn suggests that transformation by *myb* may be directly mediated by increasing the expression of other cellular genes.

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