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Transcriptional Control by *myb* Oncogene Product

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ISHII, S., NOMURA, T., KANEI-ISHII, C., NAKAGOSHI, H., SUDO, T. and SAWAZAKI, T. *Transcriptional Control by myb Oncogene Product.* Tohoku J. Exp. Med., 1992, **162** (2), 189-194 —— Structure and function of two domains of c-Myb were analyzed. We show that a leucine zipper structure is a component of the negative regulatory domain, because its disruption markedly increases both the transactivating and transforming capacities of c-Myb. Our results suggest that an inhibitor which suppresses transactivation binds to c-Myb through the leucine zipper, and that c-Myb can be oncogenically activated by mis-sense mutation. We also proposed a model, the "tryptophan cluster", for the structure of the Myb DNA-binding domain, in which the three tryptophans form a cluster in the hydrophobic core in each repeat. The results of NMR analysis of repeat 3 revealed that the conserved tryptophans play a key role to make the hydrophobic core. ———— sequence-specific DNA-binding protein; negative regulatory domain; leucine zipper; NMR

Proto-oncogene *myb* encodes a sequence-specific DNA-binding protein (c-Myb) that can activate transcription (Biedenkapp et al. 1988; Ness et al. 1989; Nishina et al. 1989; Weston and Bishop 1989; Nakagoshi et al. 1990). While c-Myb is involved in the regulation of normal haemopoiesis, truncated forms are associated with transformation of haemopoietic cells (Luscher and Eisenman 1990; Sheng-Ong 1990). Analysis of mouse c-Myb has revealed three functional domains responsible for DNA-binding, transcriptional activation, and negative regulation, respectively (Sakura et al. 1989). Deletion of the latter domain, which is largely removed from the proteins encoded by v-*myb* genes, a rearranged c-*myb* gene, and transforming recombinant murine retroviruses, increases transcriptional activation markedly. Interestingly, the negative regulatory domain contains a potential leucine zipper structure. The leucine-zipper motif was originally identified as mediating dimerization of several DNA-binding proteins such as C/EBP (Landshulz et al. 1988) and Jun/Fos (Kouzarides and Ziff 1988; Sassome-Corsi et al. 1988; Gentz et al. 1989; Turner and Tjian 1989), but may

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also mediate interactions between other proteins including certain membrane proteins. We show here that the leucine repeat structure of c-Myb is essential for the function of the negative regulatory domain and that it can bind specifically to other cellular proteins.

In the DNA-binding domain of the *myb*-related proteins including the A-*myb* and B-*myb* gene products (Nomura et al. 1988; Mizuguchi et al. 1990) and the C1 protein of *Zea mays* (Paz-Ares et al. 1987), there are 3 perfectly conserved tryptophans in each repeat with an interval of 18 or 19 amino acids, and the tryptophans between adjacent repeat are separated by 13 amino acids (Anton and Frampton 1988). Moreover, in a DNA-binding domain of the BAS1 protein (Tice-Baldwin et al. 1989), which is required for activation of GCN4-independent *HIS4* transcription in yeast, and also in a region highly conserved between the products of the chicken c-*ets*-1 and c-*ets*-2 genes (Boulukos et al. 1988), which are all presumptive DNA-binding proteins, there is a conserved triplet of tryptophans with spacing similar to that of c-Myb. To examine the role of the conserved tryptophans in the Myb DNA-binding domain, we have undertaken a mutagenesis analysis (Kanei-Ishii et al. 1990). Our resutls show that these tryptophans are essential for the DNA binding and can be replaced by hydrophobic amino acids. Raman spectroscopic study has indicated that these tryptophans are buried in the protein core. Based on these results and on previous data, we proposed a model for the structure of the Myb DNA-binding domain which involves a cluster of typtophans in the hydrophobic core. To clarify the role of three tryptophans as well as three repeats, we have examined the three-dimentional structure of the third repeat in aqueous solution by nuclear magnetic resonance (NMR).

Results

The negative regulatory domain of c-Myb lies within the C-proximal portion of the protein, between amino acids 326–500, and contains a potential leucine zipper structure comprising amino acids 375–403. This region is predicted to form an amphipathic α helix and contains characteristic hydrophobic residues at every seventh position (Fig. 1). To examine whether the putative leucine zipper is involved in negative regulation, we introduced single and multiple amino acid substitutions into this region (Fig. 1). The effects of these mutations on the transactivating capacity of c-Myb were assayed by cotransfection experiments using a reporter plasmid pA10CAT6MBS-I in which the SV40 early promoter and six tandem repeats of a Myb-binding site (MBS-I) were linked to the CAT gene as described (Nishina et al. 1989; Nakagoshi et al. 1990). Replacement of the leucines at the third and fourth positions by proline residues (mutant L34P) would be expected to distort the α-helix and reduce hydrophobicity. As shown in Fig. 1, this resulted in 3-, 10-, and 17-fold increases in transcriptional activation (compared to wild-type c-Myb) in CV-1, NIH3T3, and HeLa cells, respectively.

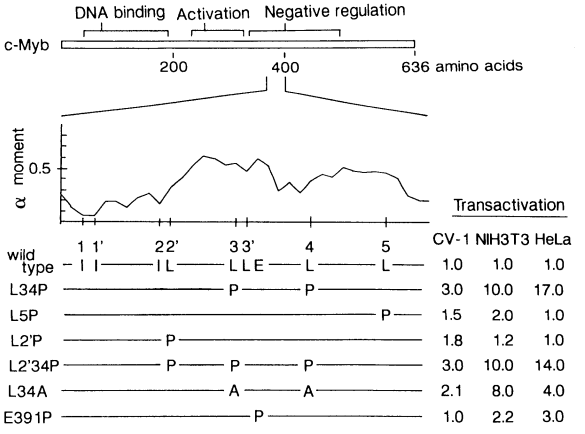

Fig. 1. Mutations introduced within the leucine zipper region of c-Myb. The three functional domains of mouse c-Myb are shown at the top. The probability of c-Myb residues 373 to 407 assuming an α-helical structure of is also indicated by the α moment. The leucine zipper is shown below where the heptad repeat hydrophobic amino acids of the zipper are numbered 1 to 5. The hydrophobic amino acids of another leucine zipper-like structure are also numbered 1′ to 3′. The mutants are named according to the position of the mutation and the substituted amino acids. The results of transactivation assays are indicated on the right, and activities are expressed relative to that of the wild-type protein.

This effect was not due to an increase in protein stability, since the levels of wild-type and L34P protein in transfected CV-1 cells were not appreciably different. Taken together, the effects of the mutations illustrated in Fig. 1 on transactivation imply that both the hydrophobicity of the heptad repeat leucine residues and the (predicted) α-helical structure are required for full activity of the negative regulatory domain, although some perturbation of secondary structure can apparently be accommodated.

We also examined the effect of disrupting the putative leucine zipper on transforming capacity. L5P, L34P and L2′34P mutations clearly increase the transforming capacity of Myb to levels comparable to those of the carboxyl truncations. The data reveal a correlation between increased transactivation and transformation by these mutants, in agreement with our previous report (Hu et al. 1991).

The results presented here indicate that two activities of c-Myb — transactivation and transformation — are negatively regulated through the putative leucine zipper and imply the existence of an inhibitor of c-Myb, which is likely to be important in the normal regulation of its activity. Clearly, isolation and biochemical characterization of the inhibitor are necessary to validate and elaborate on such models. The variation in both the degree of transactivation by wild-type c-Myb and in the effect of disruption of the putative leucine zipper

between cell types, may reflect differing levels of the putative inhibitor(s), as also reported for inhibitors of c-Jun and AP-1.

To dissect the structural basis of the c-Myb DNA-binding domain, we introduced single and multiple amino acid substitutions in the periodic tryptophans of mouse c-Myb. Mutant c-Mybs were expressed in *E. coli* and used for DNase I footprinting. A single or multiple mutation of the periodically arrange tryptophans in c-Myb to hydrophilic amino acids or alanines abolishes or greatly reduces the sequence-specific DNA-binding activity, but the hydrophobic amino acids can replace these tryptophans without significant loss of DNA-binding activity. We have constructed a model for the structure of the Myb DNA-binding domain which is consistent with most of the available data (Fig. 2). In this model, each repeat consists of an arm in the N-terminal side and three α-helices in the C-terminal side and the three tryptophans form a cluster in the hydrophobic core. Therefore, we named this model structure the "tryptophan cluster". A connection between the three repeats 1, 2, and 3 can be made from the extended arm to the third helix without disruption of each repeat structure nor steric clash between them. This leads to a head-to-tail tandem repeat structure, In repeat 2, ten basic residues are localized on the second helix side. These results suggest that repeat 3 interacts with DNA in a sequence-specific manner through the helix 3 in the most C-terminal region, and repeat 2 and 1 may bind to DNA nonspecifically via electorostatic interactions between positively-charged surfaces

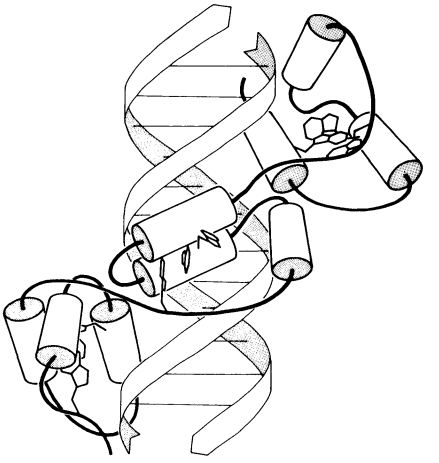

Fig. 2. A model of a DNA-binding mode of Myb. In this model, the three repeats of the Myb DNA-binding domain bind to DNA in tandem such that the orientation of adjacent repeats is almost perpendicular with each other. In each repeat, one of the helices fits into the major groove of DNA. In this figure, helix 3 (the one at the C-terminal end) in each repeat faces DNA, with its helix axis running parallel with the major groove. In the case of repeat 2 (the unit in the center), it is likely that helix 2, rather than helix 3, faces DNA, considering the charge distribution of the surface.

and negatively-charged phosphate backbones.

To confirm whether the DNA-binding domain of Myb has the "tryptophan cluster" structure, we have determined the structure of repeat 3 by NMR. Results of NMR analysis revealed that repeat 3 consists of three helices maintained by a hydrophobic core which includes the three conserved tryptophans, together with two histidines. The three dimensional structure obtained here may help to elucidate how a group of proteins including Myb and Ets binds to DNA and regulates transcription. The structure of Myb DNA-binding domain will also be helpful to clarify the mechanism by which the phosphorylation inactivates the DNA-binding of c-Myb, since recently the phosphorylation of c-Myb at an N-terminal site near its DNA-binding domain casein kinase II was demonstrated to inhibit the DNA-binding of c-Myb (Luscher et al. 1990).

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