Transcriptional *trans*-repression by the *c-myb* proto-oncogene product

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Received May 30, 1989; Revised and Accepted August 22, 1989

ABSTRACT

We report that the *c-myb* protein binds to another site, MBS-II, in the SV40 enhancer with low affinity. In co-transfection experiments with a *c-myb* expression plasmid, tandem repeats of the sequence containing the MBS-II site induced *c-myb*-dependent transcriptional repression. Results of mutational analyses of the sequence around the MBS-II site suggested that the *c-myb* protein represses transcription by competing with another *trans*-activator. These results indicate the *c-myb* protein can regulate transcription not only positively but also negatively.

INTRODUCTION

The *c-myb* gene is the cellular homolog of the viral oncogene *v-myb* carried by the chicken leukemia viruses AMV and E26 (1, 2), which is involved in cellular transformation of hemopoietic cells (3, 4). High expression of *c-myb* is observed in immature hemopoietic cells and decreases during differentiation (5–8). This cell-type specificity of the *c-myb* expression is probably reflected in the ability of retroviruses carrying *v-myb* oncogene to transform myeloid cells but not fibroblasts. However, expression of the *c-myb* gene has been detected in IL-2-stimulated peripheral T-cells, some non-hemopoietic cell lines, and some malignant tissues (9–13).

Both the *c-myb* and *v-myb* proteins are localized in the nucleus and bind to DNA directly (14, 15). Analysis of the purified p135^{gag-myb-ets} protein from mutants of E26 virus, temperature-sensitive for myeloblast transformation, showed that DNA binding is important for the biological activity of *v-myb* (14). Both *v-myb* and *c-myb* protein have a sequence-specific DNA-binding activity and recognize the sequence 5'-(C/Pu)(Py/A)PyAACPyPu-3' (16, Nakagoshi *et al.*, manuscript submitted). We have found that the mouse *c-myb* protein bound to the MBS-I site, which overlaps the GT-I and GT-II motifs, in the SV40 enhancer with high affinity, and the MBS-I sequence functioned as a *c-myb*-dependent enhancer element (Nakagoshi *et al.*, manuscript submitted).

The SV40 enhancer is composed of multiple sequence motifs that act synergistically to stimulate transcription from the SV40 early promoter (17–21). These sequence motifs are organized in two functional domains, A and B, whose multimerization generates enhancer activity (17). The mechanism by which the interdigitated control elements of the SV40 enhancer operate to trigger RNA synthesis is still unknown, but it is evident that an important step toward unraveling the function of these complex regulatory sequences is the characterization of the cellular factors that are responsible for recognizing and interacting with them. Analysis of fractionated cell extracts showed that the SV40 enhancer is recognized by specific DNA-binding proteins such as AP-1 (22), which is encoded by the *c-jun* proto-oncogene (23), and AP-2 (24).

Here we report the identification of a second *c-myb*-binding site, MBS-II, in the SV40 enhancer and the discovery that tandem repeats of the sequence containing MBS-II can be a *c-myb*-dependent silencer.

MATERIALS AND METHODS

Plasmid construction. To generate the reporter plasmid pMFcolCAT6SV-II containing six tandem repeats of SV-II sequence, the double stranded-oligonucleotide SV-II shown in Fig. 1 was chemically synthesized, annealed, phosphorylated with T4 polynucleotide kinase, and ligated with *Bam*HI-digested pUC19. A plasmid containing three tandem repeats of SV-II was isolated (pUC3SV-II). The *Hind*III-*Sma*I fragment containing three tandem repeats of SV-II was isolated from pUC3SV-II and inserted into the *Hinc*II and *Hind*III sites of pUC3SV-II to generate a pUC plasmid containing six tandem repeats of SV-II (pUC6SV-II). The *Eco*RI-*Hind*III fragment containing six tandem repeats of SV-II prepared from pUC6SV-II was then inserted into the *Bam*HI site of the pMFcolCAT-SVE plasmid, which is about 100 bp downstream of the poly A addition signal for CAT mRNA, using a *Xho*I linker (Fig. 1B). The plasmid pMFcolCAT-SVE contains a sequence of the mouse α 2(I) collagen gene from approximately 2000 bp upstream of the start of transcription (+1) to 159 bp downstream of this site, fused to the CAT gene (25). The plasmid pMFcolCAT6SV-III containing the six repeats of SV-III was constructed similarly. SV-II and SV-III were oligomerized 6 times head-to-tail in the plasmids pMFcolCAT6SV-II and pMFcolCATSV-III. The plasmid pUC1SVE containing the SV40 enhancer, which covers the sequence between +113 and +270 of the SV40 map, was described elsewhere (Nakagoshi *et al.*, manuscript submitted).

To construct the reporter plasmids pMFcolCAT6MBS-I, pMFcolCAT6SV-II_{m1}, pMFcolCAT6SV-II_{m2}, pMFcolCAT6SV-II_{m3}, pMFcolCAT6SV-II_{m4}, and pMFcolCAT6SV-II_{m5}, the oligonucleotides MBS-I (shown in Fig. 1), SV-II_{m1}, SV-II_{m2}, SV-II_{m3}, SV-II_{m4}, and SV-II_{m5} (shown in Fig. 4) were chemically synthesized, annealed, and phosphorylated with T4 polynucleotide kinase. After ligation of each double-stranded DNA, products were separated on a polyacrylamide gel and a DNA fragment containing six tandem repeats of each sequence was isolated. Each DNA fragment was then inserted

Figure 1. Delineation of sequences within the SV40 enhancer responsible for *c-myb*-induced *trans*-repression. (A) Structure of the DNA segments SV-II, SV-III, and MBS-I. SV-II, SV-III, and MBS-I contain sequences from the wild-type SV40 from positions 184–218, 257–290, and 246–267, respectively (BBB numbering system, ref. 36). L, late- coding strand; E, early-coding strand. (B) Schematic representation of the reporter plasmids containing tandem repeats of SV-II, SV-III, and MBS-I. Tandem repeats of the synthetic oligonucleotide SV-II, SV-III, or MBS-I were inserted into the *Bam*HI site of the plasmid pMFcolCAT-SVE in which the mouse α 2(I) collagen promoter was linked to the CAT gene (25). (C) Repression of CAT activity by *c-myb*. Mixtures of the indicated reporter plasmid DNA, effector plasmid DNA (pact1 [–] or pact-*c-myb* [+]), and pRSV- β -gal plasmid DNA for internal control were transfected into CV1 cells. In the effector plasmid pact-*c-myb*, a murine *c-myb* cDNA (8) was linked to the chicken β -actin promoter, while the effector plasmid pact1 lacks *c-myb* sequences (27). The plasmids pMFcolCAT6SV-II, pMFcolCAT6SV-III, and pMFcolCAT6MBS-I contain 6 repeats of SV-II, 6 repeats of SV-III, and 6 repeats of MBS-I, respectively, at the *Bam*HI site of pMFcolCAT-SVE. In the case of the reporter plasmid pMFcolCAT6SV-III, 6-fold more amount of extracts were used for CAT assays than the case of other reporter plasmid, because the level of CAT activity expressed from this reporter plasmid was low. The level of CAT activity (as percent conversion) are also shown below each lane. (D) Primer extension analysis of CAT RNA. Mixtures of the indicated reporter plasmid DNA, effector plasmid DNA (pact1(–) or pact-*c-myb*(+)), and pRSV- β -gal plasmid DNA were transfected, and RNA prepared. Primer extension analysis was done using a primer complementary to the CAT mRNA. Correctly initiated transcripts from the mouse α 2(I) collagen promoter are indicated by arrow. ³²P-labeled *Hinf*I-digested pBR322 was used as a size marker.

Figure 2. Binding of the bacterially synthesized *c-myc* protein to the MBS-II site. Results of DNase I footprint analyses obtained using the DNA fragment containing the SV40 enhancer or six tandem repeats of SV-II are shown. The appropriate DNA fragments were ^{32}P -end-labeled on the late strand (see *Materials and Methods*). The ^{32}P -labeled DNA fragment was incubated with 30 ng (lane 2), 60 ng (lane 3), 120 ng (lane 4), or 200 ng (lane 5) of the bacterially synthesized *c-myc* protein. In control experiments, ^{32}P -DNA fragment was incubated without any protein (lanes marked -). A+G refers to the adenine and guanine marker obtained by partial digestion of the same end-labeled DNA fragment. The positions of the protected regions are indicated at the side of each figure.

into the *Bam*HI site of the plasmid pMFcolCAT-SVE using *Bam*HI linkers after blunt-ending with Klenow polymerase. In these reporter plasmids, each of the oligonucleotides was oligomerized 6 times head-to-tail.

DNA transfection, CAT assay and primer extension analysis. Mixtures of 6 μg of reporter plasmid DNA, 6 μg of effector plasmid DNA and 2 μg of pRSV- β -gal plasmid DNA (26)

Figure 3. Schematic representation of the results of DNase I footprinting experiment using the SV40 enhancer. Top, the SV40 early promoter region containing two tandem repeats of the 72-bp sequence. Location of enhancer sequence motifs (32) and three *c-myb* protein binding sites (one MBS-I and two MBS-II sites) are indicated. The sequences of the late-coding strand protected by the *c-myb* protein are also shown.

were transfected into CV1 cells, and CAT assay was done as described (27). The amounts of cell extract used for the CAT assays were normalized with respect to β -galactosidase activity. Forty hours after transfection, RNA was isolated by the guanidium thiocyanate method (28), and at the same time β -galactosidase activities were measured in a sample of cells to confirm that transfection efficiencies were similar. Primer extension analysis was done with 50 μ g of total RNAs as described previously (29). A synthetic 24 base oligonucleotide complementary to nucleotides 26–49 of the coding sequence of the CAT gene was used as a primer.

Purification of the bacterially synthesized *c-myb* protein. The insoluble material containing the *c-myb* protein was prepared from BL21(DE3) bacteria carrying the *c-myb* expression plasmid pAR2156*myb* as described (30), and dissolved in urea buffer (50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 6 M urea, 5% [v/v] glycerol, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], and 0.1 mM dithiothreitol [DTT]). After standing on ice for 2 h, the sample was centrifuged at 100,000 g for 2 h, and the supernatant was dialyzed against TDE buffer (10 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM EDTA). The *c-myb* protein in this dialyzed sample was about 20 to 30% of total proteins. This protein sample containing about 500 μ g of *c-myb* protein was mixed with 300 μ l of an affinity matrix that was made using the synthetic MBS-I oligonucleotide as a ligand as described by Kadonaga and Tjian (31), and left at 4°C for 8 h. After the matrix was packed in a column and washed with buffer Z (25 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM DTT, 5% [v/v] glycerol, 0.1% Nonidet P40 [NP40]) containing 0.1 M NaCl, the bound material was eluted with 1.0 M NaCl (volume of each fraction was 0.3 ml), dialyzed against TDE buffer, and used for DNase I footprint analysis.

DNase I footprint analysis. For DNase I footprint experiments, the *EcoRI-PstI* fragment of the plasmid pUC6SV-II or the *HindIII-SacI* fragment of the plasmid pUC1SVE was 32 P-labeled at the 5' end of the late-coding strand. DNA-binding reactions and DNase I

digestions were done in a 50- μ l volume with 3–5 ng (about 10–20 fmol) of an end-labeled DNA fragment and variable amounts of purified *c-myb* protein synthesized in *E.coli*, in a final buffer concentration of 25 mM Hepes, pH 7.9, 0.5 mM EDTA, 50 mM KCl, 10% (v/v) glycerol, 0.5 mM PMSF, and 0.5 mM DTT. Reactions were incubated for 20 min at 25°C and then digested for 60 sec at 25°C with 1–5 μ l of freshly diluted 5 μ g/ml solution of DNase I (Takara Shuzo Co.) after addition of MgCl₂ to a final concentration of 5 mM. The digestion products were separated on a denaturing 6% polyacrylamide gel and visualized by autoradiography.

RESULTS

SV-II sequence is responsible for the c-myb-induced trans-repression. Using a co-transfection experiment with a *c-myb* expression plasmid, we had found that the tandem repeats of the SV40 enhancer are responsible for the *c-myb*-induced transcriptional *trans*-activation (27). The SV40 enhancer region consists of multiple enhancer motifs such as P, Sph-I, and GT-I (Fig. 1A), each of which binds specific *trans*-activators (32). To identify the exact position of the sequence responsible for the *c-myb*-induced *trans*-activation in the SV40 enhancer, we had chemically synthesized segments of double stranded-DNA each of which contained sequences corresponding to various portions of the SV40 enhancer. Six tandem repeats of each segment were inserted into the *Bam*HI site of the plasmid pMFcolCAT-SVE in which the CAT gene was linked to the mouse α 2(I) collagen promoter (Fig. 1B), and these plasmid DNAs, such as pMFcolCAT6SV-II, were transfected into CV1 cells together with the *c-myb* expression plasmid (pact-*c-myb*) or the control plasmid (pact1). When the reporter plasmid contained six tandem repeats of the segment containing the MBS-I (*myb*-binding site-I), the level of CAT activity was stimulated about 26-fold by *c-myb* (Fig. 1C, lanes 1 and 2, and Nakagoshi *et al.*, manuscript submitted). In contrast, *c-myb* repressed the level of the CAT activity to about 1/3 in the case of the reporter plasmid containing six copies of SV-II (Fig. 1C, lanes 3 and 4). The level of CAT gene expression from the reporter plasmid containing six copies of SV-III, which did not contain the MBS-I site and did not overlap SV-II, was not affected by *c-myb*, although the level of CAT activity expressed from this reporter plasmid was low (Fig. 1C, lanes 5 and 6).

To check whether the CAT enzyme activities were correlated with the level of correctly initiated RNA, primer extension analysis was done with a primer complementary to a segment of CAT mRNA. If CAT RNA starts at the correct site in the mouse α 2(I) collagen promoter, the extended primer should run as a doublet of 246–247 nucleotides (162 nucleotides of α 2(I) collagen mRNA plus 85 nucleotides of CAT mRNA). In CV1 cells transfected with pMFcolCAT6SV-II, the intensity of the 246-nucleotide band was decreased to about 1/4 by *c-myb* (Fig. 1C), indicating that *c-myb* repressed specific and correct initiation at the α 2(I) collagen promoter. In the cells transfected with pMFcolCAT6SV-III, the intensity of the 246-nucleotide band was not affected by *c-myb*, indicating that the levels of CAT RNA were correlated with those of CAT enzyme activities.

Sequence-specific binding of the bacterially synthesized c-myb protein to the MBS-II site. We had made a mouse *c-myb* expression plasmid in *E.coli* using a T7 expression vector and obtained large quantities of the *c-myb* protein (30). The *c-myb* protein synthesized in *E.coli* contains 25 amino acid residues derived from the vector and part of the 5' untranslated region of the *c-myb* cDNA clone fused to the 636 amino acid residues of the complete *c-myb* protein. This bacterially synthesized *c-myb* protein has been shown to bind to the MBS-I site in a sequence-specific manner and has been purified by a sequence-

specific affinity column in which the MBS-I oligonucleotide was used as a ligand. To examine whether the *c-myb* protein binds directly to the SV-II sequence, this highly purified *c-myb* protein and the DNA fragment containing six tandem repeats of SV-II was used for DNase I footprint analysis. A 15-bp sequence (MBS-II), which overlaps the P and Sph-I motifs, was protected by the *c-myb* protein (Fig. 2 and 3). The MBS-I site was completely protected by 120 ng of *c-myb* protein, but the MBS-II site in 6SV-II was only partially protected by 200 ng of *c-myb* protein (compare Fig. 2, lanes 1 and 4). Therefore, the bacterially synthesized *c-myb* protein bound to the MBS-II site with lower affinity than to the MBS-I site. When the sequences of MBS-I and MBS-II sites were compared, obvious homology was observed and 9 of the 15 bp were identical (Fig. 3).

Mutational analysis of SV-II sequence. To delineate the sequence responsible for the *c-myb*-induced *trans*-repression more precisely, we introduced a series of mutations into the SV-II sequence (Fig. 4). Six tandem repeats of each mutated sequence were inserted into the *Bam*HI site of the pMFcolCAT-SVE plasmid, in which the mouse $\alpha 2(I)$ collagen promoter was linked to the CAT gene, and these plasmids were used as before in co-transfection experiment. The level of CAT activity expressed from the reporter plasmid containing six copies of the normal SV-II sequence was repressed to 1/3 (Fig. 4B). When the reporter plasmids containing mutations 1 and 3, which are located in the Sph-II and the Sph-I motifs, respectively, were used, *c-myb* repressed the level of CAT activity to 1/5 and 1/2, respectively (Fig. 4B). On the other hand, the level of CAT activity was not affected by *c-myb* in the case of mutations 4 and 5, which are located in the P motif (Fig. 4B). Prevention of the *trans*-repression by *c-myb* with mutations 4 and 5 is consistent with the observation that both mutants failed to bind the bacterially synthesized *c-myb* protein (data not shown). Interestingly, *c-myb* stimulated the level of the CAT activity expressed from the reporter plasmid containing mutation 2, which is located in the octamer motif, although the mutation decreased the basal level (Fig. 4B). These results suggest that at least two enhancer-binding factors, the binding or function of which are prevented by mutations 2 and 4, are necessary for the expression of the CAT gene linked to tandem repeats of SV-II. Moreover the results suggest that the interaction or competition for binding between these factors and the *c-myb* protein might mediate *trans*-repression by *c-myb*.

DISCUSSION

We have demonstrated that in addition to the MBS-I site the bacterially synthesized *c-myb* protein binds to another site, MBS-II, which overlaps the P and Sph-I motifs, in the SV40 enhancer, and that the SV-II sequence containing the MBS-II site is a *c-myb*-dependent silencer element. It is worth noting that enhancer activity was dominant in the case of tandem repeats of the SV40 enhancer, and in fact led to high levels of *myb*-induced CAT activity (27). So far, two examples of *trans*-activators that can also function as negative regulators have been reported. The glucocorticoid receptor was shown to negatively regulate expression of the human glycoprotein hormone α -subunit gene through interference with a cAMP responsive enhancer (33). The second example is the thyroid hormone receptor which inhibits estrogen-dependent *trans*-activation (34). It was speculated that these two *trans*-activators can repress transcription by competing with the cAMP response element-binding protein and the estrogen receptor, respectively, because the binding sites of two *trans*-acting factors overlap (33, 34). In the case of *trans*-repression by *c-myb*, the same kind of mechanism is possible. It is unlikely that binding of the *myb* protein to MBS-II site reduces progression of RNA polymerase II through this region, or impede the

Figure 4. Mutational analysis of SV-II sequences responsible for *c-myb*-induced *trans*-repression. (A) Structure of DNAs containing tandem repeats of the mutated SV-II sequence. Each mutated fragment contains the same sequence as SV-II except for the 5 altered nucleotides shown in addition to flanking region containing *Xho*I restriction enzyme sites. Six tandem repeats of each DNA were inserted into the *Bam*HI site of the plasmid pMFcolCAT-SVE. (B) Transient expression of CAT activity. Mixtures of each reporter plasmid shown below, the effector plasmids (pactI [-] or pact-*c-myb* [+]), and pRSV- β -gal plasmid were transfected into CV1 cells, and CAT activity assayed. In the right of the figure, the results obtained using 3- (pMFcolCAT6SV-IIIm2 reporter plasmid) or 6-fold (pMFcolCAT6SV-IIIm4 reporter plasmid) more amount of extract were shown. The level of CAT activity are also shown below each lane as percent conversion.

polyadenylation process, since the *c-myb*-induced transcriptional repression was not observed when tandem repeats of MBS-I were placed in the *Bam*HI site of pMFcolCAT-SVE (Fig. 1C, lanes 1 and 2). Several enhancer-binding proteins such as AP-1 (22) and TEF-1 (21), which have binding sites that overlap with the *c-myb*-binding site, could be candidates for the *trans*-activator with which the *c-myb* protein competes. Moreover, because sequences outside the *c-myb*-binding site (mutation 2 in Fig. 4) are also needed for *c-myb*-induced *trans*-repression, interaction between the *c-myb* protein and the octamer-binding factor, OTF-1 (35), is also possible. It is also worth noting that transcription was not repressed by *c-myb* in the construct which had one copy of SV-II (data not shown). The level of transcription from pMFcolCAT6SV-II was about 10-fold higher than that from the construct containing one copy of SV-II (data not shown), indicating that tandem repeats of SV-II has an enhancer activity, but one copy of SV-II does not. Therefore, binding of the *c-myb* protein to MBS-II site in the construct containing one copy of SV-II may compete with other enhancer-binding factor(s), but not induce transcriptional repression, since the enhancer-binding factor(s) does not activate transcription in this construct.

It is not clear whether *trans*-activation, *trans*-repression, or both activities of *c-myb* are necessary for transformation, because both activities of the *v-myb* protein are lower than the *c-myb* protein (Ishii, *et al.* unpublished results). Therefore, it will be also important to clarify which target gene is stimulated or repressed by the *c-myb* protein. It is clear that while identification of the *in vivo* target genes will be necessary to resolve such questions, further analyses of regulation of gene expression by the *c-myb* gene product should lead to valuable insights into the mechanism of cellular transformation and differentiation.

ACKNOWLEDGEMENT

We are grateful to Drs. Thomas J. Gonda and Robert G. Ramsay for critical reading of the manuscript.

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