Phosphorylation dependent down-regulation of c-Myb DNA-binding is abrogated by a point mutation in the v-myb oncogene

Kristin Brevik Andersson¹§#, Elisabeth Kowenz-Leutz²#, Elen Margrethe Brendeford¹, Ann-Helen Herwig Tygsett¹, Achim Leutz² and Odd S. Gabrielsen¹

¹Department of Biochemistry, University of Oslo, N-0316 Oslo 3, Norway
²Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, 13092 Berlin, Germany
§ Present address: Institute for Experimental Medical Research, Ullevål Hospital, 0407 Oslo, Norway
#The two first authors contributed equally to the work.

Running title: Phosphorylation of c-Myb DBD – abrogated in v-Myb

Correspondence to:
Odd S. Gabrielsen, Department of Biochemistry, University of Oslo,
P.O.Box 1041 Blindern, 0316 Oslo, Norway
Phone no. +47-22 85 73 46; Fax no. +47-22 85 44 43
e-mail: O.S.Gabrielsen@biokjemi.uio.no
SUMMARY

The viral Myb (v-Myb) oncoprotein of the avian myeloblastosis virus (AMV) is an activated form of the cellular transcription factor c-Myb causing acute monoblastic leukemia in chicken. Oncogenic v-Myb alterations include N- and C-terminal deletions as well as point mutations. Whereas truncations in Myb cause loss of various protein modifications, none of the point mutations in v-Myb has been directly linked to protein modifications. Here we show that the DNA-binding domain of c-Myb can be phosphorylated on serine 116 by the catalytic subunit of PKA. Phosphorylation of S116 differentially destabilizes a subtype of c-Myb-DNA complexes. The V117D mutation of the AMV v-Myb oncoprotein, abolishes phosphorylation of the adjacent S116 residue. Modification of S116 was also detected in live cells in c-Myb, but not in AMV v-Myb. Phosphorylation-mimicking mutants of c-Myb failed to activate the resident mim-1 gene. Our data imply that PKA or a kinase with similar specificity negatively regulates c-Myb function, including collaboration with C/EBP, and that the leukemogenic AMV v-Myb version evades inactivation by a point mutation that abolishes a phospho-acceptor consensus site. This suggests a novel link between Myb, a signal transduction pathway, co-operativity with C/EBP and a point mutation in the myb oncogene.
INTRODUCTION

The \( v-myb \) oncogene and its cellular progenitor \( c-myb \) both encode transcription factors that are implicated in the switch between growth and differentiation of hematopoietic cells (reviewed in (1-4)). The c-Myb protein consists of a N-terminal DNA-binding domain (DBD), a central transactivation domain (TAD) and a C-terminal negative regulatory domain (NRD). The DBD of c-Myb is comprised of the three imperfect repeats: \( R_1 \), \( R_2 \) and \( R_3 \), each related to the helix-turn-helix motif (5-7). \( R_1 \) is deleted in \( v-Myb \) and the \( R_2 \) and \( R_3 \) repeats are sufficient for sequence-specific DNA binding (hereafter termed \( \text{mDBD} \), minimal DNA-binding domain). This prototype Myb mDBD is highly conserved throughout evolution in the animal and plant kingdoms (8).

A large body of evidence supports a central role for c-Myb in regulating cell growth and differentiation (reviewed in (4)), in particular in hematopoietic progenitor cells of different lineages (9-15). This function is consistent with phenotypes observed in mice with a \( c-myb \) \text{null} mutation and in homozygous null \( c-Myb/Rag1 \) chimeric mice (11, 13). The c-Myb protein has also been reported to be expressed in a variety of tissues in developing embryos and/or in adult tissues such as tooth buds, retina and epithelium of the gastrointestinal and respiratory tracts and skin (16-19). Consistent with this expression pattern, c-Myb was found to be required for colon development in mice (20).

Activated forms of c-Myb have been associated with cancer in animal models (21). In mice, retroviral insertions may result in N-terminal truncation of the c-Myb protein and deregulated expression, leading to myeloid leukemia (22). In chickens, the two retroviruses AMV and E26, which carry truncated Myb proteins, elicit myeloid leukemia and erythroblastosis/stem cell leukemia, respectively (reviewed in (23)). These viruses encode the \( v-Myb \) proteins p48\( ^{v-myb} \).
(AMV-derived) and the Myb-Ets fusion protein p135<sup>pag-myb-ets</sup> (E26-derived), respectively. Both v-Myb proteins are truncated in the N- and C-terminal ends. In addition, the p48<sup>v-myb</sup> protein carries 10 point mutations relative to c-Myb (23).

AMV v-Myb has been an instructive model for understanding oncogenic activation and the normal function of c-Myb. AMV v-<i>myb</i> is a potent oncogene that induces monocytic leukemia after very short latency (24). A distinctive feature of AMV v-Myb is its cell-type specificity inducing transformation of the macrophage lineage (23). Since the oncogenic alterations include both N- and C-terminal deletions as well as point mutations, AMV v-Myb probably utilizes multiple mechanisms that synergize in oncogenicity and cell type specificity.

Although the cellular phenotypes induced by AMV v-Myb are well characterized, much remains to be learned about the molecular mechanisms involved. Several studies have attempted to define oncogenic determinants of v-<i>myb</i>. These studies have revealed that the N- and C-terminal deletions remove several sites of protein modification. The v-Myb protein lacks N-terminal CK2 phosphorylation sites (S11 and S12) present in the wild type protein. Phosphorylation of these sites reduced specific DNA-binding (25). Substitution of the two serines to alanines increased the transcriptional activity on both the <i>mim</i>-1 and neutrophil elastase promoters (26). However, loss of the CK2 sites is not sufficient to turn <i>myb</i> into an oncogene (27, 28). A MAPK phosphorylation site (S528) in the C-terminal negative regulatory domain is also deleted in AMV v-Myb. Substitution of this serine to alanine increased the transcriptional capacity of c-Myb on some promoters but not on others (29-31). Two studies have described <i>in vitro</i> phosphorylation sites for PKA in c-Myb. PKA phosphorylation of S8 in the N-terminus resulted in partial inhibition of DNA binding (32), whereas phosphorylation of S116 in the DNA-binding domain was suggested to have no effect on DNA binding (33). Recently, c-Myb was
found to be acetylated within the C-terminal NRD by the histone acetyltransferases p300 and CBP both \textit{in vitro} and \textit{in vivo} (34, 35). The modification sites were mapped to positions K438 and K441 in mouse c-Myb and to K471, K480, and K485 in human c-Myb. The corresponding residues in chicken c-Myb (K443+ K446, and K472, K481 + K486) are all deleted in AMV v-Myb. The truncations in AMV v-Myb may also cause changes in protein-protein interactions. The N-terminal deletion permitted cyclin D to inhibit transcriptional activation in a CDK-independent fashion (36).

The AMV-specific point mutations in the second repeat R2 of the v-Myb DBD perturb normal Myb functions through altered protein-protein interactions (37). We have previously shown that functional aspects of DNA binding are also altered (38). In addition, the point mutations determine the target genes Myb regulates and the proteins it interacts with (39-41). It therefore appears that the v-Myb DBD has accumulated gain- and loss-of-function mutations to escape regulation during proliferation and differentiation (40).

Although point mutations in the AMV v-Myb DBD have been linked to alterations in function and protein-protein interactions, none of them has been found to affect Myb-protein modifications. The fact that one of the mutations, V117D, is located adjacent to a reported phosphorylation site for protein kinase A (PKA) (33) led us to re-examine phosphorylation at S116. We found that S116 was phosphorylated by PKA \textit{in vitro} and that this modification strongly affected the ability of c-Myb to bind to a subgroup of Myb recognition elements (MRE) \textit{in vitro}. Phosphorylation of the S116 site is abrogated in oncogenic AMV v-Myb, due to the V117D mutation adjacent to S116. We developed a phospho-c-Myb(S116)-specific antibody to demonstrate the presence of this modification in c-Myb and its absence in AMV v-Myb in cells. Furthermore, a mutant c-Myb protein (S116D, phosphorylation mimic) failed to cooperate with
NF-M (C/EBP\[\]) in the activation of the resident \textit{mim-1} gene. These findings implicate PKA or a kinase with similar specificity in a novel type of regulation of c-Myb function from which v-Myb has escaped.

\textbf{EXPERIMENTAL PROCEDURES}

\textit{Constructions and plasmids}

The mutations S116A, S146A, S116D, S116E, V117D, S116A/S146A were introduced into the chicken c-Myb minimal DNA-binding domain (mDBD) R\textsubscript{2}R\textsubscript{3} as previously described (5) or with the QuikChange\textsuperscript{TM} mutagenesis kit (Stratagene) using pBET-R\textsubscript{2}R\textsubscript{3} (5) as a template. The backmutation D117V was also introduced into AMV mDBD (R\textsubscript{2}R\textsubscript{3}[AMV\textsubscript{NHBD}]) (38). The mammalian expression plasmids pCIneo-ccM (encoding the chicken c-Myb protein, residue 72-443), pCIneo-AMV (encoding the AMV v-Myb protein, residue 72-440 in chicken c-Myb) and their mutant forms were constructed as follows. The relevant fragments from the chicken \textit{c-myb} gene and the AMV \textit{v-myb} gene were amplified by PCR and inserted into the PCIneo (Promega) vector as a XhoI-NotI fragment behind the CMV (cytomegalovirus) promoter. All constructs were verified by sequencing. Two effector plasmids expressing shortened c-Myb proteins were used, pCIneo-ccM-[72-349] and pCIneo-ccM-[72-443] where the encoded regions are given in parentheses.

\textit{Expression of recombinant proteins.}

Wild type and mutant minimal DNA-binding domains (R\textsubscript{2}R\textsubscript{3}), termed mDBD, were expressed in \textit{E. coli} and purified as previously described (5, 42). The AMV mutant version of R\textsubscript{2}R\textsubscript{3} has previously been characterized (38). The protein concentration of each mDBD domain was measured by optical density at 280 nm (predominantly tryptophan absorption), Bradford
assay and by fluorescence quenching (43) to ensure equal amounts of each protein in the
experimental protocol.

The mDBD domain contains six highly conserved tryptophan residues, which makes the
protein suitable for conformational analysis by fluorescence emission spectrum analysis.
Spectroscopic analyses were performed as described (38, 43).

*In vitro phosphorylation of mDBD proteins*

For phosphorylation site mapping experiments, recombinant wild type and mutant mDBD
c-Myb proteins (8 pmol) were incubated in PKA buffer (20 mM HEPES pH 7.9, 60 mM KCl, 5
mM MgAc, 1 mM dithiothreitol), 5 Ci (3.3 pmol) [γ-32P]ATP (3000 Ci/mmole), 2.5 µg BSA and
3.8 U PKA Cα (Roche) in a final volume of 120 µl. Reactions were incubated at 30 °C for 15
minutes. Reactions were chased with 1.4 µmol ATP and placed on ice before loading on a 15%
SDS-PAGE gel and autoradiography. An alternative buffer (10 mM MgAc, pH ≈ 8.5) +/- 4 U
PKA Cα (Promega) was used for [γ-32P-ATP] labeling of mDBD proteins for phosphoamino
acid analysis. 250 µM ATP was added for quantitative phosphorylation of mDBD proteins for
MS analysis. Phosphoaminoacid analysis of 32P-phosphorylated wild-type and mutant S146A
mDBD domains was performed as described (44). A MALDI-TOF Mass spectrometer (Voyager-
RP DE, Applied Biosystems) was employed to measure the molecular mass of PKA-
phosphorylated wild-type and mutant mDBD domains. All experiments were carried out with the
mass spectrometer in the linear positive ion mode. The total acceleration voltage was 25 kV. The
voltage on the first grid and the delay time between ion production and extraction were adapted
to the mass of the different samples. 100 single scans were accumulated for each spectrum. The
matrix, 3,5-dimethoxy-4-hydroxycinnamic acid (Sigma, D-7927), was dissolved at a
concentration of 15 mg/ml in a mixture of 1:1 acetonitrile/0.1% aqueous TFA. 0.5 µl of sample
and 1.5 µl of matrix were mixed together on the sample plate and air-dried. All data were calibrated using an external calibration standard mixture (Applied Biosystems).

For the determination of Km of PKA for mDBD, various amounts of recombinant mDBD (final concentrations 5 5.6, 5.8, 6.2, 7.6, 10, 15 and 16.6 µM) was incubated in PKA buffer with 500 µM ATP, 3.3 pmol (5 µCi) [γ-32P]ATP, and 46 U PKA C enzyme (Promega) in a final volume of 50µl. The concentration of 500 µM ATP was sufficient for saturating the PKA catalytic subunit. The reactions were terminated by adding 2 µl 0.5 M EDTA at 0, 2, 4 and 6 minutes. Samples were spotted in triplicate onto Whatman P81 paper, washed in 75 mM H3PO4 and dried in 96% EtOH before scintillation counting. The 2-minute time point (in the linear range) was chosen for the calculation of Km.

Quantitative phosphorylation of mDBD proteins for EMSA analysis was performed with 10 µM (200 pmol) mDBD protein in PKA buffer with 500 µM ATP and 80 U PKA C enzyme (Promega) (optimized) in 20 µl at 30 °C for 30 minutes. Reactions were terminated by addition of 2 µl 0.5 M EDTA. Reproducible quantitative phosphorylation of mDBD was >75%, as estimated by incorporation of [32P]-phosphate and by mass spectrometry analysis. Note that the unit definitions for the two PKA enzyme sources used in the study differ: substrates for activity are caseine for the Promega enzyme and Kemptide (45) for the Roche enzyme. One caseine unit is equivalent to 1,6x10⁵ Kemptide units (Roche).

**Electrophoretic Mobility Shift Assay (EMSA).**

DNA binding was monitored by electrophoretic mobility shift assay as described in (5, 42). In general, recombinant mDBD protein (20 fmol) was incubated with 5'-[γ-32P]-labeled MRE oligo probe (10 fmol) in a total volume of 20 µl for 10 minutes at 25 °C before electrophoresis. Binding reactions were run on 6% 0.5xTBE, 5%glycerol PAGE gels at 4°C. Decay EMSA was
performed as described (38). Briefly, protein and \(^{32}\)P-labeled oligo were mixed and incubated at 25 °C for 15 minutes before competition with an excess of unlabeled competitor oligo for various times. Samples were then immediately loaded and run on an EMSA gel. For binding experiments with phosphorylated proteins, proteins were first phosphorylated \textit{in vitro} by PKA C\(_\text{a}\) catalytic subunit (described above) before used in EMSA analysis.

The oligos used for Myb-binding are based on the MRE A site in the \textit{mim-1} promoter (46). All oligo variants of the Myb recognition element (MRE) have been previously described (47) and table 1 in (48). The sequence of the basic \textit{mim-1}A MRE oligo (core Myb binding site in italics) is 5´-A \textit{TAACGG} TCTTTTAGCGC-3´. The GG, TG, GT and TT variants refer to positions 5 and 6 in the core recognition sequence (underlined) and all carry a C to T substitution in position 8 (bold). The non-specific oligo (NSO) 5´-GCATTA \textit{TCAAGC} TTTTTTAGCGC–3´ is a triple mutant variant of the GG MRE oligo. All oligos were end-labelled and purified through a G-25 spin column. Cold double-stranded oligos for competition were similarly annealed. Variant MRE oligos were labelled to the same specific activity (47) and purified through a G-25 spin column.

\textit{Cell Lines}

The c-Myb expressing erythroid cell line HD3, quail QT6 fibroblasts, and the HD11 cell line expressing \textit{v-myc} have been described (49). All cells were grown in Dulbecco’s modified Eagle medium supplemented with 8% fetal bovine serum, 2% heat-inactivated chicken serum, 15 mM HEPES, penicillin, and streptomycin. Cells were maintained in 5% CO\(_2\) atmosphere at 39°C.
Immunoprecipitation and Western analysis

A synthetic peptide coding for the human/chicken c-Myb internal twelve amino acids (C+LVQKYGPKRWSV), in which the second last serine was phosphorylated, was conjugated and used for immunization using the antibodies service of Eurogentec Bel S.A.

For immunoprecipitation 6 x 10⁶ QT6 cells were seeded 5 hrs before transfection in 10 ml tissue culture medium. Cells were subsequently transfected with 10 µg of expression vectors as indicated, using a calcium phosphate coprecipitation technique (50). Cells were harvested 16-18 hr post transfection. For HD3 immunoprecipitation 2x10⁷ cells were used. Cell lysates were prepared in 0,3 ml lysis buffer (20mM Tris pH 7.5, 150 mM NaCl, 1mM EDTA pH8, 10% glycerol, 0,8% NP40, 0.1% Deoxycholate, 1mM DTT, 1 mM PMSF, 10 µg/ml of each aprotinin, pepstatin and leupeptin).

Expressed proteins were precipitated with a pre-immune rabbit serum, a Myb specific rabbit immune serum and a Myb PKA-site specific rabbit immune serum as indicated for 2 hrs at 4 °C. A-Sepharose (Pharmacia) was added, incubated for 1 hr, and washed extensively with lysis buffer. Immunoprecipitates were separated by 10% SDS-PAGE, and transferred to PVDF membranes (Millipore). Precipitated proteins were revealed with a mouse monoclonal antibody directed against c-Myb (MYB2-7.77; ATCC), appropriate peroxidase conjugated second antibodies and subsequently revealed using a chemiluminescence kit (ECL, Amersham).

Transient Transfections, Reporter Assay, and Resident Gene Activation Assay

Plasmids used for transfections were purified twice on CsCl gradients. QT6 and HD11 cells were transfected using DEAE-dextran as described previously (51). To monitor reporter gene activation, cells were harvested after 24 hr, whole cell lysates were prepared, and luciferase
activity was determined as previously described (51, 52). Activation of endogenous genes was determined as previously described ((53, 54).

**RESULTS**

*A PKA phosphorylation site in the c-Myb mDBD (R₂R₃)*

Two putative PKA sites were identified in c-Myb by a Prosite motif search (55), namely S116 (KRWSV) and S146 (KKTSW). Both residues reside in the R₂R₃ minimal DNA-binding domain (mDBD) and conformed well to the PKA consensus motif RRXS/TY (45). An alignment of vertebrate Myb proteins (Fig. 1a) shows that both sites are highly conserved. Mapping of these two sites onto the published NMR structure of mouse mDBD (7) and the recent crystal structure (37), showed that they are both on the surface of the protein and hence should be accessible for interaction with other proteins (Fig. 1b). Note the close proximity of S116 to the DNA sugar-phosphate backbone in the model.

A Myb fragment, consisting of the N-terminus and all three repeats (amino acids 1-192) has been shown to be a phosphorylation target of PKA (56). Pilot biochemical experiments with recombinant c-Myb mDBD domain corresponding to the AMV-myb version (amino acids 89-192) showed that this domain was also readily phosphorylated *in vitro* by PKA catalytic subunit (data not shown). In order to map the PKA phosphorylation site(s), mutant mDBD domains carrying the mutations S116A, S146A and the double mutant S116A/S146A were expressed in *E.coli* and purified by affinity and ion-exchange chromatography (5). To exclude indirect structural effects of the mutations introduced, the overall conformations of the mutant mDBD proteins were compared with wild type mDBD by fluorescence quenching spectroscopy as
described (38). We did not detect any gross structural changes as a consequence of introducing mutations at the S116 and S146 positions of mDBD (data not shown).

Wild type and mutant mDBD domains were used in phosphorylation site mapping experiments with purified PKA C\(\alpha\) catalytic subunit and [\(\gamma\)\(^{32}\)P]ATP. The results in Fig. 2a showed that the S116A mutation (lanes 3, 5) completely abolished the mDBD phosphorylation (lane 2), whereas the S146A mutation (lane 4) had no effect. An SDS-PAGE analysis of the mDBD proteins demonstrated equal amounts of protein in the phosphorylation reactions (Fig 2a, lower panel). Phosphoaminoacid analysis of wild-type and S146A mutant mDBD domains confirmed phosphorylation on serine residues only (Fig. 2b). Mass spectrometry analysis showed the addition of one phosphate group in wild-type and S146A mDBD, but not in S116A mDBD (Fig. 2c). In conclusion, of the three serines present in c-Myb mDBD (S116, S146 and S187), only S116 is a PKA target site.

The Michaelis constant (Km) of mDBD as a substrate for PKA was determined using purified bacterially expressed mDBD, PKA C\(\alpha\) and [\(\gamma\)\(^{32}\)P]ATP as described in the Materials and Methods (Fig. 3). The Km was calculated to be 10 µM. This value is in good accordance with other well-defined substrates for PKA. The determination of S116 in c-Myb as a high affinity site for PKA is consistent with conclusions by Ramsay and co-workers, who reported that S116 in C-terminally truncated recombinant Myb proteins was phosphorylated by PKA in vitro (33).

The oncogenic V117D mutation in AMV-Myb abrogates PKA mediated phosphorylation

In AMV v-Myb, the V117D point mutation located next to the S116 residue abolishes the PKA recognition motif (as defined by an extended consensus, Fig. 1a). To examine whether the V117D mutation potentially interferes with S116 phosphorylation, experiments were performed with recombinant wild type and mutant mDBD proteins and PKA C\(\alpha\) as shown in Figure 4. In
contrast to wild type mDBD, the AMV mDBD (R2R3 carrying the three AMV mutations I91N, L106H, and V117D) (38) was not phosphorylated by PKA (Fig. 4, lanes 2 and 3). Introduction of the single AMV-Myb point mutation V117D in c-Myb also abrogated the PKA phosphorylation of the domain (lane 4). In the reciprocal experiment, back-mutation of the D117 to V117 restored PKA phosphorylation of the AMV-Myb mDBD (Fig. 4 lane 7). We therefore conclude that c-Myb but not v-Myb can be phosphorylated at S116.

*Phosphorylation of S116 affects the stability of c-Myb / DNA interaction*

In the NMR and crystal structures of c-Myb mDBD, the S116 residue is in close proximity to the sugar-phosphate backbone of the DNA helix (see Fig. 1b and (7, 37)). A predicted consequence of phosphorylation would therefore be interference with DNA binding due to electrostatic repulsion. We tested this idea by phosphorylating wild type mDBD in vitro with PKA Cα, and assaying DNA binding on EMSA gels using the mim-1A binding site (MRE) (46) as DNA probe. Quantitative phosphorylation was consistently >75%, as calculated by 32P incorporation and mass spectrometry. As shown in Fig. 5a, the DNA binding activity of phospho-mDBD was reduced (lanes 1 and 3) compared to non-phosphorylated mDBD (lanes 2 and 4). Phosphorylated mDBD migrated also slightly faster in the gel than non-phosphorylated mDBD. Phosphorylation of mDBD diminished DNA-binding, but did not totally abolish complex formation. We confirmed that phospho-mDBD was able to bind to the cognate MRE sequence by running EMSA reactions with [γ-32P]ATP-labeled mDBD protein and unlabeled DNA oligo (Fig. 5b, lanes 2, 3), side by side with ordinary EMSA reactions with unlabeled phospho-mDBD and 32P-labeled DNA oligo (lanes 4, 5). Again, note the reduced binding of phospho-mDBD to the MRE oligo compared to wild type mDBD (lanes 4 and 5).
We have previously shown that subtle differences in DNA binding for c-Myb proteins may be detected by analysis of decay of protein-DNA complexes in EMSA (“decay-EMSA”) (38, 47, 48). After the formation of a protein-DNA complex, a large excess of unlabelled specific DNA oligo is added, and the time course of complex dissociation is followed by analyzing the remaining complexes at different time points by EMSA. Decay-EMSA was performed on wild type mDBD, a S116D mutant (a phosphorylation mimic) and an *in vitro* phosphorylated mDBD proteins (n=4). A typical experiment is shown in figure 5c. Note that threefold more phospho-mDBD protein than wild type or S116D mutant mDBD was loaded onto the gel in order to visualize the protein/DNA complexes. PKA-phosphorylation of the mDBD protein led to a dramatic increase in the dissociation rate of the protein/DNA complex (Fig. 5c, lanes 12-16) compared to the non-phosphorylated mDBD (lanes 2-6). The dissociation rate of the mDBD S116D mutant from the complex was also increased compared to wild type mDBD (compare lanes 7-11). The protein/DNA dissociation rates were estimated to be approximately >20 min, 2-5 min and <1 min for wild type, S116D and phospho-S116 mDBD proteins, respectively (n=4). Note that the S116D mutation in mDBD did not fully mimic PKA-phosphorylated mDBD with regard to DNA binding properties. An alternative mutant, S116E mDBD, exhibited similar properties as the S116D mutant. The neutral S116A mDBD mutant exhibited a similar decay rate as wild type mDBD (n=2, data not shown), thus excluding a general effect of mutations at S116.

*PKA phosphorylation differentially affects c-Myb binding to various MRE sites*

In previous studies, we have developed an EMSA system for evaluating the selectivity of both wild type and mutant forms of Myb for different DNA recognition elements. The differences detected with the set of variant MRE oligos in EMSA studies are reflected both in yeast and in animal cell transactivation systems (38, 47, 48). The effect of PKA phosphorylation of mDBD on
MRE site selectivity was investigated using a set of 4 variant MRE oligos (core sequence TAACGG, TAACGT, TAACTG and TAACCTT) (47, 48). The oligos are named after the bases in positions 5 and 6 in the core sequence (see details in ref (48)). As shown in Fig. 6a, phosphorylation of mDBD led to a dramatic decrease in the ability of mDBD to bind to the tested cognate DNA sequences compared to the wild type protein. The binding of phospho-mDBD to the GG oligo (lanes 1-4) was similar to the results obtained with the MRE oligo (Fig. 5). Binding to the GT and TG oligos was almost completely abrogated (lanes 5-8 and 9-12, respectively). Phosphoimager analysis revealed that reduction of DNA-binding was approximately 60%, 89% and 88% for the GG, GT and TG oligos, respectively (n=4). The TT oligo (lanes 13-16), which is not bound by c-Myb, was included as a negative control.

*DNA bound Myb is barely accessible to PKA phosphorylation*

We have previously reported that the R₂ repeat (in which S116 resides) undergoes a disorder-to-order transition upon binding to DNA (43, 57, 58). Experiments were therefore performed to investigate whether this transition could affect the accessibility of S116 in a free mDBD versus in an mDBD-DNA complex. Phosphorylation reactions with PKA C₁ subunit were performed on free mDBD protein, on mDBD pre-incubated with a specific DNA oligo (MRE) or mDBD preincubated with an unspecific DNA oligo (NSO). The pre-formation of a mDBD/MRE complex severely reduced the phosphorylation of mDBD compared to free mDBD protein (Fig. 6b, lanes 7-12 compared with lanes 1-6). In contrast, preincubation of mDBD with a non-specific oligo (NSO) barely affected PKA-mediated mDBD phosphorylation (Fig. 6b, lanes 13-18). This demonstrates that sequence specific DNA-binding severely reduces the accessibility of the S116 site. This could be due to direct shielding by DNA or be a consequence of a DNA-induced conformational change in R₂. Irrespective of the mechanism, the results suggest that S116 can be
present in an “open” or “closed” conformation, being accessible and efficiently modified in the free protein, but changing into a site with poor accessibility for the kinase in the protein-DNA complex.

c-Myb, but not AMV v-Myb, is phosphorylated at S116 in intact cells

An important question to ask was whether a steady-state pool of S116-phosphorylated c-Myb existed in cells. Antibodies against an S116-phosphorylated c-Myb were generated to elucidate this possibility. As shown in Figure 7A, the antiserum recognizes PKA-phosphorylated mDBD, but not non-phosphorylated protein (Fig. 7A, lanes 1 and 2), S116A-mutated mDBD (lanes 3 and 4) nor AMV v-Myb (lanes 7 and 8). It did, however, crossreact with an S116D phospho-mimicking mutant version of mDBD (lanes 5 and 6), suggesting requirement of a negative charge at position 116. Moreover, PKA-phosphorylated back-mutated AMV v-Myb D117V was recognized (lanes 9 and 10), indicating that restoration and phosphorylation of the PKA site restored binding of the phospho-c-Myb (S116)-specific antibody.

The antibody was subsequently used to determine whether endogenous c-Myb is phosphorylated at S116 in vivo. HD3 erythroblasts were chosen as they express high levels of c-Myb. As shown in Figure 7B, the phospho-c-Myb (S116)-specific antibody immunoprecipitated a fraction of c-Myb in live cells. To analyze whether point mutations in the c-Myb DBD affect its phosphorylation, we choose quail QT6 cells that lack endogenous c-Myb and that are an established cell line to determine Myb functions. As shown in Fig. 7C, the phospho-c-Myb (S116)-specific antibody recognized a fraction of transfected full-length or of truncated c-Myb (lanes 8 and 11), but not of the S116A mutant (lane 14). Similarly, AMV v-Myb was not recognized by the phospho-c-Myb (S116)-specific antibody whereas back-mutation of the critical D117 in AMV v-Myb to wild-type V117 that restored the phosphorylation site also restored
antigenicity (lanes 17 and 20). We conclude that c-Myb can be phosphorylated at S116 and that the V117D mutation present in AMV v-Myb abolishes this phosphorylation site.

Introduction of charge in S116 affects resident Myb-target gene expression

In effector-reporter experiments we failed to observe significant differences between wild type c-Myb, S116A, and phospho-mimicking S116D, S116E mutations (data not shown). However, different results were obtained when the activation of the resident mim-1 gene by Myb and C/EBP was examined. The chicken macrophage cell line HD11 was chosen for these experiments because it constitutively expresses C/EBP/NF-M but not c-Myb. (53, 59). Wild type S116 c-Myb constructs induced transcription of the mim-1 resident gene as assayed by Northern blotting (lanes 4 and 6). The activation was only slightly affected by the neutral S116A mutation (compare lanes 6 and 7). or by the V117D mutant (lane 9). However, both the phospho-mimicking mutants S116D and S116E severely inhibited mim-1 mRNA induction (lanes 8 and 10). This suggests that a negative charge at position 116 abrogates expression of a resident chromosomal c-Myb target gene. Similar observations were made in QT6 cells transfected with both Myb and C/EBP expression vectors (data not shown). The latter experiments also showed that a C/EBP-only target gene (designated #126, see (60)) was unaffected by the coexpression of various Myb-mutants, excluding indirect effects (data not shown). Co-expression of the PKA catalytic subunit strongly reduced mim-1 expression, as shown in Fig. 8 (upper panel, lane 5). This was not due to reduced c-Myb expression, as shown in Fig. 8 (middle panel). However, cotransfection of PKA negatively regulates the induction of mim-1 also by wt S116 and by the S116A Myb mutant, as well as the expression of Myb independent C/EBP target genes (data not shown). This suggests pleiotropic effects of the catalytic subunit of PKA on other factors.
involved in Myb- and C/EBP-induced gene activation and, beyond the analysis of Myb mutants examined, currently precludes further analysis of PKA-specific effects on Myb.

**DISCUSSION**

Here we show that c-Myb is phosphorylated by the catalytic subunit of PKA on S116 in the DNA-binding domain. Phosphorylation of wild type (S116) mDBD severely affects binding of Myb to DNA. Phosphorylation of Myb at S116 by PKA or by a kinase with similar specificity may link the proto-oncoprotein to a novel regulatory signal transduction pathway. A specific point mutation of the AMV oncogene (V117D) abolishes phosphorylation at S116, implying that escape from such a pathway contributes to leukemogenicity.

AMV v-Myb carries 10 point mutations in comparison to c-Myb (23). Four of these mutations are conservative replacements (I181V, V267I, V270I, R438K) and hence not primary candidates for altered structure or function. Two mutations (L199P, L207P) introduce additional prolines in a P-rich putative linker region between DBD and TAD. Of the remaining four mutations (I91N, L106H, V117D and N285T) those located in the DBD all have been identified as important for the transformed phenotype (39). The crystal structures of c-Myb DBD, AMV DBD, C/EBP and DNA complexes confirm the importance of both the I91N and L106H mutations. These mutations break specific contacts with the C/EBP transcription factor, contribute to a shift in the 2 helix in the R2 domain, and weaken the interaction with the DNA phosphate backbone (37). The question about the significance of the third point mutation at position 117, however, remained to be solved.

The concerted function of the three mutations have been linked to both gain- and loss-of-function phenotypes of AMV v-Myb and to signaling pathways (40). Biochemical analysis
showed that position 117 lies in a consensus site (RRXS/TY) for PKA phosphorylation and that this consensus site actually represents a high affinity substrate for PKA with a Km of approximately 10 µM (45, 61). Intriguingly, the PKA consensus sequence and the phosphaacceptor function are lost in v-Myb, due to the adjacent V117D mutation. A similar PKA consensus site in A-Myb (KRWS^{111}L) differs from the c-Myb S116 site (KRWS^{116}V) only in the +1 position, and was not efficiently phosphorylated by PKA (56). Thus, the amino acid present in the neighboring residues outside of the established consensus PKA site (45) influences the ability to function as a bona fide PKA target site.

Ramsay and coworkers located two PKA phosphorylation sites in c-Myb by phosphopeptide mapping of a C-terminal truncated c-Myb protein revealing S8 and S116 as substrates for PKA in vitro (33). However, in contrast to our observations, no functional effects of the phosphorylation of S116 were reported. Our data show that phosphorylation of S116 has biochemical and functional consequences. Phosphorylation of S116 destabilizes Myb binding to DNA and a S116D phosphorylation mimic protein failed to activate the resident mim-1 gene. Whether this effect is due to destabilization of DNA-interaction, or loss of co-operativity with C/EBP is currently difficult to resolve. However, we can infer that the loss-of-function of AMV v-Myb to cooperate with C/EBP, is at least in part due to the mutation at V117D. Backmutation of D117V in AMV v-Myb regained some of the functional C/EBP/Myb-dependent gene activation, while constitutive activation of GBX2 was severely diminished (40). This back mutation also changed the transformed phenotype towards the granulocytic lineage and displayed reduced leukemogenicity in retroviral transformation studies (39). However, the reciprocal single mutation V117D in wt Myb setting was not sufficient to abrogate C/EBP collaboration entirely or to induce constitutive GBX2 activation (E. K.-L. and A. L., unpublished observations). This is in
keeping with the recent structural data, where I91 and L106 in wt c-Myb directly interacted with C/EBP, whereas V117 indirectly affects C/EBP interaction (37). Accordingly, the point mutation at V117D required the other R2 v-Myb mutations to achieve the complete v-Myb phenotype. Taken together, these data suggest that the phosphorylation at S116 or abrogation of this phospoacceptor site by the AMV v-Myb specific mutation has multiple effects on Myb function, including a direct influence on the interaction of the DBD of Myb with its co-factors.

Our data show that phosphorylation at S116 leads to decreased binding of Myb to MREs. The destabilization of the Myb-DNA complex upon PKA phosphorylation could be a consequence of electrostatic repulsion since the S116 residue is in close proximity to the DNA sugar-phosphate backbone (Fig. 1c). However, additional effects of the introduced phosphate may be postulated since a substitution of S116 with aspartic acid or glutamic acid to mimic a negative charge in this position showed a less profound inhibition of DNA binding compared to phosphorylated mDBD. Additional effects of the phosphate group could be either steric hindrance and/or an effect on the conformational transition that occurs upon DNA-binding.

PKA has been reported to exert a negative effect on the DNA binding activity of several transcription factors that contain a PKA-phosphorylation site located within or close to their DNA binding domain, such as the transcription enhancer factor TEF-1 (62) and the Wilms’ tumor gene product WT1 (63). A peculiar feature of the negative effect on complex stability observed with c-Myb was that the severity of the inhibition depends on the particular MRE sequence. The experiments were performed with mDBD domains due the inaccessibility of purified full-length c-Myb. Nevertheless, we have previously reported that there are only subtle differences observed in in vitro assays between the DNA sequence preference of the mDBD and full-length c-Myb expressed in COS cell lysates (48), suggesting that conclusions drawn from
experiments with the mDBD are applicable to the full-length protein. Hence, our data are consistent with the notion that S116 phosphorylation might down-modulate Myb-target genes in a differential fashion and thus alter the spectrum of genes activated by c-Myb in vivo. We showed that the accessibility of the S116 phosphorylation site depends on the interaction of Myb with specific target sites, a phenomenon consistent with a reported conformational transition of the DBD upon binding to DNA (43). Thus, it is possible that only the pool of c-Myb that is not bound to DNA is accessible to specific phosphorylation on S116. This could severely limit the window in which c-Myb acts as a kinase-substrate.

The development of a specific phospho-c-Myb (S116) antibody demonstrated that a fraction of c-Myb is phosphorylated at this particular site in live cells. In an approach to determine the consequences of Myb S116 phosphorylation in cells, we cotransfected an expression vector encoding the catalytic subunit of PKA. However, overexpression of PKA has effects on Myb as well as on C/EBP target genes (data not shown) making such experiments difficult to interpret. It should be emphasized, however, that although PKA phosphorylates S116 in vitro and although phospho-S116 can be detected in vivo, we do not imply that PKA is the relevant kinase in vivo. It is equally conceivable that different kinases with similar recognition peptide specificities are involved in regulating c-Myb functions in vivo. Such kinases still have to be identified.

The AMV v-Myb displays both gain- and loss-of-function phenotypes due to the combined action of the DBD mutations (40). Induction of GBX2 expression by the c-Myb protein requires activation of an upstream signaling cascade whereas the mutations in the DNA binding domain of AMV-Myb render the protein independent of such signaling events (40). Although there is currently no evidence that phosphorylation of S116 is involved directly in GBX2
activation it is possible that stabilization of v-Myb DNA binding contributes to the activation of distinct Myb target genes. Alternatively, phosphorylation of S116 might affect protein-protein interactions such as with co-activators or co-repressors. Finally, this phosphorylation might affect other processes linked to gene regulation such as removal of transcriptional activation to prevent constitutive gene expression (64-66).

What could be the role of PKA regulation of c-Myb in the hematopoietic system? It is well known that while cAMP usually is mitogenic and a stimulator of hormone secretion in endocrine cells, it generally delivers an “off”-signal for proliferation in cells of the immune system (67, 68). Thus, a PKA-induced decrease in the activity of c-Myb, a factor associated with proliferation, would be a consistent response in immune cells. Among the targets of c-Myb in T-cells is the RAG-2 (69), a protein required for recombination of B and T cell antigen receptor chains. It is intriguing that the rearrangement of the TCRβJ locus is completely inhibited upon activation of the cAMP signaling pathway (70) and that one of the components of the V(D)J recombinase is also a bona fide Myb-target, having an MRE sequence of the PKA-sensitive type (TAACTG) (69). Whether PKA inhibition of c-Myb contributes to this effect and whether and how other genes are affected by S116 phosphorylation, however, remains to be investigated.

ACKNOWLEDGEMENTS

This work was supported by The Norwegian Cancer Society (K.B.A., E.M.B. and O.S.G), the Norwegian Research Council (O.S.G.), Anders Jahre Foundation (O.S.G), The Blix Foundation for Medical Research (K.B.A.) and the Deutsche Forschungsgemeinschaft, DFG (E.K.L and A.L.). We wish to thank A.C. Østvold (Institute for Medical Biochemistry, UiO) for assistance with phosphoaminoacid analysis and Dimitrios Mantzilas (Department of Biochemistry, UiO) for help with MS measurements.
REFERENCES


37. Tahirov, T. H., Sato, K., Ichikawa-Iwata, E., Sasaki, M., Inoue-Bungo, T., Shiina, M.,
Kimura, K., Takata, S., Fujikawa, A., Morii, H., Kumazaka, T., Yamamoto, M., Ishii, S.,


1289-1297


interactions. John Wiley & Sons Ltd, Chichester

43. Myrset, A. H., Bostad, A., Jamin, N., Lirsac, P. N., Toma, F., and Gabrielsen, O. S.
(1993) EMBO J 12, 4625-4633


1042


Oncogene 13, 1043-1051


**FOOTNOTES**

- AMV, avian myeloblastosis virus; DBD, DNA-binding domain; EMSA, electrophoretic mobility shift assay; mDBD, minimal DNA-binding domain; MRE, Myb recognition element;
NRD, negative regulatory domain; PKA, protein kinase A; TAD, transactivation domain; v-Myb, viral Myb
FIGURE LEGENDS

Figure 1: PKA consensus sites in the DNA-binding domains of Myb family members

A) A comparison of PKA consensus sites in mammalian c-Myb, B-Myb and A-Myb and viral AMV v-Myb proteins. The numbering is according to chicken c-Myb. B) The putative PKA targets S116 and S146 in the mDBD are shown in the published crystal structure of the c-Myb DBD (space filling) complexed with DNA (sticks) (7). C) The AMV v-Myb DBD/DNA complex (37) is shown, and the three mutant amino acids (I91N, L106H, V117D) are highlighted together with S116. B and C were drawn using RASMAC and the 1h89 and 1h8a coordinate files (PDB).

Figure 2. In vitro mapping of the PKA phosphorylation site in c-Myb mDBD.

A) Recombinant wild type (wt) and mutant mDBD (S116A, S146A and S116A/S146A) domains (8 pmol) were incubated with purified PKA C\(_{\alpha}\) [3.8 U (Roche)] and \^[\text{32}P]ATP for 15 minutes. Reactions were seperated on a 15% SDS-PAGE gel and detection by autoradiography. Arrows denote phosphorylated proteins and free \^[\text{32}P]ATP. Lower panel: Coomassie-staining of an SDS-PA gel with parallel samples of the same proteins as in upper panel. Lanes 1 to 4 contain wt, S116A, S146A amd S116A/S146A mDBD proteins, respectively. B) Phosphoaminoacid analysis of \^[\text{32}P]-radiolabeled wt and S146A mDBD proteins. Outlines of the ninhydrin staining of unlabeled phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Thr) standards are indicated. C) Molecular mass measurements of wt, S116A and S146A mDBD proteins incubated in the absence or presence of PKA-C\(_{\alpha}\) are given in Daltons. Molecular masses calculated from the primary sequence (calculated), the increase in molecular mass in the presence of PKA (\[\Box\]) and the calculated mass increase of one phosphate group addition are also shown.
Figure 3. Km determination of PKA for Ser116 in c-Myb mDBD.

Recombinant mDBD protein (0, 5, 5.6, 6, 7.6, 10, 15, 16.6 or 20 µM) was phosphorylated for 0, 2, 4 and 6 minutes with 500 µM ATP, 3.3 pmol [\(^{32}\)P]ATP and PKA C\(_3\) subunit [46 U (Promega)] as described in the Materials and Methods section. The reactions were stopped by the addition of 2 [m] 0.5 M EDTA and spotted on P81 phosphopaper, washed and counted by liquid scintillation counting. The Km of PKA for R\(_2\)R\(_3\) was calculated in the linear reaction range at 2 minutes by linear regression analysis (n=3).

Figure 4. The effect of the AMV V1117D mutant on S116 phosphorylation in c-Myb mDBD. Upper panels) Recombinant wt and mutant mDBD proteins (AMV, V117D, AMV/D117V) as indicated, were incubated with purified PKA C\(_3\) [3.8 U (Roche)] and [\(^{32}\)P]ATP for 15 minutes and analyzed as in Fig. 2. Lower panel) Coomassie staining of the same gel as in the upper left panel.

Figure 5. Effects of phosphorylation on the DNA-binding activity of c-Myb mDBD

A) Twentyfive or 10 fmol of phosphorylated (+) or non-phosphorylated (-) mDBD was bound to \(^{32}\)P-labeled MRE and run on an EMSA gel. B) Complexes containing phospho-mDBD/\(^{32}\)P-labeled MRE oligo or \(^{32}\)P-mDBD/ unlabeled MRE oligo were compared on an EMSA gel. Lanes 2 and 3: \(^{32}\)P-phosphorylated mDBD alone (lane 2) or bound to unlabeled MRE (lane 3). Lanes 4 and 5: non-radioactive phospho-mDBD (lane 4) or non-phosphorylated mDBD (lane 5) bound to \(^{32}\)P-labeled MRE oligo. Arrows show the position of the mDBD/DNA complex, the free MRE oligo (lane 1) and free [\(^{32}\)P]ATP (lane 2, 3). C) Decay EMSA where 20 fmol wild type mDBD
(lanes 2-6), S116D mutant mDBD (lanes 7-11) or 60 fmol phospho-mDBD (lanes 12-16) was first bound to the MRE oligo (10 fmol), and then competed with unlabeled MRE (750 fmol) for the times indicated before loading on the EMSA gel. Note that for phospo-mDBD, three times the standard amount of protein was used in each reaction in order to visualize the phospho-mDBD/DNA complexes. One representative experiment of 4 is shown.

**Figure 6** MRE variants respond differentially to PKA phosphorylation of c-Myb mDBD.

A) The ability of phospho-mDBD to distinguish between MRE family Myb binding sites. Four Myb binding site oligos were used for comparing the selectivity of phospho-mDBD to non-phosphorylated mDBD. The MRE-GG, -GT, -TG and -TT (negative control mutant) oligos refer to specific positions in the core MRE sequence, (see Materials and Methods). Recombinant mDBD (-) or *in vitro* PKA phosphorylated mDBD (+) (20 and 35 fmol each) was bound to MRE oligos MRE-GG, -GT, -TG and -TT (10 fmol each) and run on EMSA gels. The gel was dried, autoradiographed and signals quantified in a phosphoimager. One representative experiment of 4 is shown.

B) Accessibility of mDBD for phosphorylation by PKA. Recombinant mDBD alone (mDBD) (0.8 mM) (lane 1-6), mDBD in complex with *mim-1A* oligo (mDBD/MRE) (0.8 mM each) (lane 7-12) or in complex with non-specific oligo (mDBD/NSO) (0.8 mM each) (lane 13-18) was incubated with PKA C in the presence of [*-32P]ATP for 0 to 30 min. as indicated, before SDS-buffer was added to terminate the reactions. Reaction mixtures were separated by SDS-PAGE, and the gel was autoradiographed.
Figure 7. S116 phosphorylation occurs in vivo.

(A) Specificity of a phospho-c-Myb (S116)-specific antibody. Recombinant wild type (wt) and mutant mDBD (S116A, S146D, AMV and AMV[D117V]) purified domains were either subjected to mock (“-“, lanes 1, 3, 5, 7, 9) or PKA Cα (“+“, lanes 2, 4, 6, 8, 10) phosphorylation before loading on a 15% SDS-PAGE gel. Subsequent Western blot analysis was performed using either a phospho-c-Myb (S116)-specific polyclonal antibody (upper panel) or the 5E11 monoclonal anti-Myb antibody (lower panel).

(B) Immunoprecipitation of a fraction of endogenous c-Myb by the phospho-c-Myb (S116) specific antibody from unstimulated live HD3 erythroblasts. Proteins were precipitated from cell lysates with a pre-immune rabbit serum, a Myb specific rabbit immune serum and a phospho-c-Myb (S116)-specific antiserum, as indicated. Immunoprecipitates were separated by 10% SDS-PAGE, transferred to PVDF membranes and proteins revealed with a mouse monoclonal antibody directed against c-Myb (MYB2-7.77; ATCC).

(C) Immunoprecipitation of Myb from QT6 fibroblasts depends on the integrity of the PKA consensus sequence. QT6 cells were transfected with the indicated expression constructs. Samples from the lysates were also loaded as expression control.

Figure 8. S116 modification affects resident mim-1 expression in vivo.

The HD11 cell line was transfected with the indicated expression constructs. RNA was extracted after 24 hr. Blots with polyA-enriched RNA were sequentially probed with labeled mim-1 (upper panel), c-myb (middle panel), and GAPDH (lower panel) fragments, the latter serving as loading control.
**Fig. 1**

**A**

<table>
<thead>
<tr>
<th>PKA consensus</th>
<th>S116</th>
<th>S146</th>
</tr>
</thead>
<tbody>
<tr>
<td>chicken c-Myb</td>
<td>VQKYGP</td>
<td>LNPEVKTE</td>
</tr>
<tr>
<td>human c-Myb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>murine c-Myb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bovine c-Myb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>canine c-Myb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xenopus c-Myb</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| chicken AMV v-myb |       |       |
| human AMV v-myb   |       |       |
| murine AMV v-myb  |       |       |
| Xenopus AMV v-myb |       |       |

| chicken A-Myb |       |       |
| human A-Myb   |       |       |
| murine A-Myb  |       |       |
| Xenopus A-Myb |       |       |

| chicken B-Myb |       |       |
| human B-Myb   |       |       |
| murine B-Myb  |       |       |
| Xenopus B-Myb |       |       |

**B**

**C**
**Fig. 2**

### Molecular mass (Da)

<table>
<thead>
<tr>
<th>Domain</th>
<th>PKA</th>
<th>MS</th>
<th>Δ</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>mDBD wt</td>
<td></td>
<td>12 668.05</td>
<td>—</td>
<td>12 677.62</td>
</tr>
<tr>
<td>mDBD wt +</td>
<td></td>
<td>12 749.84</td>
<td>81.79</td>
<td>12 757.60</td>
</tr>
<tr>
<td>mDBD S116A</td>
<td></td>
<td>12 656.94</td>
<td>—</td>
<td>12 661.62</td>
</tr>
<tr>
<td>mDBD S116A +</td>
<td></td>
<td>12 656.86</td>
<td>0.08</td>
<td>—</td>
</tr>
<tr>
<td>mDBD S146A</td>
<td></td>
<td>12 661.96</td>
<td>—</td>
<td>12 661.62</td>
</tr>
<tr>
<td>mDBD S146A +</td>
<td></td>
<td>12 738.18</td>
<td>76.22</td>
<td>12 741.60</td>
</tr>
<tr>
<td>Phosphate group</td>
<td></td>
<td></td>
<td></td>
<td>79.98</td>
</tr>
</tbody>
</table>
Fig. 3

\[ K_m = 10 \mu M \]
\[ v_{max} = 1.5 \mu M/min \]
Fig. 4

[Image of a Figure showing a gel with bands labeled as PKA-α, 32P-mDBD, 32P-ATP, and mDBD. The gel has lanes labeled with different treatments: wt, AMV, and V117D. The molecular weight markers are 20.1 and 14.4 kDa.]
Fig. 5
Fig. 6

A

<table>
<thead>
<tr>
<th>MRE oligo</th>
<th>GG</th>
<th>GT</th>
<th>TG</th>
<th>TT</th>
</tr>
</thead>
<tbody>
<tr>
<td>fmol mDBD</td>
<td>20</td>
<td>35</td>
<td>20</td>
<td>35</td>
</tr>
<tr>
<td>phspho</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>mDBD</th>
<th>mDBD / DNA</th>
<th>mDBD / NSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

- mDBD/oligo
- free [32P]-oligo
- mDBD/DNA
- [32P]-ATP
Fig. 7A

<table>
<thead>
<tr>
<th>mDBD</th>
<th>PKA-C&lt;sub&gt;α&lt;/sub&gt;</th>
<th>anti-Myb (S116P)</th>
<th>anti-Myb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>10</td>
</tr>
</tbody>
</table>

**Legend:**
- wt: wild type
- S116A
- S116D
- AMV
- AMV D117V
Fig. 7B
Fig. 7C

<table>
<thead>
<tr>
<th>Lysate</th>
<th>IP</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Myb FL</td>
<td>c-Myb [72-349]</td>
</tr>
<tr>
<td>AMV v-Myb</td>
<td>AMV v-Myb D117V</td>
</tr>
<tr>
<td>pre-immune</td>
<td>anti-Myb</td>
</tr>
<tr>
<td>anti-Myb(S116P)</td>
<td>pre-immune</td>
</tr>
<tr>
<td>anti-Myb(S116P)</td>
<td>anti-Myb</td>
</tr>
<tr>
<td>anti-Myb(S116P)</td>
<td>anti-Myb</td>
</tr>
<tr>
<td>anti-Myb(S116P)</td>
<td>anti-Myb</td>
</tr>
<tr>
<td>anti-Myb(S116P)</td>
<td>anti-Myb</td>
</tr>
</tbody>
</table>

1  2  3  4  5  6  7  8  9  10  11  12  13  14  15  16  17  18  19  20

Downloaded from http://www.jbc.org/ by guest on November 19, 2017
Phosphorylation dependent down-regulation of c-Myb DNA-binding is abrogated by a point mutation in the v-myb oncogene
Kristin Brevik Andersson, Elisabeth Kowenz-Leutz, Elen Margrethe Brendeford, Ann-Helen Herwig Tygset, Achim Leutz and Odd S. Gabrielsen

J. Biol. Chem. published online November 26, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209404200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts