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Reduction of a conserved Cys is essential for Myb DNA-binding

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ABSTRACT

The human c-Myb gene product is a regulator of transcription with intrinsic DNA-binding activity located in two of three aminoterminal repeats R_2R_3. Three purified recombinant c-Myb proteins, a 42 kD protein corresponding to the amino-terminal half (HM42), and two proteins representing R_2R_3 or $R_1R_2R_3$ (HMR_{23} and HMR_{123}) have been analyzed either as purified proteins or present in bacterial extracts in gelshift analyses using a high-affinity DNA oligonucleotide. The purified proteins are inactive in DNA-binding unless supplemented with a reducing agent such as dithiothreitol (DTT) *in vitro*. Alternatively a cellular nuclear extract (Nex) from HeLa cells strongly activates the binding. This effect is dose-dependent and sensitive to heat. The Nex does not lead to changes in the Myb-DNA mobility shift assay excluding a direct association of the complex with a cellular component. Site-directed mutagenesis of the aminoacid residue 130, a single conserved cysteine in HMR_{23} to serine almost completely abolishes DNA binding. Oxidation by diamide or alkylation by N-ethylmaleimide (NEM) of the Myb-proteins *in vitro* inhibit their interaction with DNA whereby the diamide effect is reversible by addition of excess of DTT. Nex prepared from COS cells transfected with c-myb leads to Myb-DNA interaction which is not responsive to DTT but sensitive to NEM and diamide. Our data indicate that the reduced cysteine of Myb is essential for its DNA-binding and that Myb function may be regulated by a reduction-oxidation mechanism.

INTRODUCTION

The c-myb gene is the proto-oncogene of v-myb—the genetic component of the two retroviruses, avian myeloblastosis virus AMV and avian leukemic virus E26, which are responsible for transformation of myelomonocytic cells *in vitro* (1,2). The c-myb gene is an important haematopoietic control gene involved in the regulation of proliferation and/or differentiation of progenitor cells (3,4,5). Downregulation or antisense ablation of c-myb transcripts inhibits proliferation and leads to differentiation (6).

The c-Myb protein is a molecule of 75 000 dalton which is characterized by several structural domains typical of transcriptional activators. It contains at its aminoterminus three characteristic repeats with regularly-spaced tryptophan residues which might be situated in a hydrophobic core of a helix-turn-helix domain (7,8,9). Two of these repeats, repeat 2 and 3 (R_2 and R_3) are responsible for DNA-binding (10,11). A transcriptional activation domain has been mapped as a hydrophilic region in the central part of the protein (12,13,14). The carboxyterminus may represent a negative regulatory domain (13,14). The viral Myb proteins are truncated at their amino- and carboxytermini which may contribute to oncogenic properties (15,16,17).

The c- and v-Myb proteins bind specifically to DNA fragments *in vitro* containing the consensus sequence PyAACT/GG (18) which has been recently extended by additional flanking nucleotides to the nonanucleotide C(C/T)AAC(T/G/C)PuCPy (19). DNA-binding is a prerequisite for transactivation by Myb. A mutant of Myb lacking the DNA-binding domain can, however, transactivate transcription of the hsp 70 promoter (20). Myb can also act as transcriptional repressor (13).

The Myb-DNA interaction is negatively regulated by phosphorylation with the protein kinase CKII which phosphorylates serine at position 10 and 11 (21), a region missing in v-Myb. This suggests that DNA-binding may be regulated by secondary protein modifications. We had noticed that DNA binding of Myb was strongly induced by addition of cellular extracts. We ruled out that phosphorylation events were involved. We also noticed that DNA-binding of Myb was strongly stimulated by the presence of dithiothreitol (DTT). Recently it has been shown that Fos/Jun binding to DNA was induced by DTT or treatment of the recombinant proteins *in vitro* with nuclear extracts. Abate et al. (22,23) attributed this effect to reduction-oxidation (redox) mechanisms and were able to identify one specific cysteine residue in the basic DNA-binding regions of Fos and Jun as the critical amino acid that had to be reduced for their binding to DNA. This cysteine and its flanking amino acid residues exhibit sequence homology to a region in the second repeat R_2 of Myb (24). We now show that the redox-state of the homologous cysteine influences the DNA binding activity of Myb *in vitro*.

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MATERIALS AND METHODS

Cloning and expression of recombinant human c-Myb proteins

HM42 protein represents the aminoterminal 358 amino acids of the human c-Myb protein with a molecular weight of 42 kD. It was cloned into a T7 vector pET8c (25) using the restriction sites NcoI/BamHI and expressed in bacteria as described (26). HMR_{123} and HMR_{23} were cloned by use of standard PCR-techniques. The following oligonucleotides were synthesized in an Applied Biosystems DNA synthesizer:

1:5'-ACTCGGTACCATGGACTTTGAGATGTGTGA (5'-end of HMR_{123})
2:5'-ATGGTACCATGGAGCTCATCAAGGGTCCTT (5'-end of HMR_{23})
3:5'-CCTTGGATCCTACTTCCGACGCATTGTAGA (3'-end of HMR_{123} and HMR_{23})

PCR was performed in a BIOMED thermocycler using Taq polymerase from Promega according to instructions of the manufacturer. PCR fragments were inserted into pET8c vectors (25) using NcoI and BamHI restriction sites which were introduced by the PCR primers. The *E.coli* strain BL21DE3 was transformed by the resulting constructs. Protein synthesis was induced as described (27). Translation of coding sequences generates proteins with an additional methionine aminoterminal of the aspartate residue at position 17 in the case of HMR_{123} or of glutamate at position 89 (HMR_{23}). Translation stopped at lysine 192 since a TAG stopcodon was introduced by the PCR primer (numbering of amino acid residues is according to (28)). Cysteine 130 in HMR_{23} was mutated to serine in a two step PCR using oligonucleotide 5'-ATTGGAAAACAA**AGT**AGGG-AGAGGT. One adenine residue (bold letter) mismatches with a thymidine residue in the wild type sequence leading to a substitution of a cysteine codon (TGT) to serine (AGT, underlined). This oligonucleotide was used in the first PCR together with oligonucleotide 3 (see above) and plasmid pK1 as template, which contains the complete cDNA of the human c-myb gene (14). The resulting fragment was isolated and served as one of the primers in the second PCR. After addition of oligonucleotide 2 and pK1 template the final PCR product is generated which contains the correct start (oligonucleotide 2), the mutation and the stopcodon TAG (first PCR fragment). This fragment was inserted into pET8c vector, transformed into BL21DE3 bacteria and protein synthesis of HMR_{123} was induced as described for HMR_{23}. All resulting plasmids were sequenced using a Sequenase kit supplied by USB Co, USA.

Purification of c-Myb proteins

HM42 protein overexpressed in bacteria is insoluble, a property which was exploited for its isolation. HM42 expressing bacteria (20 ml) were induced with IPTG, centrifuged and lysed in lysis buffer (2 ml). This solution was applied to gel electrophoresis and HM42 electroeluted according to Hager and Burgess (29). The amount of electroeluted protein was estimated in silver stained polyacrylamide gels (30) using different concentrations of BSA (Serva Co., Germany) as standard. For preparation of bacterial extracts (Bex) containing HMR_{23}, HMR_{123} and $HMR_{23}S$, 100 ml of IPTG-induced culture were pelleted and resuspended in 4 ml buffer (10 mM Tris HCl, pH 7.6, 1 mM EDTA, 50 mM NaCl). Bacteria were lysed in a French Press and centrifuged at 100 000×g in a Beckman ultracentrifuge. The recombinant HMR_{123}, HMR_{23} and HMR_{23S} proteins were found in the supernatant as evidenced by SDS-PAGE (31) and immunoblotting (32). The rabbit serum used was directed against the aminoterminus of c-Myb (aminoacids 1 to 185) and the sheep against HM42 (aminoacid 1 to 358). HMR_{23} protein was further purified using FPLC (Pharmacia Co., Sweden) and heparin Sepharose column chromatography. Crude lysates of HMR_{23} containing bacteria were loaded onto the column and the proteins were eluted in two steps. The bulk of the proteins were removed with 500 mM NaCl. The HMR_{23} protein was eluted in the 1 M NaCl fractions. Fractions were pooled, proteins precipitated with a saturating amount of ammoniumsulfate and dialyzed against DNA binding buffer (see below).

Preparation of nuclear extracts

Transfection assays of COS recipient cells with the HM42 protein-expression construct pHM5 were performed as described by Zobel et al. (26). Cells (2×10^6) at 40% confluency were transfected by the calcium phosphate precipitation method (33). After 36 hrs cells were harvested, trypsinized and the nuclear extract prepared as described by Dignam et al. (34) except that the high salt extract was diluted in 3 volumes of buffer D instead of dialyzing yielding a protein content of 1.7 $\mu g/\mu l$. Deproteinization of the nuclear extract was performed using Strataclean resin from Stratagene Co., La Jolla, USA, as described by the manufacturer.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides 5'-TACAGGCATAACGGTTCCGTAGTGA 3' and its complementary counterpart were annealed and end-labeled using T4-polynucleotide kinase (Boehringer). This probe contains the high affinity binding site TAACGGTT. Myb proteins used for DNA-binding assays were either purified or present in bacterial extracts. The specificity of the Myb-DNA interaction was identical in both cases (not shown). The proteins were incubated with $7-10\times10^3$ cpm of radiolabeled oligonucleotide and DNA-binding buffer (10 mM Tris HCl, pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol) for 20 min at room temperature in a final volume of 20 μl. Poly[d(I-C)] (1 μg) and unrelated single-stranded DNA-oligonucleotide (100 ng) served as non-specific competitors. Unless otherwise indicated all binding reactions contain 10 μg BSA (Serva). DNA-protein complexes were separated by electrophoresis through polyacrylamide gels (29:1 acrylamide/bisacrylamide) in 0.5×TBE (44 mM Tris-borate, 1 mM EDTA). 5% gels were used for HM42 and COS-cell extracts, 10% for HMR_{123}, HMR_{23} and $HMR_{23}S$. The double-stranded oligonucleotide which was used in competition experiments shown in Figure 6 contains the sequence TATCGGTT with one point mutation compared to the high affinity site TAACGGTT. Myb containing COS-cell extracts were preincubated either with antisera (1 μl) or unlabeled oligonucleotides (100-fold excess) for 15 min at 25°C in binding buffer and supplemented with the non-specific inhibitors poly[d(I-C)] and single-stranded oligonucleotide before radiolabeled oligonucleotide was added.

RESULTS

Expression and purification of recombinant Myb proteins

The full length Myb protein of 75 kD synthesized in bacteria is unstable and leads to low levels of protein expression (data not shown). A carboxyterminal deletion mutant coding for the first 358 amino acids of the aminoterminus of Myb (numbering as in ref. 28) expressed in a T7 expression vector was more stable and resulted in a 42 kD protein designated as human Myb protein

HM42. It acts as a specific DNA-binding protein ((26) and see below), transactivates appropriate reporter plasmids (26) and contains the aminoterminal target sites for the CKII protein kinase (21). The known functional sites of HM42 are schematically indicated in Figure 1.

The minimal portion of Myb that is capable of specifically binding to DNA is a peptide consisting of repeats R_2 and R_3 (10,11) (Figure 1). We cloned this region, designated as HMR_{23}, by use of PCR technique and expressed it in a T7 vector (for details see Materials and Methods). HMR_{23} contains only one cysteine at position 130 which is conserved among all Myb proteins. It is located in R_2 in a basic region which exhibits some homology to proteins of the Fos/Jun and CREB super family (24). Mutagenesis of this cysteine to serine (C130S) resulted in a mutant construct designated as $HMR_{23}S$ (see Figure 1). Since the function of R1 in the DNA binding activity of Myb is unclear, we wanted to analyze its influence by comparing the binding properties of HMR_{23}, which contains R_2 and R_3, with those of a protein containing all three repeats, named HMR_{123} (Figure 1). It lacks, however, 17 aminoterminal aminoacids with the CKII phosphorylation site.

For Myb-DNA-binding studies we either used purified proteins or bacterial extracts containing the overexpressed proteins. The HM42 protein was isolated from bacteria which were induced with IPTG according to the instructions of Studier and Moffat (27). Since the protein was insoluble it was purified according to the denaturation-renaturation procedure described by Hager and Burgess (29) except that DTT was omitted from the dilution buffer. The concentration of HM42 protein after purification (15 ng/μl) (Figure 2A) was estimated from silver-stained SDS-polyacrylamide gels using bovine serum albumin (BSA) as standard.

HMR_{23}, $HMR_{23}S$ and HMR_{123} are expressed in *E.coli* at similar levels (Figure 2B). These proteins are soluble and remained in the supernatant of the lysed bacteria as evidenced by Coomassie staining of SDS polyacrylamide gels or immunoblotting using a monoclonal antibody (MAB 3/7) directed against R_3 (Figure 2B).

One of the proteins, HMR_{23} was further purified using FPLC and Heparin Sepharose column chromatography. The protein elutes at 1 M NaCl and is about 70% pure (Figure 2C). An unknown protein with a molecular weight of about 6 kD copurified. It was probably not a degradation product of HMR_{23} because it did not react with different Myb-specific antisera in immunoblots (Figure 3C). HMR_{123} or $HMR_{23}S$ proteins were not purified further.

Reduction activates DNA-binding of the purified Myb proteins HM42 and HMR_{23}

Treatment of the HM42 protein with DTT induces DNA-binding activity (Figure 3A). HM42 (1 pmole corresponding to 30 ng) was incubated with increasing amounts of DTT in binding buffer

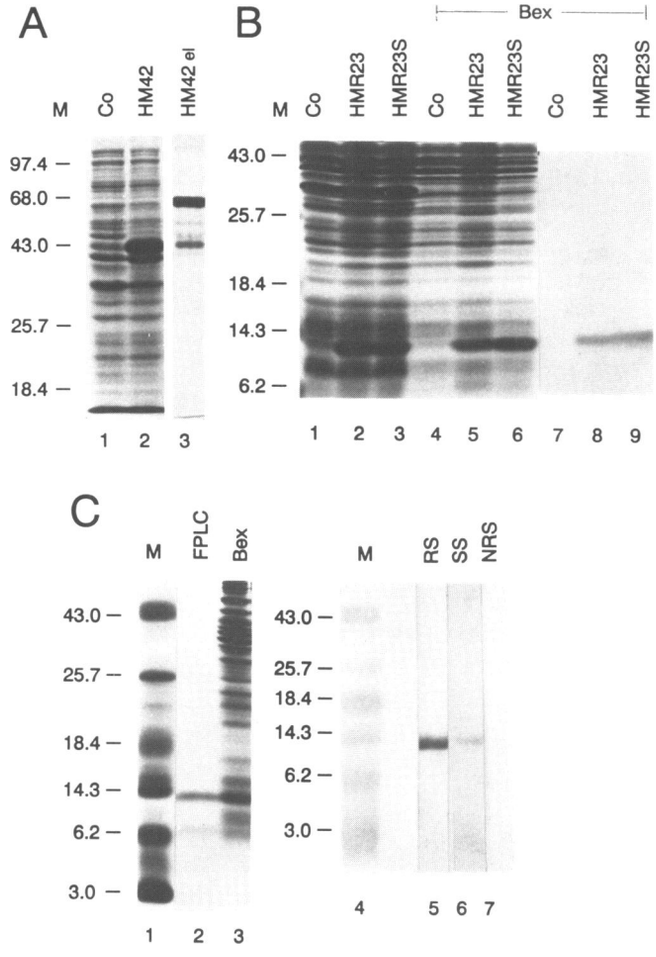

Figure 2. Expression and purification of c-Myb proteins. **(A)** SDS polyacrylamide gel (10%) electrophoresis (PAGE) of total proteins stained with Coomassie blue from bacteria expressing HM42 protein (lane 2) or from control bacteria which were transformed with the empty vector (lane 1). An aliquot of the purified electroeluted HM42 protein (HM42el) is shown in lane 3 (silver stain). The 68 kDa protein represents BSA which had been added to the renatured protein. Numbers indicate molecular mass standards (M) in kDa. **(B)** SDS-PAGE of control lysates (lanes 1,4 and 7) or of lysates containing HMR_{23} (lanes 2,5 and 8) or $HMR_{23}S$ proteins (lanes 3,6 and 9). Induced cells were pelleted and an aliquot representing 150 μl culture was boiled in sample buffer and electrophoresed in a 15% SDS-PAGE (lanes 1–3). Aliquots of bacterial extract supernatants (Bex) after centrifugation at 100 000 ×g are shown in lanes 4–9. One part of the gel was stained with Coomassie brilliant blue (lanes 1–6), the other part (lanes 7–9) was used for Western blot analyses with monoclonal antibody 3/7 which is directed against the third repeat. **(C)** SDS-PAGE of FPLC purified HMR_{23} protein. 2 μg of purified HMR_{23} protein (lane 2) or the equivalent of 150 μl original HMR_{23} expressing bacteria culture (lane 3) were electrophoresed in a 15% gel which was stained with Coomassie blue. 1 μg of purified HMR_{23} was analyzed in Western blot assays using either rabbit serum (lane 5; RS), sheep serum (lane 6; SS) or a pre-immune rabbit serum (lane 7; NRS) as first antibody.

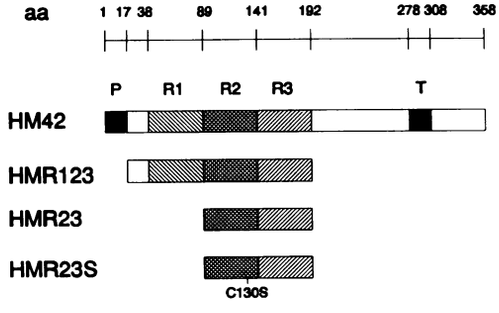

Figure 1. Schematic illustration of the c-Myb proteins used in this study. Aminoterminal phosphorylation sites (P), the tripartite repeat structure ($R_1R_2R_3$), whereby R_2R_3 represent the DNA binding domain, and the transactivation domain (T) are indicated. Numbers on the scale indicate amino acid residues (aa). Mutation of cysteine at position 130 to serine is indicated as mutant $HMR_{23}S$.

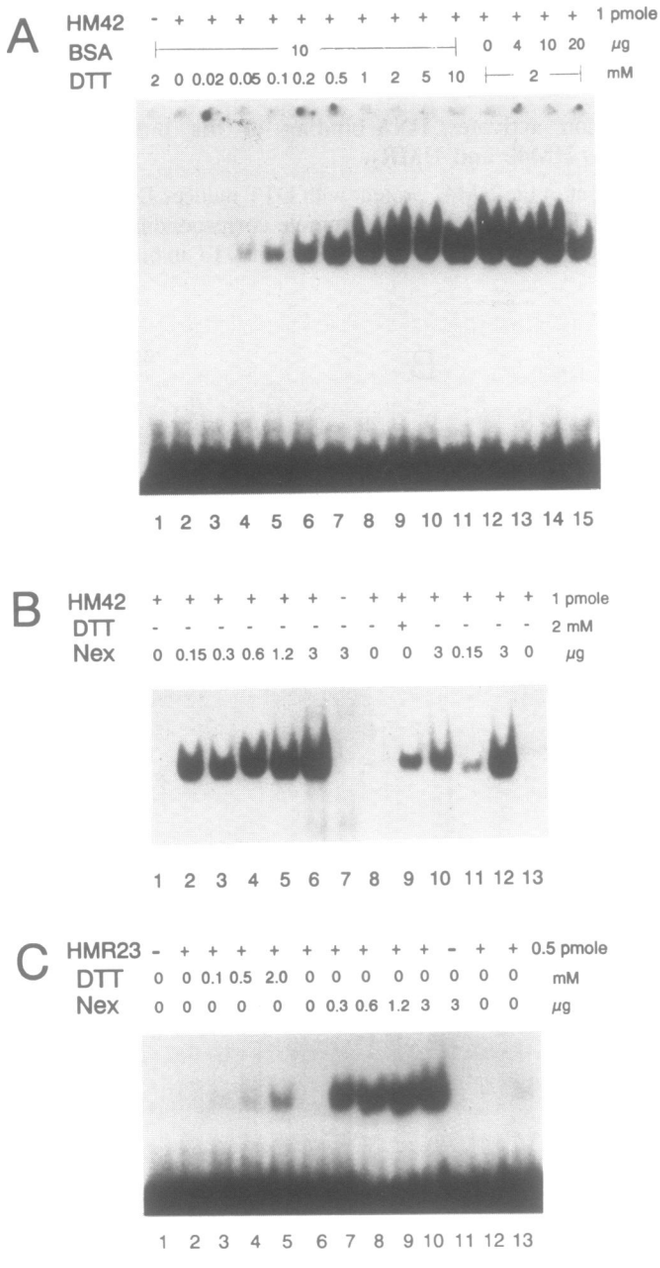

Figure 3. Induction of Myb binding after treatment with DTT or nuclear extracts. **(A)** Electrophoretic mobility shift assay of 1 pmole purified HM42 protein and 200 pg radiolabeled oligonucleotide. Increasing amounts of DTT were added to the reaction mixtures as indicated in lanes 3 to 11. Binding reactions shown in lanes 12 to 15 contained the constant amount of 2 mM DTT. In control reactions no HM42 protein (lane 1) or no DTT (lane 2) were added. In addition to 0.5 µg BSA present as protein stabilizer, 10 µg BSA (Serva) was added to binding assays shown in lanes 1 to 11. The amount of added BSA was varied as indicated in lanes 12–15. **(B)** Nuclear extracts (Nex) induced Myb binding activity. 30 ng HM42 protein was preincubated with various amounts of Nex in buffer D prepared from HeLa cells for 15 min at 37°C before binding buffer and radiolabeled probe were added (lanes 2 to 6). Myb did not bind without addition of Nex (lane 1). Different controls were performed: HM42 protein was omitted (lane 7), buffer D was used instead of Nex (lane 8), or Nex was treated with Strataclean Resin (lane 13). Incubations with 2 mM DTT or 3 µg Nex performed at 4°C (lanes 9 and 10) or incubations with 150 ng and 3 µg Nex which was heated at 65°C for one hour (lanes 11 and 12). **(C)** Effect of DTT or Nex on FPLC-purified HMR_{23} protein. 0.5 pmole HMR_{23} protein was preincubated with the indicated amounts of DTT (lanes 2–5) or Nex (lanes 6–10) for 15 min at 37°C before adding radiolabeled probe. No HMR_{23} protein was added in lane 1 and 11. Effect of buffer D and Strataclean resin-treated Nex are shown (lanes 12 and 13).

for 15 min at room temperature, then a radiolabeled DNA-oligonucleotide containing the high affinity binding site TAA-CGGTT was added and incubation continued for 20 min at room temperature. Samples were subsequently analyzed using the electrophoretic mobility shift assay. HM42 does not bind to DNA in the absence of DTT (Figure 3A, lane 2). Maximal binding is achieved with 2 mM DTT (Figure 3A, lane 9). Binding assays as shown in lanes 1–11 routinely contain 10 µg BSA in addition to 0.5 µg BSA which originate from the renaturation buffer of the HM42 protein. However, varying the final amount of BSA which serves as stabilizing agent, from 0.5 to 20.5 µg, was without effect on the binding properties of Myb (Figure 3A, lanes 12–15).

Nuclear extracts of HeLa cells (34) are able to stimulate DNA-binding *in vitro* which leads to a similarly efficient binding of purified HM42 to DNA as DTT (Figure 3B). Its effect is concentration dependent (Figure 3B, lanes 1–6). All reactions contained poly[d(I-C)] (1 µg) and single-stranded DNA oligonucleotide (0.1 µg) as non-specific competitors. Nex itself induces no DNA-binding interaction (Figure 3B, lane 7). DTT (25 µM) contributed by the Nex-containing buffer D was without effect (Figure 3B, lane 8). All reactions contained BSA (10 µg) which is in excess compared to the protein content of the Nex (150 ng, lane 2) excluding non-specific protein protection. Deproteinization of the Nex with Strataclean resin (see Materials and Methods) completely abolishes DNA-binding (Figure 3B, lane 13) indicating that proteins but not other components are responsible for stimulation of the HM42-DNA interaction.

Stimulation of Myb-DNA binding activity by treatment with DTT or Nex is temperature-dependent and decreases when incubations are performed at 4°C instead of 37°C (Figure 3B, lanes 6, 9 and 10). Heating of the Nex to 65°C for 1 hr reduces the stimulatory activity (Figure 3B, compare lanes 2 and 6 with 11 and 12) without completely destroying it, suggesting that a temperature sensitive component is involved. It has been shown recently (35) that the cellular component—presumably a reductase—which activates DNA-binding of Fos/Jun proteins is also sensitive to heat. This activity is destroyed after incubation at 90°C but, similar to our results, is only reduced when heated to 60°C. The DNA-binding activity of the soluble FPLC-purified HMR_{23} (0.5 pmole or 5 ng) protein is also stimulated by DTT or the Nex (Figure 3C). Controls similar to those shown in Figure 3B indicate again that a protein present in the Nex, activates Myb binding to DNA (Figure 3C, lanes 11–13). We have ruled out that this effect was due to phosphorylation or dephosphorylation (data not shown).

Mutation of cysteine 130 to serine inhibits DNA binding

Our results resemble those reported for Fos/Jun which are also stimulated to bind to DNA by a reducing agent. This effect was attributed to a reductase (23). On the basis of that report we speculated that a reductase was also responsible for enhancement of Myb-binding to DNA. To test this we chose the shortest DNA-binding Myb protein, HMR_{23}, which contains only one cysteine at position 130 that could be a target for a reducing agent. We mutated this cysteine to serine (see Figure 1), prepared bacterial extracts shown in Figure 2 and compared the DNA-binding properties of the extracts containing wild-type HMR_{23}, the mutant $HMR_{23}S$, and HMR_{123}. The latter one contains an additional cysteine at position 78 (numbering see Figure 1). Strong DNA-binding is observed with the HMR_{23}- and

HMR_{123}-containing bacterial extracts (Figure 4A, lanes 1−3 and 4−6) without the requirement for a reducing agent suggesting that the Myb proteins are in a reduced state under these conditions. No DNA binding was observed with control lysates even at fivefold higher concentrations (data not shown). In contrast, the mutant protein $HMR_{23}S$-containing extract binds to DNA only poorly (Figure 4A, lanes 7−9). This result indicates that the cysteine 130 located in the basic region of R2 is necessary for DNA-binding. The importance of this basic region has been demonstrated by Gabrielsen et al. (11) who mutagenized basic residues of this motif and abolished the DNA binding ability of Myb. HMR_{23} and HMR_{123} proteins may be in a reduced state in the extract either because bacterial proteins protect Myb from oxidation or Myb proteins are not oxidized during preparation of the extracts, a procedure, which is very rapid in contrast to purifications which involve FPLC or denaturation-renaturation steps. It can be ruled out that purifications removed a Myb-specific reductase present in bacterial extracts, since incubation of purified HM42 with a bacterial control lysate did not induce binding (data not shown).

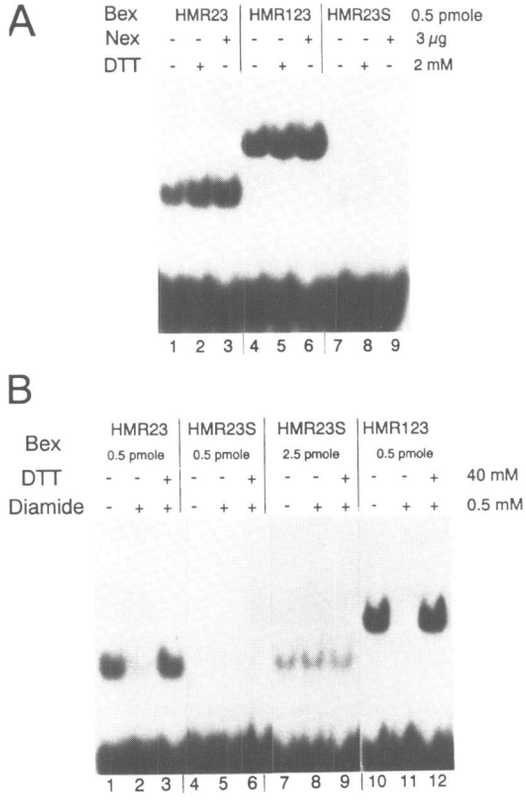

Figure 4. Diamide effect on Myb DNA-binding. **(A)** Bacterial extracts (Bex) containing 0.5 pmole HMR_{23} (lanes 1−3), HMR_{123} (lanes 4−6) or $HMR_{23}S$ protein (lanes 7−9) were incubated with 2 mM DTT (lanes 2,5,8), 3 µg Nex (lanes 3,6,9) or as control with water (lanes 1,4,7) for 15 min at 37°C, binding buffer and radiolabeled probe were subsequently added and binding of Myb was analyzed in EMSAs. **(B)** Bacterial extracts (Bex) containing 0.5 pmole HMR_{23} (lanes 1−3), $HMR_{23}S$ (lanes 4−6), HMR_{123} (lanes 10−12) or 2.5 pmole $HMR_{23}S$ (lanes 7−9) were treated with 0.5 mM diamide (lanes 2,3,5,6,8,9,11 and 12) for 30 min at 37°C. 40 mM DTT was added to the samples shown in lanes 3,6,9 and 12, incubations proceeded for another 30 min before buffer and probe were added and EMSAs performed.

Oxidation with diamide reversibly inactivates DNA-binding of Myb

Since reduction of Myb enhances its DNA binding ability it was of interest to investigate whether this effect could be reversed by an oxidizing agent such as diamide which oxidizes free sulfhydryl groups (36). This effect can be reversed again by excess of reducing agents. To test this possibility, bacterial extracts containing either HMR_{23} or HMR_{123} were treated without and with 0.5 mM diamide for 30 min at 37°C (Figure 4B). To some of the samples excess DTT (40 mM) was added (Figure 4B, lanes 3,6,9 and 12) and incubation continued for 30 min at 37°C. Subsequently the radiolabeled oligonucleotide was added and a shift assay performed. The data show that treatment with diamide strongly reduces the Myb DNA-binding affinity. This effect is completely reversible by addition of DTT (in the case of HMR_{23} and HMR_{123}).

The mutant $HMR_{23}S$-containing extract does not bind to DNA when used at the same concentrations as the wild-type HMR_{23} (0.5 pmole, Figure 4B, lanes 4−6). Only if the amount of protein is increased 5-fold, weak binding can be achieved (Figure 4B, lanes 7−9). Thus, the mutant protein has not lost its binding activity completely. This residual activity can, however, not be modulated by diamide or DTT. It therefore differs from the corresponding wild-type protein. In a published study on Fos/Jun it has been demonstrated that diamide treatment can lead to protein-protein cross-linking (23). To rule out this possibility, purified HMR_{23} protein or bacterial extracts containing

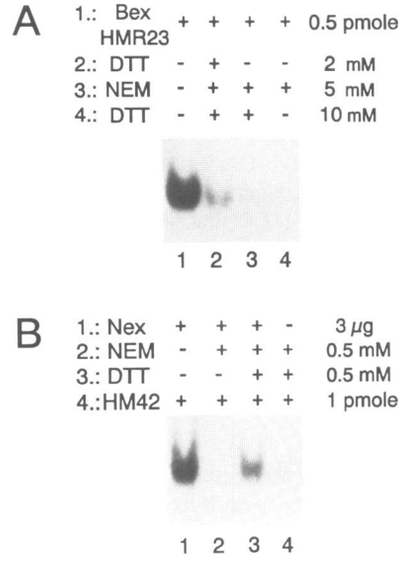

Figure 5. Alkylation of Myb. **(A)** Bacterial extracts (Bex) containing HMR_{23} protein were treated in consecutive steps with reducing or alkylating agents, binding buffer and probe were added and probes were analyzed in DNA mobility shift assays. All incubations were performed at 37°C for 15 min. Bex-containing 0.5 pmole HMR_{23} protein were pretreated with 2 mM DTT (lane 2) or as control with water (lanes 1,3 and 4) before 5 mM NEM was added (lanes 2−4). In the last step 10 mM DTT was added to reactions shown in lanes 2 and 3. **(B)** NEM treatment inhibits stimulation of HM42 DNA-binding by nuclear extracts of HeLa cells (Nex). 3 µg Nex was incubated with water (lane 1) or 0.5 mM NEM (lanes 2 to 4), NEM was then removed with 0.5 mM DTT (lanes 3) or alternatively left intact by using water instead of DTT (lane 2). HM42 protein, binding buffer and probe were added last. As control, Nex was omitted from the reactions (lane 4).

overexpressed HMR_{23} were treated with diamide (1 mM) and analyzed on non-reducing polyacrylamide protein gels in which formation of multimers should be detectable. No such forms were observed (data not shown). Alternatively, diamide treatment can result in the formation of oxidized compounds (36). These may have formed here.

The results obtained with diamide treatment of the Myb-containing bacterial extracts confirm the data described above for the purified HM42 and HMR_{23} proteins that Myb can only bind to DNA if the cysteine at position 130 is reduced.

Alkylation with NEM irreversibly abolishes Myb-DNA binding activity

To further evaluate the role of the cysteine 130 in DNA-binding we took advantage of the compound N-ethylmaleimide (NEM) which alkylates free sulfhydryl groups (37). HMR_{23}-containing extracts are inactivated by NEM treatment through alkylation (Figure 5A). Since Cys 130 is reduced in HMR_{23} proteins present in bacteria extracts, further reduction is not required for reactivity with NEM, resulting in loss of binding-activity (Figure 5A, lanes 1–3). This alkylation is irreversible as demonstrated by adding excess of DTT (compare lanes 3 and 4). We have shown that the Nex exerts a stimulatory effect on Myb binding to DNA. This could be explained by the action of a reductase activity, since reduction of cysteine 130 by DTT also activates DNA-binding of Myb (Figures 3 and 4). Free sulfhydryl groups are not only required for Myb DNA-binding but also for alkylation by NEM. We therefore investigated, whether the Nex was capable of rendering the HM42 protein sensitive to NEM. Indeed, addition of NEM to the purified HM42 Myb protein plus the Nex inhibits DNA binding (Figure 5B, lane 2). This reaction could be due to NEM affects on Nex or HM42 or both. To test this we took advantage of the fact that DTT can remove NEM from a reaction. The Nex was therefore allowed to react with NEM and subsequently DTT was added to inactivate the residual NEM again. This treatment only slightly reduced the stimulating effect of the Nex on Myb-DNA binding, about two- to threefold (Figure 5B, lane 3). Activation of Myb-binding is not caused by residual amounts of DTT that escaped reaction with NEM (Figure 5B, lane 4). The result indicates that NEM apparently affects predominantly the Myb-protein itself. Since this can only occur if the Myb protein contains a free sulfhydryl group we postulate that the Nex exerted a reducing effect on the HM42 which makes the Myb protein vulnerable to NEM. Alkylation of the cysteine of HM42 then completely blocks DNA-binding irreversibly.

Nuclear extracts of transfected COS cells show Myb-specific DNA binding activity which is sensitive to oxidation and alkylation

Instead of using Myb-containing bacterial lysates or supplementing recombinant Myb protein by the Nex *in vitro* we extended our study by using Myb-containing cellular extracts from mammalian cells. Therefore COS cells were transiently transfected with an effector plasmid coding for the HM42 protein under control of SV40 promoter/enhancer sequences (26) or with the parental pECE vector. HM42 protein is expressed in transfected COS cells at high levels as has been shown by Western blotting (data not shown). Nuclear extracts were prepared (34) and used to perform DNA-mobility shift assays (Figure 6). Only Nex prepared from HM42—but not from mock-transfected cells lead to DNA-protein complexes (Figure 6A, lanes 1 and 2). To prove that the DNA-binding was Myb-specific we competed the reaction with a 100-fold excess of the specific non-radioactive oligonucleotide. In contrast, a mutated oligonucleotide containing only one point mutation did not interfere with complex formation (Figure 6A, lanes 3 and 4). Two different Myb antisera, sheep serum 33 and monoclonal antibody (MAB) 6/22 again interfered with the binding. Sheep serum 33 which was directed against amino acids 1–358 of Myb inhibited DNA/protein complex formation, whereas interaction with MAB 6/22 recognizing the first repeat, which is dispensible for binding (10), resulted in a supershift of the complex (Figure 6A, lanes 9 and 10). A control serum did not influence the binding (lane 8). As further controls, incubation of these sera without Nex (data not shown) or with mock-transfected Nex did not produce any DNA/protein complexes (lanes 5–7). These reactions prove that the DNA/protein complex is indeed induced by Myb-DNA interaction. As expected, HM42-containing Nex lead to a DNA/protein complex independent of DTT in the reaction (Figure 6B, lanes 1 and 2). The cell derived Myb protein was then subjected to diamide as well as NEM treatment *in vitro* and their effects tested in DNA-mobility shift assay. As can be seen, both diamide, as well as NEM, completely block Myb-DNA interaction (Figure 6B, lanes 3, 5 and 6). The diamide effect is reversible by DTT. These results indicate that the COS cell Nex

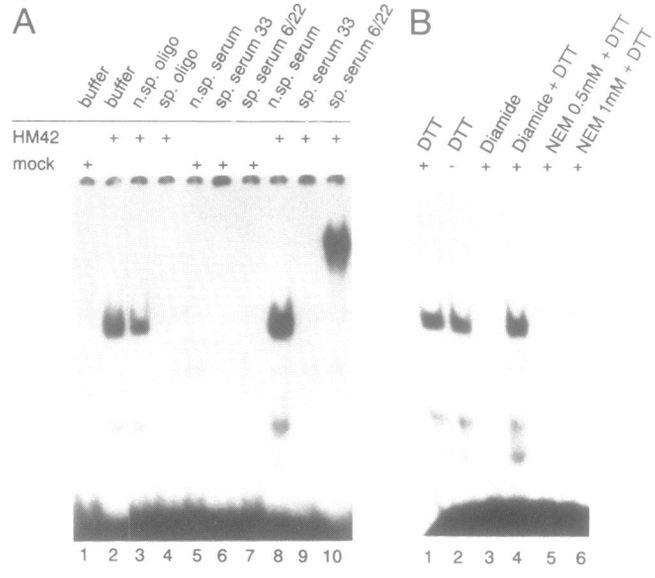

Figure 6. Myb-specific binding activity present in nuclear extracts of transfected COS cells is sensitive to oxidation and alkylation. **(A)** Transfected COS cells exhibit Myb-specific binding activity. 4 μg of nuclear extracts from mock- or HM42-transfected COS cells were incubated with radiolabeled probe for 20 min at room temperature. The resulting DNA/protein complexes were analyzed by gel mobility shift assay (lanes 1 and 2). Preincubation of HM42 transfected Nex with 100-fold excess of non-specific (n.sp., lane 3) or specific (sp., lane 4) oligonucleotide and preincubation of mock- (lanes 5–7) or HM42-transfected Nex (lanes 8–10) with one non-specific (n.sp., lanes 5 and 8) or two specific (sp., lanes 6, 7, 9 and 10) antisera proved the specificity of binding. **(B)** Oxidation and alkylation inhibit DNA-binding of transfected Myb. 4 μg of nuclear extracts from HM42-transfected COS cells were incubated with probe for 30 min at 37°C in the presence or absence of 10 mM DTT (lanes 1 and 2). Pre-treatment of nuclear extracts with 1 mM diamide (lane 3) or with 0.5 mM or 1 mM NEM (lanes 5 and 6) for 30 min at 37°C abolished Myb binding activity. Only the diamide effect is reversible by an additional incubation with 10 mM DTT for 30 min at 37°C (lane 4).

contains a component—presumably a reductase—which reduces HM42 intracellularly and thus allows its binding to DNA. DTT is not required under these conditions, whereas it is essential for purified HM42 protein. Alkylation and oxidation inhibit DNA-binding of COS cell derived Myb resembling the Myb proteins expressed in bacteria.

DISCUSSION

This paper describes that reduction of the cysteine 130 of the DNA-binding domain of the Myb protein is essential for DNA binding. Reduction is exerted by the cellular milieu and is maintained in the Myb-containing nuclear extract. The recombinant purified Myb protein requires supplementation of Nex in order to bind to DNA. The amount of lysate is small, 150 ng of Nex is sufficient, suggesting a strong activating activity. Lysates from other mammalian cell types have the same effect, whereas bacterial extracts are inactive. This rules out unspecific protein protection effects. Heating reduces and deproteinization of the Nex destroys its effects. Thus it appeared likely that a protein with enzymatic function was involved. The stimulating protein does not bind to the Myb protein directly in such a way that altered mobility of Myb-DNA complexes could be detected.

The stimulating effect of Nex can be mimicked by reducing agents such as DTT. The fact that addition of DTT and Nex stimulate Myb binding raises the possibility that a reductase present in the Nex activates Myb DNA-binding. Proteins that were purified require reduction for DNA-binding presumably because cysteine residues are oxidized during the purification procedure. Since recombinant Myb proteins in bacterial extracts do not require pretreatment by DTT or Nex we suppose that they are in a reduced state. These proteins loose their ability to bind DNA after oxidation of cysteine residues with diamide. DNA-binding of these oxidized proteins can then be induced again with DTT. Alkylation of cysteine residues by NEM also inhibits DNA-binding of Myb. Nex and DTT reduce oxidized cysteine residues raising free sulfhydryl groups which can be alkylated. Since mutant HMR_{23} contains only one cysteine at position 130 we identify this cysteine as the target for redox-manipulations and alkylation. This cysteine was found to be essential for DNA-binding since its mutation to serine almost completely abolished this property. Cysteine 130 is located in a basic motif of the Myb protein. Surrounding basic aminoacids have been mutagenized and also been shown to be essential for DNA binding (11). Thus, this site plays an essential role in the function of the Myb protein. The basic surrounding increases the reactivity of the cysteine and makes it more easily accessible to oxidation. Within the Myb protein family the cysteine is conserved in mice (38), chicken (1), *Drosophila* (39), maize (40) and yeast (41) as well as in human A-Myb and B-Myb proteins (42) and in the homologous *Xenopus* proteins Xmyb1 and Xmyb2 (43). It is also present in the two retroviral Myb proteins from AMV (1) and E26 (2). Mutation of cysteine to serine as described here does probably not disrupt the helix structure of this part of the protein since the two helix-loop-helix proteins Cro and Rep of phage 434 (44) contain a serine at the analogous site (11).

A reductase has recently been implicated in regulation of binding of Fos-Jun to DNA. DNA binding of the Fos-Jun heterodimer was modulated by reduction-oxidation of a single conserved cysteine residue in the basic DNA-binding domains of the two proteins (23). The reductase has been identified very recently (45). The purified protein is able to induce binding activity of Fos/Jun proteins *in vitro*. The high degree of similarity between the results presented here on Myb and the ones published for Jun/Fos is further supported by the fact that the basic motif containing Cys 130 exhibits some homology to Fos/Jun proteins (24). However, mutation of the homologous cysteine residue to serine in Fos/Jun proteins leads to increased binding properties of the mutant proteins (23). In contrast, $HMR_{23}S$ binds to DNA with reduced affinity. Therefore, the function of the conserved cysteine residue for DNA/protein interaction may be different. Although the DNA-protein interaction of Fos/Jun and Myb proteins is molecularly different, both proteins may be regulated by redox mechanisms that modulate a single cysteine in a conserved region.

No significant difference in DNA-binding properties of Myb HMR_{23} and HMR_{123} is detectable. Moreover, we show that both proteins are sensitive to redox manipulations. These two proteins differ by the R_1 region, the role of which is still unknown. It has been shown not to be directly involved in DNA-binding (10). Therefore it is not surprising that no effect of R_1 can be detected here either.

Redox modifications have also been considered as modulators of other transcription factors. Regulation of DNA and RNA binding by redox mechanisms has been described for several proteins such as the bacterial protein OxyR which changes its binding properties and activates oxidative stress genes only when oxidized (46). Reduction is also necessary for human iron response element binding protein which only in its reduced state is able to bind to mRNAs of ferritin and transferrin receptor (47). H_2O_2 treatment of the HIV-DNA provirus-containing Jurkat cells induces virus production, an effect which can also be provoked by NF$\varkappa$B (48). Redox manipulations may also affect NF$\varkappa$B binding directly (49).

Modification of DNA-binding of the Myb protein has previously been described by phosphorylation of the aminoterminus by CKII which negatively regulates binding to low affinity fragments (21). CKII phosphorylation sites are deleted in HM_{23} and HMR_{123} proteins. Moreover, these proteins are not phosphorylated *in vitro* by purified CKII kinase or by cellular extracts which stimulate DNA-binding (data not shown). The effect described here positively regulates Myb binding and was determined with a DNA oligonucleotide containing the high affinity binding site TAACGGTT which is present on the upstream region of the target gene mim-1 (50) and the c-*myc* gene (26). Redox manipulations and alkylation *in vitro* modulate not only recombinant bacterial Myb proteins but also Myb proteins present in transfected COS-cell extracts. Thus, changes in the redox state of a cell may have consequences on Myb-regulated gene expression.

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