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The importance of the linker connecting the repeats of the c-Myb oncoprotein may be due to a positioning function

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ABSTRACT

The DNA-binding domain of the oncoprotein c-Myb consists of three imperfect tryptophan-rich repeats, R_1, R_2 and R_3. Each repeat forms an independent mini-domain with a helix–turn–helix related motif and they are connected by linkers containing highly conserved residues. The location of the linker between two DNA-binding units suggests a function analogous to a dimerisation motif with a critical role in positioning the recognition helices of each mini-domain. Mutational analysis of the minimal DNA-binding domain of chicken c-Myb (R_2 and R_3), revealed that besides the recognition helices of each repeat, the linker connecting them was of critical importance in maintaining specific DNA-binding. A comparison of several linker sequences from different Myb proteins revealed a highly conserved motif of four amino acids in the first half of the linker: LNPE (L138 to E141 in chicken c-Myb R_2R_3). Substitution of residues within this sequence led to reduced stability of protein–DNA complexes and even loss of DNA-binding. The two most affected mutants showed increased accessibility to proteases, and fluorescence emission spectra and quenching experiments revealed greater average exposure of tryptophans which suggests changes in conformation of the proteins. From the structure of R_2R_3 we propose that the LNPE motif provides two functions: anchorage to the first repeat (through L) and determination of the direction of the bridge to the next repeat (through P).

INTRODUCTION

The c-*myb* proto-oncogene is expressed in a limited range of differentiating cell types and has a critical role in early stages of hematopoiesis (1,2). The c-Myb transcription factor plays a role in the balance between proliferation and differentiation in these cells. Aberrant overexpression of the transcription factor inhibits differentiation of hematopoietic precursor cells (3) while loss of c-Myb expression inhibits their proliferation (4–6).

The c-*myb* gene encodes a 75 kDa sequence-specific DNA-binding transcription factor with at least three functional domains (2). The DNA-binding domain (DBD) is located near the N-terminus and is a highly conserved tryptophan-rich region composed of three imperfect repeats (R_1, R_2 and R_3) each with a helix–turn–helix related motif (7–9). Each repeat has a distinct function and is folded independently of the others. R_3 is tightly folded in solution and mainly responsible for the sequence-specific recognition of the AAC-core in the binding site (9,10). R_2 is a more flexible unit with a cavity inside the hydrophobic core (11), and seems to undergo slow conformational fluctuations and specific conformational changes upon binding to DNA (11–14). The function of R_1 is thought to be stabilisation of the protein–DNA complex through electrostatic interactions (15–17). The minimal region in Myb giving sequence-specific DNA-binding has been delimited to the R_2 and R_3 repeats (18–20). The R_2R_3 of Myb binds to the major groove of DNA continuously (10) similar to transcription factor IIIA-type zinc fingers (21–23).

The majority of eukaryotic transcription factors analysed so far use motifs that insert an α-helix into the major groove of the target DNA to obtain specific binding. However, due to the curving of the DNA surface, a single straight α-helix can only contact 4–6 base pairs, which is too short an interaction surface to achieve necessary specificity (24). Therefore, sequence-specific DNA-binding usually requires two or more subdomains to get a sufficiently large interaction surface in the major groove. Many transcription factors achieve this by forming homo- or heterodimers like, for example, leucine zipper proteins, helix–loop–helix proteins and nuclear receptors (25). In addition to the primary function of binding monomers together, the dimerisation domain is also responsible for positioning the two DNA-recognition units in a correct distance and direction.

Certain families of transcription factors bind their target as monomers but still use multiple recognition units to contact DNA. The best studied example is the large family of zinc finger proteins which binds DNA through covalently linked mini-domains. Another example is the covalently linked tryptophan-rich repeats in the DNA-binding domain of the Myb family of transcription factors. In these transcription factors with repeated motifs the relative positioning of the mini-domains might be directed by the linker sequences joining them. If so, these linkers would have a role equally important as the dimerisation domains in dimeric transcription factors. Accordingly, the linkers in these transcription factors contain distinct conserved residues. The evolutionary conservation of these linkers in Myb- and zinc finger proteins suggest a distinct functional role in the activity of the protein.

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Choo and Klug (26) studied the influence on DNA-binding of linker sequences connecting zinc fingers and found that a replacement of individual amino acids could reduce binding by factors up to 24.

In the present work we addressed the question whether the linkers between the repeats in the Myb proteins might be of similar importance. We report that certain single amino acid substitutions in the linker sequence of c-Myb R_2R_3 impair the ability of the protein to bind DNA. The weakening or loss of binding is accompanied by changes in conformation of the protein as seen from protease cleavage patterns, fluorescence emission spectra and quenching experiments.

MATERIALS AND METHODS

Expression and purification of c-Myb R_2R_3 proteins

The minimal DNA-binding domain of the chicken c-Myb protein, R_2R_3 and derived mutants were expressed in *Escherichia coli* using the T7 system (27). Proteins were purified as described by Gabrielsen *et al.* (8).

Mutagenesis

The following mutations were introduced in the R_2 region of c-Myb R_2R_3 by site-directed *in vitro* mutagenesis: T96R, Q101A, R102A, E105A, PK112/113A, H121A, K123A, R125A, Q129A, R131A, R133A and N136A, and in the linker region: L138A, N139A, P140A, P140G and E141A (amino acids numbered from the first residue in the chicken c-Myb protein). The mutants Q101A, R102A, Q129A, R133A, N136A and N139A have been described previously (8).

Electrophoretic mobility shift assay

DNA-binding was monitored by the electrophoretic mobility shift assay (EMSA) (28), with the modifications described in Gabrielsen *et al.* (8). The MRE probe was a 23mer duplex oligo derived from the c-Myb recognition element (MRE) in the upstream region (site A) of the *mim*-1 gene (29).

MRE

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5′-GCATTATAACGGTTTTTTAGCGC-3′
3′-CGTAATATTGCCAAAAAATCGCG-5′
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Protease treatment of c-Myb R_2R_3 proteins

Trypsin and chymotrypsin solutions were made fresh each time from powder dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Purified c-Myb proteins were diluted in TE-buffer and incubated with or without protease at 37°C for 15 min and the reaction stopped by addition of SDS loading buffer. The samples were heated at 95°C for 2 min and loaded on a 10–20% polyacrylamide gradient gel containing SDS according to Laemmli (30).

Fluorescence spectroscopy

A Perkin-Elmer LS-50B Luminescence Spectrometer and a Perkin Elmer Luminescence Spectroscopy Cell of 120 µl were used for the fluorescence experiments. The exitation wavelength was 295 nm, and exitation slit 15 nm. Emission spectra were recorded between 310 and 400 nm with an emission slit of 5 nm,

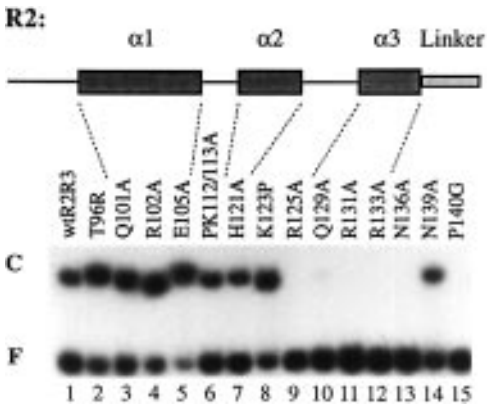

Figure 1. DNA-binding of R_2- and linker mutants of the minimal DNA-binding domain of chicken c-Myb. Recombinant *E.coli* crude protein extracts of the various mutants were diluted either 1:1000 (Q101A, R102A, PK112/113A, H121A, K123P, R125A, Q129A, R131A, R133A, N136A, N139A, P140G) or 1:50 (T96R and E105A) due to differences in induction levels as judged by SDS–PAGE analysis. One µl was incubated with 10 fmol MRE probe at 25°C for 10 min. Purified wild-type R_2R_3 (10 fmol) was included as control (lane 1). The complexes formed were analysed by the electrophoretic mobility shift assay as described in Materials and Methods. The positions of the mutations relative to secondary structure elements in R_2 (12) are indicated. C, protein–DNA complex; F, free DNA.

and a scanning speed of 500 nm/min. Each recording was made as an average of three accumulated scans. Samples were prepared from purified proteins in TE buffer at a concentration of 1–2 µM. In the quenching experiments (31), each protein was measured at its emission maximum. Immediately before recording, 126 µl diluted protein was added to a test tube containing 14 µl of either TE buffer, 1.5, 3, 4.5, 6 or 7.5 M acrylamide solution to obtain final concentrations of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 M acrylamide. Fluorescence values were corrected with respect to background fluorescence and the inner filter effect of acrylamide according to Parker (32). For denaturation, proteins were incubated with 6 M guanidinium chloride for 20–30 min at room temperature before recording.

RESULTS

In a screening study of a series of single amino acid substitutions dispersed throughout the second repeat (R_2) in the DNA-binding domain of chicken c-Myb and the linker connecting the second and third repeats, we observed two regions especially affected by mutations. When performing simple EMSA studies on crude bacterial extracts containing these mutants, we found that mutations in the third α-helix of R_2 abolished or severely reduced DNA-binding (Fig. 1), as would be expected from previous reports (7,8). However, we also observed that one mutation in the linker connecting R_2 and R_3 abolished binding while another did not (N139A and P140G in Fig. 1). This suggested that the linker region between the repeats R_2 and R_3 might have an equally important role in binding as the third helix of R_2, and we decided to investigate more closely the importance of the linkers.

To compare linker sequences for the conserved residues, we defined the 'linker' sequence of the c-Myb DNA-binding domain to span from the end of the third α-helix in one repeat to the beginning of the first α-helix in the next repeat (for chicken c-Myb that is N136 to T148), assuming conservation of these

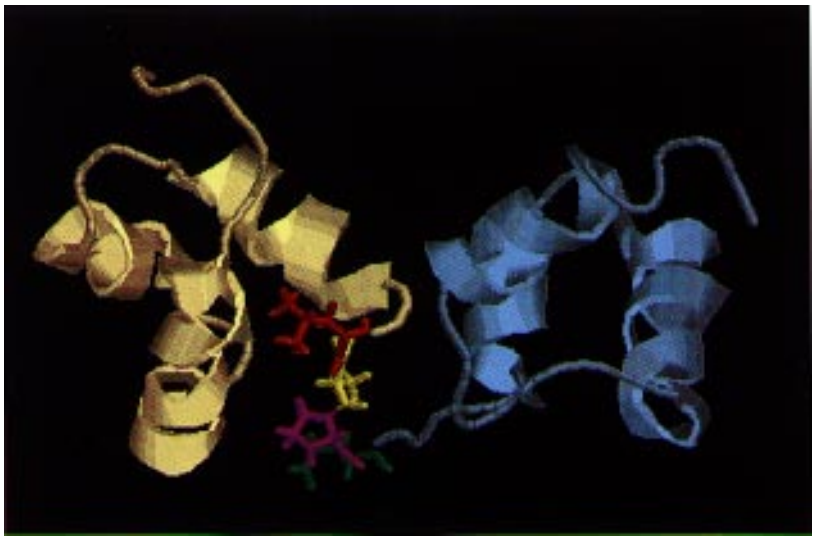

Figure 2. Three-dimensional structure of mouse c-Myb R_2R_3 in complex with DNA. The structure of Ogata *et al.* (10) was displayed using the RasMac v.2.6 Molecular Visualisation Program (Roger Sayle). R_2 is displayed in pale yellow, R_3 in light blue, L138 in red, N139 in yellow, P140 in magenta and E141 in green.

secondary structure elements. A comparison of linkers connecting R_1 and R_2 and linkers connecting R_2 and R_3 in Myb proteins from both humans, higher mammals, frog, plants, insects and yeast gave the following conserved linker residues: x x L_{100} N_{82} P_{100} E_{67} x x K_{97} x x W_{97} T_{97} (numbers referring to percent conservation). This shows a highly conserved sequence of four amino acids in the first half of the linker: LNPE, and a single conserved lysine closer to R_3. In the chicken c-Myb the conserved amino acids are L138, N139, P140, E141 and K144. The 3-D location of the LNPE motif according to the NMR structure of Ogata *et al.* (10) is shown in Figure 2. Frampton *et al.* (7) performed a mutational analysis of c-Myb R_2R_3 where they substituted K144 with isoleucine and observed a severe decrease in binding. They also substituted the entire triplet NPE (139–141) for RRK found in the C-terminal of R_3 and observed a complete loss of DNA-binding.

To obtain more detailed experimental evidence for the importance of this conserved linker motif, we mutated each residue in the LNPE motif and performed a detailed biochemical analysis of these five R_2R_3 linker mutants: L138A, N139A, P140A, P140G and E141A. The mutant proteins were expressed in *E.coli*, purified and analysed for DNA-binding by the electrophoretic mobility shift assay. The P140G mutant was chosen in addition to the alanine mutant because glycine is the only amino acid able to adapt to the chain-breaking conformation of proline. As shown in Figure 3, the P140G mutant abolished complex formation while the N139A, P140A and E141A seemed to be marginally affected compared with wild-type R_2R_3. L138A had reduced affinity compared with wild-type. About four times more L138A protein than wild-type protein was needed to give the same amount of binding to the probe. Thus the two most conserved positions (L138 and P140) also seemed to be the most important for maintaining an active DNA-binding structure. It is though noteworthy that P140G did not bind to the MRE probe while the alanine mutant P140A bound with an affinity indistinguishable from wild-type R_2R_3.

In our experience direct EMSA with purified protein does not discriminate clearly between complexes that have moderate

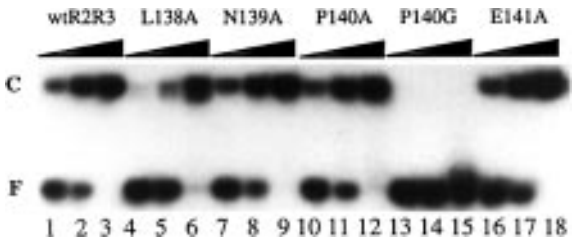

Figure 3. DNA-binding of c-Myb R_2R_3 proteins mutated in the linker connecting the repeats. Purified wild-type R_2R_3 or linker mutant proteins (5, 10 and 50 fmol) were incubated with 10 fmol MRE probe at 25°C for 10 min and analysed by the electrophoretic mobility shift assay as described in Materials and Methods. C, protein–DNA complex; F, free DNA.

differences in stability, although such differences may have large effects *in vivo* (33). Excluding the P140G mutant that did not bind DNA, the remaining proteins mutated in the LNPE motif were analysed with regard to the stability of the specific complexes. First the protein–MRE complexes were titrated with increasing amounts of poly(dI–dC) (Fig. 4A). The L138A mutant was titrated out at much lower concentrations of poly(dI–dC) than the wild-type. Also the titration of P140A occurred at lower concentrations of poly(dI–dC) than for the wild-type protein. The N139A and E141A mutant proteins were titrated almost in parallel with the c-Myb R_2R_3 wild-type. Similar differences were observed when the preformed specific complexes were subjected to a 75-fold excess of unlabelled specific DNA and the decay of specific complexes were followed for a period of 30 min (Fig. 4B). The L138A and P140A mutant proteins in complex with DNA had a very rapid decay. While there was still detectable amounts of labelled complexes with the wild-type protein left after 30 min of competition, these two mutants were almost competed out after 2 min. The N139A mutant was competed out after 10 min suggesting slightly reduced stability, while the E141A mutant behaved very similar to the wild-type protein. These studies show that there were distinct differences in stabilities between the

Figure 4. Analysis of mutant c-Myb R_2R_3–DNA complexes. **(A)** Titration with poly(dI–dC). Purified wild-type R_2R_3 or linker mutant proteins (N139A, P140A, E141A) (20 fmol), or 40 fmol of the L138A protein, were incubated with 10 fmol MRE probe and 0, 0.125, 0.25, 0.5, 1.0 or 2.0 µg poly(dI–dC) respectively (lanes 1–6) and analysed by the electrophoretic mobility shift assay as described in Materials and Methods. **(B)** Decay of mutant c-Myb R_2R_3–DNA complexes. Purified wild-type R_2R_3 or linker mutant proteins (N139A, P140A, E141A) (15 fmol), or 40 fmol of the L138A protein, were incubated with 10 fmol MRE probe at 25°C for 10 min. Then the specific complexes were exposed to an excess of 750 fmol unlabelled MRE oligo and further incubated for 0, 2, 5, 10 or 30 min before analysis by the electrophoretic mobility shift assay as described in Materials and Methods.

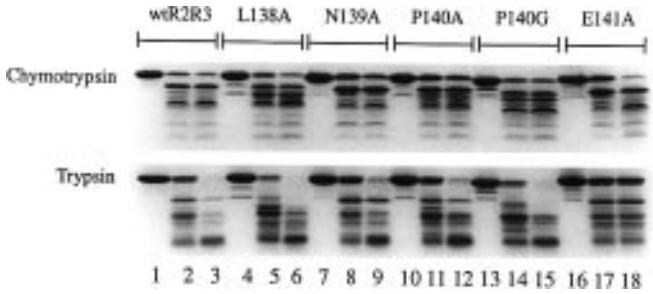

Figure 5. Protease fingerprinting of mutants. Purified wild-type R_2R_3 or linker mutant proteins (6 µg) were incubated with 0, 6 or 24 ng chymotrypsin or 0, 4.5 or 12 ng trypsin at 37°C for 15 min and then analysed on an SDS–polyacrylamide gradient gel, 10–20%, as described in Materials and Methods.

various specific complexes and that the linker sequence indeed has an important impact on the DNA-binding properties of R_2R_3.

Using a combination of proteolytic fingerprinting and fluorescence spectroscopy, we have in previous studies of the chicken c-Myb DNA-binding domain seen that changes in conformation can be correlated with altered DNA-binding activity (13). To get an indication of whether there was differences in overall conformation in the various linker mutants the purified proteins were subjected to limited proteolysis by chymotrypsin and trypsin (Fig. 5), two proteases whose specificity should not be affected by the amino acid replacements in our mutants. The pattern of bands after cleavage revealed distinct differences in degree and pattern of proteolysis among the different mutants. The L138A and P140G mutants gave patterns which differed from wild-type protein, while the cleavage pattern of N139A, P140A and E141A were more similar to wild-type R_2R_3. This suggested to us that mutants most affected in DNA-binding also had induced changes in conformation.

To obtain more evidence for a possible change in conformation induced by linker mutations, we performed a series of fluorescence analyses of the mutants. Quenching experiments with the neutral quencher acrylamide monitored if mutations in the linker induced any alteration in the average exposure of tryptophans in the R_2R_3 protein. As seen by the plot in Figure 6A, the L138A and P140G mutants were quenched significantly more than the wild-type R_2R_3 suggesting an altered conformation, while the N139A, P140A and E141A mutants gave slopes almost identical to wild-type. In addition, a series of emission scans of native proteins showed that the L138A and P140G mutants had significant higher quantum yield and a shift in emission maximum towards longer wavelength (352 nm compared with 338 nm for wild-type R_2R_3), supporting the conclusion drawn from the quenching experiments (Fig. 6B). All proteins gave the same emission spectra after denaturation with 6 M GuHCl where the tryptophans are fully exposed to the solvent (emission maximum = 358 nm).

We conclude that mutations of conserved amino acids in the linker region connecting the repeats in the DBD of c-Myb gives proteins with different abilities to bind DNA. We can range the mutants according to their increasing affinity/stability when complexed to DNA as follows: P140G < L138A << P140A < N139A ≤ E141A = wild-type R_2R_3. The two weakest mutants, L138A and P140G, also shows altered protein conformation.

DISCUSSION

In this work we have investigated the importance of the linker sequence between the R_2 and the R_3 repeats in the DNA-binding domain of the chicken c-Myb protein. We have shown that certain mutations in the highly conserved LNPE-motif in the first half of the linker severely affects the proteins ability to bind DNA. Mutations of the invariant residues L138 and P140 were found to have great impact on both DNA-binding and conformation of the protein. Point mutation in the highly conserved K144 was shown previously to severely affect DNA-binding as well (7).

The Myb protein is a transcription factor with a repeated DNA-binding motif. The DNA-binding domain is made up from

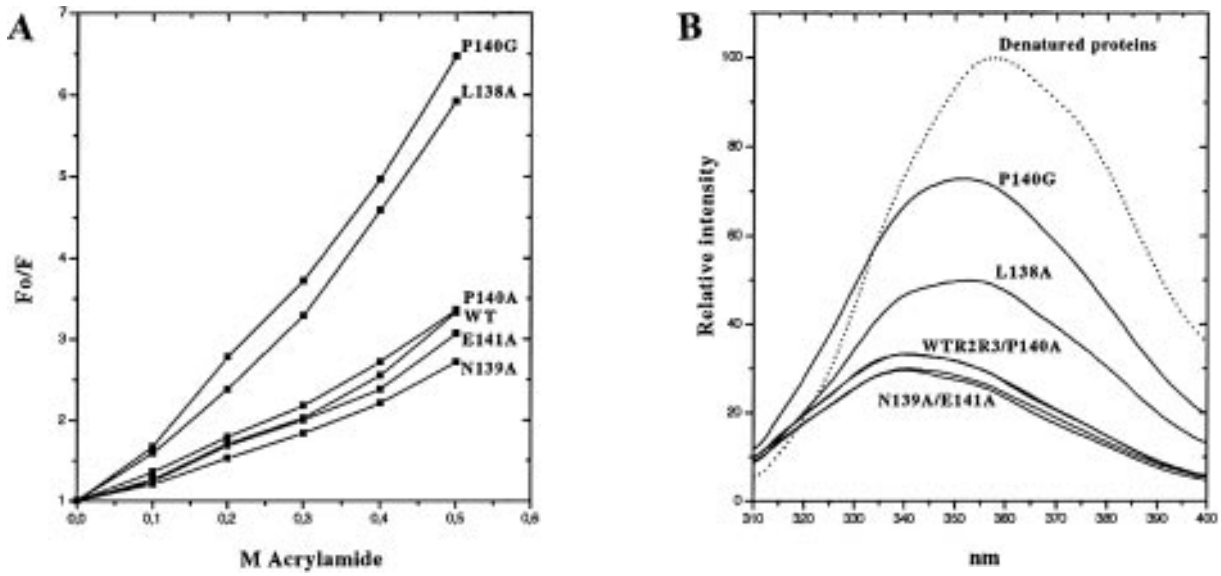

Figure 6. Fluorescence analysis of wild-type R_2R_3 and linker mutants. **(A)** Quenching of the fluorescence by acrylamide. The emission of 2 µM protein were quenched by increasing concentrations of acrylamide (0, 0.1, 0.2, 0.3, 0.4 and 0.5 M) and emission recorded at the emission maximum for each protein. The results are presented as the ratio of unquenched and actual fluorescence (Fo/F) as a function of acrylamide concentration, as described in Materials and Methods. **(B)** Emission spectra of the proteins in denatured and native form. Samples containing 1 µM protein were prepared with or without 6 M guanidinium hydrochloride, and emission spectra measured as described in Materials and Methods. The spectra of the denatured forms were used for normalising.

three imperfectly repeated mini-domains: R_1, R_2 and R_3, connected by short linker sequences. We see only one reasonable explanation why the linker sequence should be of critical importance for DNA-binding and that is if it had a role in positioning the two DNA-interacting subdomains, R_2 and R_3, relative to each other to achieve an optimal DNA-binding surface. This explanation would define the linker as a functional analogue of the various dimerisation domains found in transcription factors forming homo- or heterodimers, since many of them also have a critical role in mediating an optimal relative orientation of the DNA-binding monomers. We have not provided conclusive evidence for this hypothesis but all our observations can easily be rationalised within such a framework. The high conservation of specific residues would not have been expected if the linker was only a passive chain holding two autonomous domains physically together. Our mutational analysis also demonstrates that they are conserved because they are necessary to achieve stable protein–DNA complexes. Furthermore, increasing the flexibility of the linker by replacing a stiff proline residue with a glycine, abolished DNA-binding. In our collection of nearly 20 single amino acid replacements in R_2R_3 this is the only mutation outside the recognition helices that has such a large negative effect on DNA-binding.

An NMR-derived 3-D model of the mouse R_2R_3–DNA complex allows us to rationalise our observation. Displaying this structure revealed that the leucine in position 138 in the linker of R_2R_3 seems to make close contact with the hydrophobic core and thereby the tryptophans of R_2 (Fig. 2). It appears to be a kind of last anchoring, and then the proline in position 140 changes the direction of the peptide chain which makes the linker break away from R_2. A comparison of a wide range of linker sequences from Myb proteins in many organisms confirmed that these two amino acids are invariant in all linker sequences we analysed. The loop from glutamate 141 to serine 146 have some flexibility, since Ogata *et al.* (11) observe some flexibility in this connecting loop when measuring NMR relaxation parameters. Accordingly, the orientation of R_2 and R_3 are not fixed in solution, while upon binding to DNA their relative orientation becomes fixed (34). At the R_3 side the flexibility ends with the docking of the tryptophan and start of the first helix (E149). To keep our hypothesis consistent with these observations we have to assume that the conserved residues in the linker limits the relative orientation of the two subdomains to an allowed range of conformations and that upon DNA-binding the final fixed orientation is achieved.

Mutations in the four conserved amino acids LNPE in the first half of the linker sequence of R_2R_3 gives rise to specific protein–DNA complexes with great variation in binding affinity and stability. The L138A mutant binds to the MRE probe with approximately one fourth the strength of the wild-type complex, and was severely destabilised under competitive conditions. It was also more sensitive to proteases, and together with fluorescence emission and quenching studies this suggests that the mutation had caused an increase in the average exposure of the peptide chain and the tryptophans to the solvent. These changes might be due to the alanine failing to make as extensive contact with the hydrophobic core and the tryptophans of R_2 as leucine because of its smaller side chain, thereby leaving one tryptophan more exposed to solvent than in the wild-type R_2R_3 protein.

Two mutations were made in position 140: P140A and P140G. The idea was that a glycine residue would be the only amino acid able to mimic the conformation of the peptide bond made by proline and thereby letting the linker bend in the same way as in the wild-type protein. However, the P140G mutant was found to be unable to bind to MRE while the P140A mutant bound to MRE but with reduced complex stability. Studies of the conformation revealed that the alanine mutant had the same average exposure of tryptophans as wild-type protein, and the same degree of quenching, which makes us conclude that the overall conformation is not severely altered. The glycine mutation on the other hand, had a much higher average exposure of tryptophans with increased

accessibility to proteases and to quenching by acrylamide, which indicates changes in conformation of the protein. Because of the lack of side chain in glycine the protein should have increased freedom of rotation in the mutated region and the impact that this has on the overall conformation may be too severe for the protein to be able to recover to an active DNA-binding conformation. The linker might just be too flexible and leave the protein outside acceptable ranges of conformations resulting in loss of function and failure to position R_2 and R_3 correctly for DNA-binding.

The two other mutations, N139A and E141A, corresponding to highly conserved but not invariant positions, were practically unchanged with regard to DNA-binding and protein conformation except for a slightly increased off rate seen with N139A.

Zinc finger proteins represent another family of transcription factors besides c-Myb where the DBD is built up from imperfect repeats. Choo and Klug (26) reported evidence for the importance of the conserved linker sequences between the individual zinc fingers. Upon DNA-binding the zinc fingers must be positioned correctly for continuous binding, each finger contacting 3 base pairs in the major groove in a simple and repetitive manner. When heterologous linkers were inserted, binding was abolished, and single amino acid replacements led to severe reduction of the binding activity. Their results are similar to those obtained with the c-Myb linkers in our study, and clearly suggests that in these transcription factors the linkers are not only random sequences linking together autonomous domains, but they also have an active role in the overall conformation and function of the proteins.

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