J. Biochem. **134**, 583–589 (2003)
DOI: 10.1093/jb/mvg177

Mutagenesis Analysis of the Interaction between the Dorsal Rel Homology Domain and HMG Boxes of DSP1 Protein

Davy Martin, Anne Daulny, Martine Decoville and Daniel Locker*

Centre de Biophysique Moléculaire, CNRS, conventionné avec l'Université d'Orléans, rue Charles Sadron, 45071 Orléans cedex 2, France

Received June 6, 2003; accepted August 4, 2003

DSP1 is an HMG-like protein of *Drosophila melanogaster* consisting of 386 amino acids with two HMG domains at the C-terminal end. It was shown to interact with Dorsal protein through the HMG domains and to enhance its DNA binding. Each HMG domain consists of approximately 80 amino acid residues, forming three alpha helices folded into an L-shaped structure. We have compared the interaction of various truncated and mutated forms of DSP1 with the dorsal Rel homology domain (RHD). In particular, we have mutated the conserved tryptophan residue 212 or 302 in A or B boxes or the lysine-rich region (^{253}KKRK^{256}) of the A/B linker. Analysis by circular dichroism revealed that the protein tertiary structure is affected in these mutants. However, these mutations do not abolish the DSP1 binding to Dorsal, except if the two HMG boxes are altered, *i.e.*, in a double mutant or in mutant isolated domain. Finally, studies on the enhancement of Dorsal DNA binding by DSP1 revealed that the DNA affinity is maximum in the presence of wild-type DSP1, is dramatically reduced when box A is altered, and is completely abolished when box B is altered.

Key words: Dorsal, *Drosophila*, DSP1, HMGB, protein–protein interaction.

HMGB1 proteins are an abundant class of mammalian chromatin proteins, present in all vertebrate nuclei. HMGB1 proteins are characterized by three structural domains: the N-terminal A domain, the central B-domain and the terminal C-domain. The two domains A and B comprise 75–80 residues, named box A and box B respectively, with about 30% similarity in amino acid sequence. NMR data showed that boxes A and B have a similar fold with minor differences, in which three alpha helical segments form an L-shaped structure stabilized by a hydrophobic core (*1*–*3*). HMGB1 proteins display structure-specific rather than sequence- specific DNA binding. Although they bind to double-stranded DNA and facilitate the circularization of DNA restriction fragments, they interact with higher affinity with DNA already containing a bend, such as four-way junctions and cisplatin-modified DNA (*4*, *5*).

They also have the property to distort the DNA structure quite dramatically by introducing sharp bends upon binding. It is worthy of note that a recurring function proposed for HMGB1 is the enhancement of the binding of various transcription factors like Hox protein (*6*), p53 (*7*) or steroid hormone receptors (*8*). Impaired glucocorticoid receptor binding has been provided as an explanation for the pleiotropic effects on glucose metabolism observed in HMGB1-knock-out mice (*9*). These observations suggest that HMGB1 could be implicated in the formation of nucleoprotein complexes that play a role in transcription. Despite numerous studies, it is not clear whether the different domains of HMGB1 participate in recognition of specific proteins.

The *Drosophila* Dorsal switch protein (DSP1) is a member of the HMGB1/2 family (*10*, *11*). Like mammalian HMGB1 proteins, it has two HMG boxes and a C-terminal acidic tail, but also contains an additional N-terminal glutamine-rich region. The two HMG boxes of DSP1 are very similar to the two HMG boxes of HMGB1/2 (*12*, *13*). In addition, the *dsp1* and *Hmgb1* genes revealed a highly conserved structure with similar exon-intron boundaries, suggesting that they derive from a common ancestor. DSP1 was first isolated as a corepressor of the Dorsal protein, but it seems to have additional functions during *drosophila* development, as absence of DSP1 causes homeotic transformations.

Dorsal belongs to the Rel family of transcription factors. This family has been implicated in different physiological and cellular processes, such as immunity, inflammation, oncogenesis, apoptosis, and embryonic development (reviewed in Ref. *14*). The members of this family are characterized by a conserved 300 amino acid region, the Rel homology domain (RHD), located at the N-terminus (reviewed in Ref. *15*). The RHD domain mediates DNA binding, homodimer and heterodimer formation and contains the nuclear localization signal. During *drosophila* embryonic development, Dorsal can act as a transcriptional activator or a repressor depending on the promoter content.

In a recent study (*16*), we have shown by affinity chromatography and far-western experiments that DSP1 and Dorsal interact with each other *in vitro*. Analysis of the binding of several truncated DSP1 proteins has revealed two facts: firstly, the HMG boxes of DSP1 are necessary for the interaction; secondly, the basic residues between the two boxes are a prerequisite for the interaction. We have also shown by electrophoretic mobility shift assay that Dorsal binding to DNA is significantly increased by the presence of DSP1.

*To whom correspondence should be addressed. Tel: +33-2-38-25-55-82, Fax: +33-2-38-63-15-17, E-mail: locker@cnrs-orleans.fr

Table 1. **Primers* used for construction of DSP1 Dorsal RHD expressing plasmids and DSP1 mutants.**

sequence	purpose
5′GGTACGGCGAAAAGCTTCGCATATGAAGCCCTACGTAAAGATCACCGAACAACCG3′	Forward primer for Dorsal RHD
5′GGCTTGCCAAGCTTTGATCACATTGGCACGTACTCGAAGGGCAGGGCCTC3′	Reverse primer for Dorsal RHD
5′GGTTGTGGGACGCGGCGCGAAGAGGAAACAGATCAAGG3′	Forward primer for K253A
5′CCTTGATCTGTTTCCTCTTCGCGCCGCGTCCCACAACC3′	Reverse primer for K253A
5′GGTTGTGGGACGCGGCAAGGCGAGGAAACAGATCAAGG3′	Forward primer for K254A
5′CCTTGATCTGTTTCCTCgccttgccgcgtCCCACAACC3′	Reverse primer for K254A
5′GGACGCGGCAAGAAGAGGGCACAGATCAAGGACCCCAATGC3′	Forward primer for K256A
5′GCATTGGGGTCCTTGATCTGTGCCCTCTTCTTGCCGCGTCC 3′	Reverse primer for K256A
5′GGACGCGGCAAGAAGGCGAAACAGATCAAGGACCCCAATGC3′	Forward primer forR255A
5′GCATTGGGGTCCTTGATCTGTTTCGCCTTCTTGCCGCGTCC3′	Reverse primer for R255A
5′GGAAGTGCGCCGAACGGCGGAAGACAATGGTGG3′	Forward primer for W212R
5′CCACCATTGTCTTCCGCCGTTCGGCGCACTTCC3′	Reverse primer for W212R
5′GCTGGGACGAAAGCGGTCCGATGTGGATCC3′	Forward primer for W302R
5′GGATCCACATCGGACCGCTTTCGTCCCAGC3′	Reverse primer for W302R
5′GCGGTTGTGGGACGCGGCGCGGCGAGGAAACAGATCAAGGAC3′	Forward primer for K253AK254A
5′GTCCTTGATCTGTTTCCTCGCCGCGCCGCGTCCCACAACCGC3′	Reverse primer for K253AK254A
5′GTGGGACGCGGCAAGAAGGCGGCACAGATCAAGGACCCCAATGC3′	Forward primer for R255AK256A
5′GCATTGGGGTCCTTGATCTGTGCCGCCTTCTTGCCGCGTCCCAC3′	reverse primer for R255AK256A
5′GGACGCGGCGCGGCGGCGGCACAGATCAAGGACCCCAATGC3′	Forward primer for K253AK254AR255AK256A
5′GCATTGGGGTCCTTGATCTGTGCCGCCGCCGCGCCGCGTCC3′	Reverse primer for K253AK254AR255AK256A

*Primers are used on DSP1^{-162} , F33 or J11 as template except for the quadruple mutant (K253AK254AR255AK256A) where we used the K253A K254A plasmid as template.

In order to investigate the mode of interaction between Dorsal and DSP1, we undertook a mutational analysis of DSP1. Our choice of mutations predated the release of the interaction data between Dorsal and DSP1, notably on the fact that HMG boxes of DSP1 are necessary but not sufficient for the interaction. In a mutational analysis (*17*), it was reported that mutation of the tryptophan residue in the A domain of HMGB1 affects the protein tertiary structure. We have used this property to obtain DSP1 proteins with one or two unfolded domains. In this work, we have compared the interaction between Dorsal and various mutated forms of DSP1 (W^{212}, W^{302} substituted by arginine or ^{253}KKRK^{256} substituted by alanine). Our results strongly suggest that mutations altering the folding of the HMG domains altered the protein/protein interactions and the stimulation of Dorsal DNA binding.

MATERIALS AND METHODS

Cloning—The Dorsal RHD coding sequence was amplified by PCR using the pTrcHisB Dorsal vector (*16*) as template and the primers described in Table 1. The PCR product was inserted into pGEM-3Zf(+) vector using *Hin*dIII site. The Dorsal RHD coding sequence was excised using the *Nde*I and *Bcl*I sites and inserted into pET-5a vector using *Nde*I and *Bam*HI sites. For expression, *E. coli* BL21 (DE3) strain was transformed by the plasmid. The constructions were verified by sequencing.

Construction of the plasmids expressing DSP1^{-162} or DSP1 mutant proteins with different deletions has been described elsewhere (*16*) (*18*). Site-directed mutagenesis was carried out with the corresponding pET 5a DSP1^{-162}, J11 and F33 plasmids and the appropriate primers (Table 1), using the QuickChange™ Site-directed Mutagenesis kit (Stratagene). The name of the mutants was chosen according to the following guidelines: the first letter refers to the amino acid in the wild-type protein, the number refers to the position of this amino acid in the full-length protein DSP1, and the second letter refers to the amino acid introduced in the mutant (Fig. 1). All constructions were verified by sequencing. For expression of the proteins, the *E. coli* strain BL21 (DE3) was transformed by the plasmids.

Purification of Recombinant Proteins—Purification of the various DSP1 proteins from *E. coli* crude extract was performed as described elsewhere (*18*)

Far-Western Experiments—Far-western experiments were performed as described in (*16*). Synthesis of radiolabelled Dorsal RHD was performed using a pGEM3Zf(+) clone as template and the TNT coupled reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine.

Electrophoretic Mobility Shift Assay (EMSA)—Two 43mer oligonucleotides corresponding to Dorsal strong binding site were purified on a 12% polyacrylamide denaturing gel (19:1 acrylamide:bisacrylamide). The corresponding top strands were end-labeled using [γ-^{32}P]ATP and T4 kinase and hybridized with their complementary strands. The double-stranded species were purified on a 6% polyacrylamide gel followed by electroelution. Their sequences are as follows (the underlined sequences correspond to the Dorsal binding site):

dl2 5′-ATCCTCTATAACTGGGAGAAACCCAATCAATATTCGTTCACCC-3′

3′-TAGGAGATATTGACCCTCTTTGGGTTAGTTATAAGCAAGTGGG-5′

DNA (1.3 nM) was incubated with increasing quantities of the Dorsal RHD for 15 min on ice in a total reaction volume of 10 μl containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 100 μg/ml BSA (binding buffer). To this was added 2 μl of 15% Ficoll with dyes, and the mixture was loaded on a 6% polyacrylamide

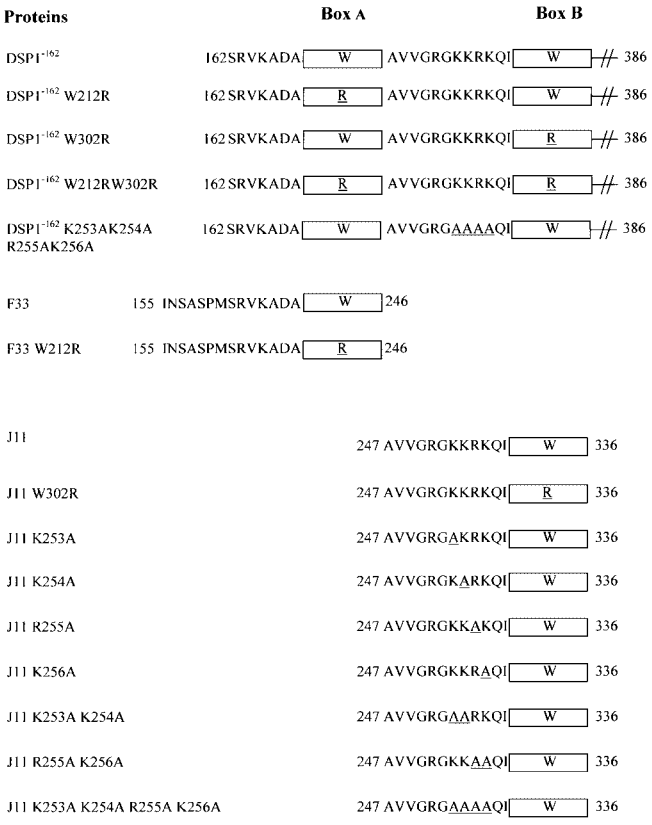

Fig. 1. **Various mutated versions of DSP1.**

gel containing 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA). Electrophoresis was performed at 4°C for 1 h at 12.5 V/cm using 0.5× TBE buffer. After migration, the gel was dried and exposed on a phosphorimager screen. Bands were quantified and the binding constants were calculated by fitting four sets of data to the following equation:

$$f = \{(1 + K_a P_T + K_a D_T) - \sqrt{[(1 + K_a P_T + K_a D_T)^2 - (4 D_T K_a^2 P_T)]}\} / 2 D_T K_a$$

Where f is the fraction of bound DNA, K_a the association constant, P_T the total concentration of protein and D_T the total concentration of DNA.

When the effect of different DSP1 proteins was analyzed, proteins were incubated together for 15 min on ice in binding buffer prior to incubation with DNA. The percentage of bound DNA and the error were calculated from a set of four independent experiments.

Circular Dichroism (CD)—Circular dichroism spectra of the recombinant DSP1^{-162} and mutant DSP1^{-162} proteins were obtained on a CD6 Jobin Yvon Dichrograph at room temperature in buffer: 10 mM NaP pH 7.5, 100 mM NaF, 0.5 mM DTT. All the spectra were recorded using a 1-mm path length quartz cell. Each spectrum was an average of three scans. The spectra were smoothed by means of standard software. The secondary structure analysis was carried out using Johnson's CDsstr software (*19*).

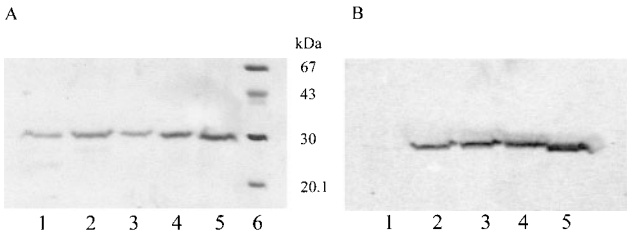

Fig. 2. **Interaction between Dorsal and mutants DSP1 proteins monitored by far-western experiment.** (A) Coomassie-stained SDS-PAGE of the purified proteins. (B) Phosphorimager scan of the membrane after transfer of the proteins and incubation with radiolabelled Dorsal RHD. Lane 1, DSP1^{-162} W212R W302R ; lane 2, DSP1^{-162} W212R ; lane 3, DSP1^{-162} W302R ; lane 4, DSP1^{-162} K253A K254A R255A K256A ; lane 5, DSP1^{-162} ; lane 6, molecular weight marker.

RESULTS

Binding of the Different DSP1 Domains to Dorsal RHD Protein—In a previous work, we have shown that the Dorsal DSP1 interaction was mediated by the HMG boxes of DSP1. However, the HMG box was not sufficient for full recognition, since the recognition was abolished when the flanking sequences were absent at the N-terminus. In particular, the box B of DSP1 (or HMGB1) lacking the N-terminal ^{253}KKRK^{256} sequence (*16*) was unable to interact with Dorsal protein. One explanation was that these N-terminal residues help to stabilize the extremities of the boxes. In order to ascertain the role of the HMG box structure in Dorsal DSP1 recognition, we compared the binding of Dorsal with different mutated DSP1 proteins.

First, we substituted the highly conserved tryptophan residue in box A or B (W^{212} and W^{302} respectively) by an arginine. Teo *et al*. (*17*) have shown that mutation of this residue in the HMGB1 box A largely destroys the tertiary structure. As shown in Fig. 2, DSP1^{-162}, DSP1^{-162} W212R and DSP1^{-162} W302R bind to Dorsal RHD. This result was not surprising, as we can hypothesize that the substitution altered only one HMG box structure, and we have previously shown that a single HMG box is sufficient to mediate the interaction. In contrast, no binding was detectable with the double mutant DSP1^{-162} W212R W302R, suggesting that an intact HMG box is necessary for the interaction between Dorsal and DSP1. Then, to compare the interaction of DSP1^{-162}, DSP1^{-162} W212R and DSP1^{-162} W 302R with Dorsal RHD, we performed a semi-quantitative analysis using a slot blot experiment. Known quantities of each protein were loaded onto a nitrocellulose sheet and incubated with radiolabelled Dorsal RHD as in the far-western experiment. The quantification of the signal revealed that the interaction with Dorsal RHD was not identical for each protein (data not shown). From these results, the three proteins can be ordered according to the efficiency of their interaction with Dorsal RHD: DSP1^{-162} > DSP1^{-162} W212R > DSP1^{-162} W 302R. These results suggest that a single HMG box correctly folded is sufficient to allow the interaction with Dorsal RHD. However, it appears that the two HMG boxes are not strictly equivalent.

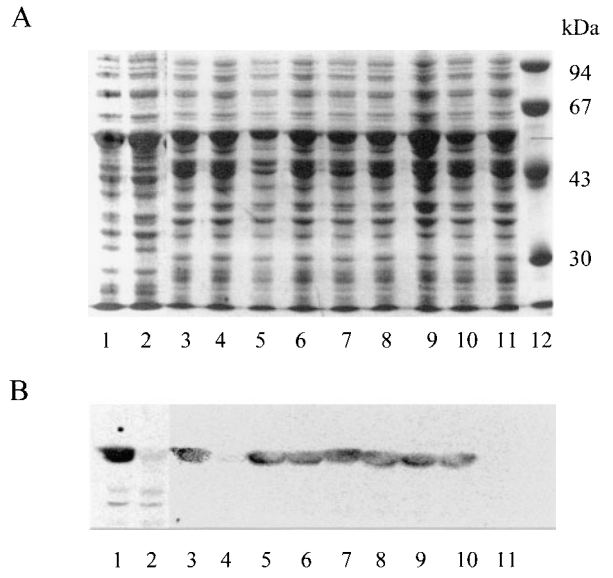

Fig. 3. **Interaction between Dorsal and mutant HMG boxes of DSP1 monitored by far-western blotting.** (A) Coomassie-stained SDS-PAGE of the lysates. (B) Phosphorimager scan of the membrane after transfer of the proteins and incubation with radiolabelled Dorsal RHD. Lane 1, F33 ; lane 2, F33 W212R ; lane 3, J11 ; lane 4, J11 W302R ; lane 5, J11 K253A ; lane 6, J11 K254A ; lane 7, J11 R255A ; lane 8, J11 K256A ; lane 9, J11 K253A K254 A ; lane 10, J11 R255A K256A ; lane 11, J11 K253A K254A R255A K256A ; lane 12, molecular weight marker.

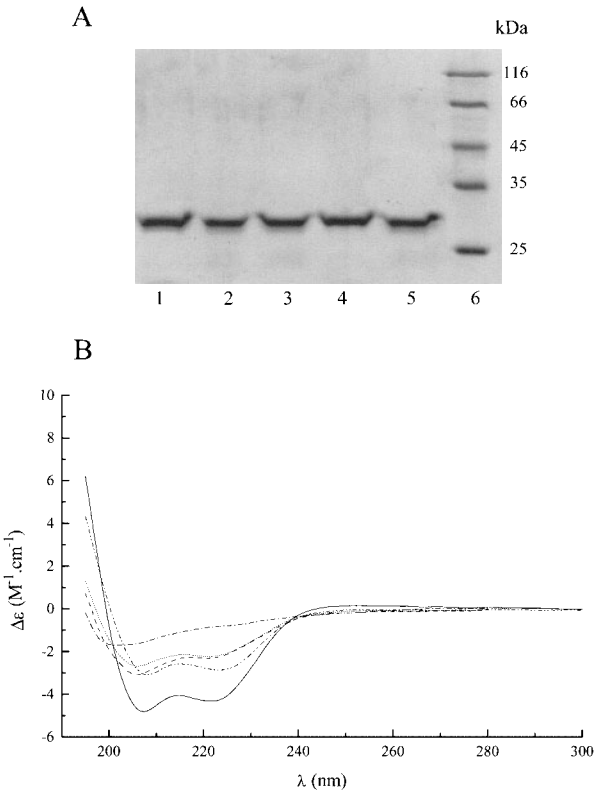

Fig. 4. **CD Spectra.** (A) 10% SDS-PAGE gel of the purified recombinant proteins stained with Coomassie-Brilliant blue. Lane 1, DSP1^{-162}; lane 2, DSP1^{-162} W212R; lane 3, DSP1^{-162} W302R; lane 4, DSP1^{-162} W212RW302R; lane 5, DSP1^{-162} K253A K254A R255A K256A; lane 6, molecular weight marker. (B) Far-UV CD spectra of DSP1^{-162} (—), DSP1^{-162} W212RW302R (-·-·-), DSP1^{-162} W212R (···), DSP1^{-162} W302R (– –), and DSP1^{-162} K253A K254A R255A K256A (–··–). The spectra were recorded at 20°C with measurements every 0.5 nm.

To confirm these results, we decided to test isolated domain A (F33) and B (J11) and their mutant forms (Fig. 1). Far-western blot assays with the B (J11 W302R) or A (F33 W212R) domains revealed that specific binding to Dorsal RHD was not retained as for the double mutant (Fig. 3).

Second, the four basic residues of the N-terminal strand of the DSP1 B domain (253KKRK256) were specifically targeted for mutagenesis (Fig. 1). When the four residues were substituted by alanine in DSP1^{-162}, the interaction with Dorsal was not affected (Fig. 2). This can be explained if the structure of the HMG box B is the only one altered. Then the same experiment was performed with the isolated B domain (J11) mutated at one, two or four lysines. As shown in Fig. 3, binding of DSP1 to Dorsal was not compromised with B domain in which one or two K residues were replaced by alanine. On the contrary, when the four residues were changed, binding to Dorsal was severely compromised, since no complex was detected.

Analysis of Recombinant DSP1 Proteins by Circular Dichroism—In view of the differences in the Dorsal binding properties of the various mutated forms of DSP1, we examined the structural consequences of the mutations. DSP1 polypeptides were expressed in *E. coli* BL21 (DE3) and purified by FPLC chromatography to near homogeneity as judged by coomassie blue staining of the electrophoresis (Fig. 4A), and we compared the CD spectra of DSP1^{-162} and of its mutated forms (Fig. 4B). Compared with DSP1^{-162}, the two single mutants DSP1^{-162} W212R and DSP1^{-162} W302R showed a decrease in residues implicated in α-helices (43 aa and 38 aa versus 94 aa for DSP1^{-162}), suggesting that one of the two HMG boxes is correctly folded and the other completely unfolded. The large decrease of the negative peak at 222 nm in the CD spectrum of the double mutant DSP1^{-162} W212RW302R indicates extensive loss of α-helix, although some remains (<10%), suggesting that the two HMG boxes are unfolded. Like DSP1^{-162} W212R and DSP1^{-162} W302R, the quadruple mutant (DSP1^{-162} K253A K254A R255A K256A) in the linker between the two HMG boxes retained some residues involved in α-helicity (72 aa) providing an evidence for an uncorrect folding of the protein. This can be explained by the alteration of the fold of only one of the two HMG domains or both.

Interactions between DSP1, Dorsal RHD and Dorsal DNA Binding Site—In vitro, full-length DSP1 increases Dorsal binding to DNA, but little is known about the mechanism. We have investigated, using EMSA, the role of each HMG box in this phenomenon, using truncated proteins having only one HMG box and mutated proteins having unfolded HMG boxes.

First, we studied the effect of DSP1^{-162} on the DNA binding of Dorsal RHD (Fig. 5A). In the absence of Dorsal RHD, DSP1^{-162} alone is able to bind to DNA giving C1 (one DSP1^{-162} bound) and C2 (two DSP1^{-162} bound) complexes. However, the presence of DSP1^{-162} slows down migration of the Dorsal-DNA complex (C3), suggesting that a ternary complex is formed (C4). For the higher

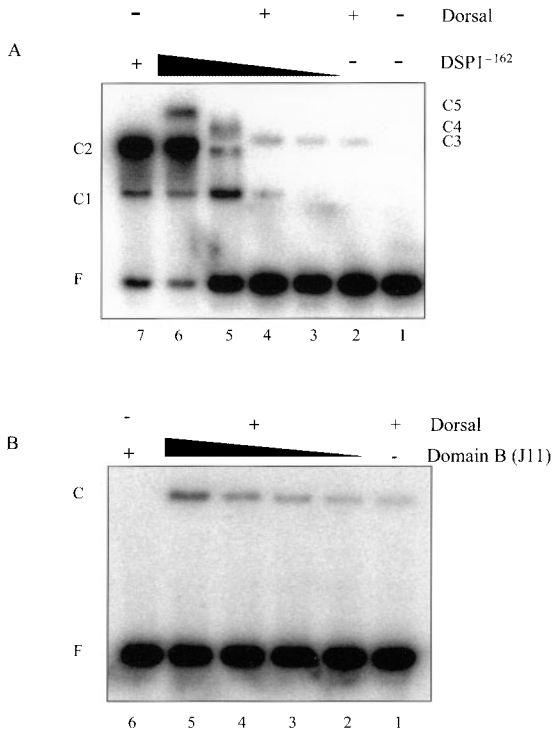

Fig. 5. **Effect of DSP1^{-162} or box B of DSP1 on Dorsal DNA binding.** (A) Phosphorimager scan of EMSA experiment whereby Dorsal RHD was incubated with DSP1^{-162} prior to incubation with Dorsal strong site DNA. EMSA with 1 nM Dorsal RHD and increasing concentrations of DSP1^{-162}: lane 2, 0 nM; lane 3, 4 nM; lane 4, 20 nM; lane 5, 40 nM; lane 6, 100 nM; lane 7, 100 nM DSP1^{-162} alone. Lane 1: no protein was added. Unbound DNA (F); DSP1^{-162} -DNA complexes (C1 one DSP1^{-162} bound, C2 two DSP1^{-162} bound), Dorsal-RHD-DNA complex (C3), DSP1^{-162} -Dorsal-RHD-DNA complexes (C4 one DSP1^{-162} bound, C5 two DSP1^{-162} bound). (B) Phosphorimager scan of EMSA experiment whereby Dorsal RHD was incubated with J11 (box B) prior to incubation with Dorsal strong site DNA. EMSA with 1 nM Dorsal RHD and increasing concentrations of J11: lane 1, 0 nM; lane 2, 4 nM; lane 3, 20 nM; lane 4, 40 nM; lane 5, 100 nM; lane 6, 100 nM J11 alone.

DSP1^{-162} concentration (Fig. 5A, lane 6), a slower complex is observed (C5), which could correspond to two DSP1 proteins associated with Dorsal RHD and DNA. DSP1^{-162} causes a significant increase in the amount of DNA bound by Dorsal, since when DSP1^{-162} is 100 times more concentrated than Dorsal RHD, the affinity constant of Dorsal RHD for its binding site is almost 40 times higher than when DSP1^{-162} is not added to the solution (Fig. 6). A similar effect was observed with J11, which contains a single HMG box B with an N-terminal extension (Fig. 5B), but in this case no ternary complex was observed. However, for the same Dorsal RHD /DSP1 ratio, the increase of Dorsal RHD affinity for its binding site is 5 times less than the one generated by DSP1^{-162} (Fig. 6). The difference observed between these two DSP1 truncated proteins may be explained by the fact that the two HMG boxes are necessary to mediate the enhancement of Dorsal binding on its site. Another explanation is that the size of J11, which contains only the HMG box B with an N-terminal extension, does not allow the interaction between Dorsal and DNA.

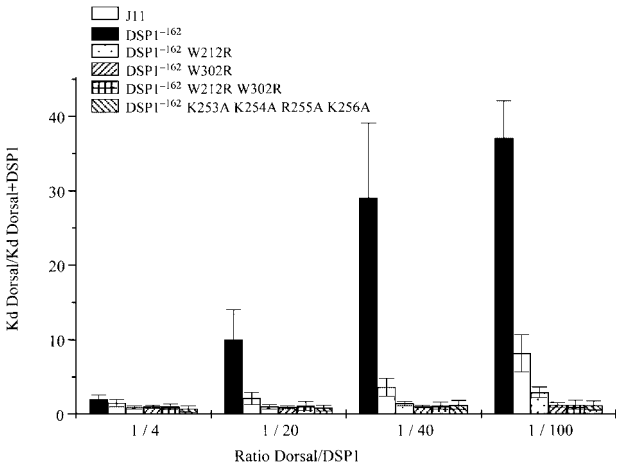

Fig. 6. **Graphic representation of enhancement of Dorsal RHD DNA binding by DSP1^{-162}, box B or mutated forms of DSP1^{-162}.** Histogram shows the ratio of Kd binding of Dorsal with and without DSP1 plotted against the ratio of Dorsal/DSP1 proteins. K_d values are the mean values obtained from four experiments and the error bars correspond to the standard deviation.

To discriminate between these two possibilities, we tested the effect of different mutations in DSP1^{-162} on the binding of Dorsal RHD to its site. In this case, the structure of a single HMG box was destroyed, but the size of the protein was not affected. Results are summarized in Fig. 6. The enhancement of the binding of Dorsal RHD to its binding site is dramatically reduced when HMG box A is destroyed (DSP1^{-162} W212R), and completely abolished when HMG box B or the two HMG boxes are mutated (DSP1^{-162} W302R or DSP1^{-162} W212R W302R). For a DSP1/Dorsal ratio of 100, the enhancement by DSP1^{-162} W212R is significantly stronger than by DSP1^{-162} W302R, suggesting that the two HMG boxes are not equivalent in stimulating DNA binding of Dorsal.

In this experiment, DSP1^{-162} K253A K254A R255A K256A behaves as DSP1^{-162} W302R, which has an unfolded HMG box B.

DISCUSSION

The aim of the work presented here was to investigate the role of HMG boxes in the Dorsal-DSP1 binding.

Dorsal DSP1 Protein–Protein Interaction—We have shown by far-western experiments that mutation of the two highly conserved tryptophan residues in HMG box A (W^{212}) and box B (W^{302}) of DSP1 abolished the interaction between Dorsal RHD and DSP1, when a single substitution did not. Analysis of the mutated forms of DSP1 by circular dichroism revealed that the tryptophan substitution partly destroys the structure of the HMG box. These results, together with those obtained with the truncated DSP1 protein J11, which contains a single HMG box, suggest that the fold of the HMG box is a prerequisite necessary for the interaction between Dorsal and DSP1.

Different studies on the binding of full-length HMGB1 or the single domain A or B to DNA have highlighted the importance of the N-terminal ^{85}TKKKFKD^{91} (*20–22*). For example, the ability of box B to bend DNA is significantly reduced in the absence of the basic N-terminal sequence.

A recent study found that only the full-length HMGB1 protein and its domain B to which the lysine-rich region is attached at the N-terminus specifically recognizes interstrand cross-linked by cisplatin (*21*). It was proposed that electrostatic interactions formed by this basic region are involved in the stabilization of HMG binding on DNA.

In a previous study, we demonstrated that the ability of the HMG box B to bind Dorsal was dependent on the presence of the ^{253}KKRK^{256} sequence between boxes A and B. Here, we have extended this latter study by showing that the B domain with the N-terminal ^{253}AAAA^{256} sequence was not able to bind Dorsal. We examined the structural consequences of these mutations by circular dichroism. Single or double mutations of the basic residues do not result in any change of α-helicity (data not shown). In contrast, mutation of the four residues reduced the folding of the protein. These results suggest that A/B linker region is required for stabilization of the HMG box B. Interestingly, the J11 K253A K254A R255A K256A mutant behaves as the J11 W302R mutant, which has an unfolded HMG box.

Stimulation of Dorsal DNA Binding—In order to understand how DSP1 stimulates the DNA binding of Dorsal, we examined the impact of the number of HMG boxes on this interaction. Our experiments clearly confirm that the two boxes (A and B) are compulsory to get the maximal effect. DSP1^{-162} stimulates the binding of Dorsal RHD to its DNA sequence much better than J11, and mutations in DSP1^{-162} altering the folding of a single HMG box reduce or abolish the enhancement of Dorsal binding on its site, depending on which HMG box is unfolded. This result is comparable to those of other teams working with HMGB1. For instance, Ellwood and coworkers showed that both boxes, A and B, of HMGB1 are necessary to enable HMGB1 and ZEBRA, the Epstein-Barr virus activator, to cooperatively bind DNA, and to increase the binding of ZEBRA to its sites (*23*). Other studies carried out with HMGB1 suggest that the two boxes do not have the same role. Stros highlights that linear DNA bending by HMGB1 is achieved by box B but that it cannot be completely done without box A (*22*). Comparison of DSP1^{-162} W212R and DSP1^{-162} W302R showed that HMG box A and box B are not equivalent in stimulating DNA binding of Dorsal. So it appears that:

– the folding of the boxes is fundamental to this interaction,
– two HMG boxes are necessary for efficient enhancement of Dorsal DNA binding
– box B is responsible for the stimulation, and box A enhances the stimulation but cannot generate it on its own.

These considerations are consistent with the ones of Thomas and Travers. They propose that, during the nucleoprotein complex formation involving HMGB1, box B binds and bends the DNA and that box A mediates protein–protein contacts (*24*).

We have noticed that DSP1^{-162} K253A K254A R255A K256A has the same functional properties as DSP1^{-162} W302R. We propose, considering the CD spectrum of the protein, that the box B structure is altered by these mutations, explaining the absence of effect on DNA binding of Dorsal RHD. It would be interesting to examine by NMR spectroscopy the consequences of the mutations on the structure of the two boxes.

However, several questions remain to be answered in terms of protein/protein/DNA binding mechanisms, such as what are the different functions of the two boxes in the DNA binding and the recognition of the two proteins? It also remains to be established whether DSP1 is able to modulate the binding of Dorsal to its target sequences *in vivo*.

REFERENCES

1. Jones, D.N., Searles, M.A., Shaw, G.L., Churchill, M.E., Ner, S.S., Keeler, J., Travers, A.A., and Neuhaus, D. (1994) The solution structure and dynamics of the DNA-binding domain of HMG-D from *Drosophila melanogaster*. *Structure* **2**, 609–627
2. Read, C.M., Cary, P.D., Crane-Robinson, C., Driscoll, P.C., and Norman, D.G. (1993) Solution structure of a DNA-binding domain from HMG1. *Nucl. Acids Res.* **21**, 3427–3436
3. Weir, H.M., Kraulis, P.J., Hill, C.S., Raine, A.R., Laue, E.D., and Thomas, J.O. (1993) Structure of the HMG box motif in the B-domain of HMG1. *EMBO J.* **12**, 1311–1319
4. JR, P.O., Norman, D.G., Bramham, J., Bianchi, M.E., and Lilley, D.M. (1998) HMG box proteins bind to four-way DNA junctions in their open conformation. *EMBO J.* **17**, 817–826
5. Ohndorf, U.M., Rould, M.A., He, Q., Pabo, C.O., and Lippard, S.J. (1999) Basis for recognition of cisplatin-modified DNA by high-mobility-group proteins. *Nature* **399**, 708–712
6. Zappavigna, V., Falciola, L., Helmer-Citterich, M., Mavilio, F., and Bianchi, M.E. (1996) HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation. *EMBO J.* **15**, 4981–4991
7. Jayaraman, L., Moorthy, N.C., Murthy, K.G., Manley, J.L., Bustin, M., and Prives, C. (1998) High mobility group protein-1 (HMG-1) is a unique activator of p53. *Genes Dev.* **12**, 462–472
8. Boonyaratanakornkit, V., Melvin, V., Prendergast, P., Altmann, M., Ronfani, L., Bianchi, M.E., Taraseviciene, L., Nordeen, S.K., Allegretto, E.A., and Edwards, D.P. (1998) High-mobility group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells. *Mol. Cell. Biol.* **18**, 4471–4487
9. Calogero, S., Grassi, F., Aguzzi, A., Voigtlander, T., Ferrier, P., Ferrari, S., and Bianchi, M.E. (1999) The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nature Genet.* **22**, 276–280
10. Bustin, M., Lehn, D.A., and Landsman, D. (1990) Structural features of the HMG chromosomal proteins and their genes. *Biochim. Biophys. Acta* **1049**, 231–243
11. Grosschedl, R., Giese, K., and Pagel, J. (1994) HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Trends Genet.* **10**, 94–100
12. Canaple, L., Decoville, M., Leng, M., and Locker, D. (1997) The *Drosophila* DSP1 gene encoding an HMG 1-like protein: genomic organization, evolutionary conservation and expression. *Gene* **184**, 285–290
13. Lehming, N., Thanos, D., Brickman, J.M., Ma, J., Maniatis, T., and Ptashne, M. (1994) An HMG-like protein that can switch a transcriptional activator to a repressor. *Nature* **371**, 175–179
14. Siebenlist, U., Franzoso, G., and Brown, K. (1994) Structure, regulation and function of NF-kappa B. *Annu. Rev. Cell. Biol.* **10**, 405–455
15. Liou, H.C. and Baltimore, D. (1993) Regulation of the NF-kappa B/rel transcription factor and I kappa B inhibitor system. *Curr. Opin. Cell Biol.* **5**, 477–487
16. Decoville, M., Giraud-Panis, M.J., Mosrin-Huaman, C., Leng, M., and Locker, D. (2000) HMG boxes of DSP1 protein interact with the rel homology domain of transcription factors. *Nucl. Acids Res.* **28**, 454–462

17. Teo, S.H., Grasser, K.D., Hardman, C.H., Broadhurst, R.W., Laue, E.D., and Thomas, J.O. (1995) Two mutations in the HMG-box with very different structural consequences provide insights into the nature of binding to four-way junction DNA. *EMBO J.* **14**, 3844–3853
18. Janke, C., Martin, D., Giraud-Panis, M.J., Decoville, M., and Locker, D. (2003) *Drosophila* DSP1 and rat HMGB1 have equivalent DNA binding properties and share a similar secondary fold. *J. Biochem.* in press
19. Johnson, W.C. (1999) Analyzing protein circular dichroism spectra for accurate secondary structures. *Proteins* **35**, 307–312
20. Stros, M. (1998) DNA bending by the chromosomal protein HMG1 and its high mobility group box domains. Effect of flanking sequences. *J. Biol. Chem.* **273**, 10355–10361
21. Kasparkova, J., Delalande, O., Stros, M., Elizondo-Riojas, M.A., Vojtiskova, M., Kozelka, J., and Brabec, V. (2003) Recognition of DNA interstrand cross-link of antitumor cisplatin by HMGB1 protein. *Biochemistry* **42**, 1234–1244
22. Stros, M. (2001) Two mutations of basic residues within the N-terminus of HMG-1 B domain with different effects on DNA supercoiling and binding to bent DNA. *Biochemistry* **40**, 4769–4779
23. Ellwood, K.B., Yen, Y.M., Johnson, R.C., and Carey, M. (2000) Mechanism for specificity by HMG-1 in enhanceosome assembly. *Mol. Cell. Biol.* **20**, 4359–4370
24. Thomas, J.O. and Travers, A.A. (2001) HMG1 and **2**, and related 'architectural' DNA-binding proteins. *Trends Biochem. Sci.* **26**, 167–174