The EMBO Journal vol.15 no.18 pp.4981–4991, 1996

HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation

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High mobility group protein 1 (HMG1) is a non-histone, chromatin-associated nuclear protein with a proposed role in the regulation of eukaryotic gene expression. We show that HMG1 interacts with proteins encoded by the *HOX* gene family by establishing protein–protein contacts between the HMG box domains and the HOX homeodomain. The functional role of these interactions was studied using the transcriptional activity of the human HOXD9 protein as a model. HMG1 enhances, in a dose-dependent fashion, the sequence-specific DNA binding activity *in vitro*, and the transcriptional activation in a co-transfection assay *in vivo*, of the HOXD9 protein. Functional interaction between HMG1 and HOXD9 is dependent on the DNA binding activity of the homeodomain, and requires the HOXD9 transcriptional activation domain. HMG1 enhances activation by HOXD9, but not by HOXD8, of the HOXD9-controlled element. Specific target recognition and functional interaction with HMG1 can be transferred to HOXD8 by homeodomain swapping. We propose that HMG1-like proteins might be general co-factors in HOX-mediated transcriptional activation, which facilitate access of HOX proteins to specific DNA targets, and/or introduce architectural constraints in the assembly of HOX-containing transcriptional complexes.

Keywords: chromatin/high mobility group/homeodomain/transcription

Introduction

High mobility group 1 protein (HMG1) is a very abundant and highly conserved nuclear protein present in all mammalian tissues and cells (Bustin *et al.*, 1990). HMG1-like proteins exist also in invertebrates, yeast, protozoa and plants (reviewed by Baxevanis and Landsman, 1995; Bianchi, 1995). HMG1 traditionally has been considered a structural component of chromatin (van Holde, 1988), and consists of two DNA binding domains and a highly acidic C-terminal tail. While an acidic segment is common to many unrelated proteins, the two DNA binding domains belong very distinctively to the HMG box class (Bianchi *et al.*, 1992; Grosschedl *et al.*, 1994). Most proteins containing HMG box domains are transcription factors: for example, UBF is required for DNA polymerase I transcription (Jantzen *et al.*, 1990), TCF-1 is involved in lymphocyte differentiation (Verbeek *et al.*, 1995) and SRY determines the formation of testes in mammalian males (Goodfellow and Lovell-Badge, 1993). Both positive and negative effects of HMG1 (and of the related protein HMG2) on *in vitro* transcription systems have been described (Tremethick and Molloy, 1986, 1988; Singh and Dixon, 1990; Ge and Roeder, 1994; Stelzer *et al.*, 1994). These effects appear to involve interactions with elements required for basal transcription, perhaps via the stabilization of activated conformations of the TFIID–TFIIA–promoter complexes (Shykind *et al.*, 1995).

Here, we have considered a possible additional role of HMG1, consistent with its inability to recognize any specific sequence in DNA, and with its distinctive ability to recognize or introduce sharp bends or kinks in the double helix upon binding (Bianchi *et al.*, 1989; Pil and Lippard, 1992; Paull *et al.*, 1993; Pil *et al.*, 1993). Accordingly, HMG1 could take part in regulated transcription by contributing geometrical information in the assembly of transcriptionally active complexes. In such a working model, HMG1 would be able to interact both physically and functionally with other transcription factors. Indeed, HMG1 is reported to stimulate binding to DNA of the progesterone receptor (Oñate *et al.*, 1994), while the related HMG2 protein interacts with POU homeodomain proteins (Zwilling *et al.*, 1995). The proteins encoded by the vertebrate *Hox* genes, because of their relaxed DNA binding specificity, seemed to us to be plausible targets for HMG1 activity as an architectural factor.

Hox genes are homologous to the *Drosophila* homeotic selector (*HOM*) genes, and encode sequence-specific transcription factors controlling the organization of the body plan during development (reviewed in Krumlauf, 1994). The HOM/Hox DNA binding domain, the homeodomain, is composed of three α-helices, and is structurally related to the helix–turn–helix motif common to several prokaryotic regulatory proteins. The HOM/Hox homeodomain and flanking amino acids play a crucial role in DNA sequence recognition and binding *in vitro*, and, at least in the case of HOM proteins, in determining their functional specificity *in vivo*. Hox proteins have been shown to regulate transcription in cultured cells through binding to specific target sequences (reviewed in Krumlauf, 1994), although all known Hox and HOM proteins show very similar DNA binding specificity *in vitro* (for a review, see Hayashi and Scott, 1990). For this reason, the specificity of action required of Hox proteins in the patterning of the vertebrate embryo is unlikely to derive from homeodomain–DNA interactions alone, and has been postulated to involve the activity of additional co-factors (Manak and Scott, 1993). The products of the *Drosophila extradenticle*

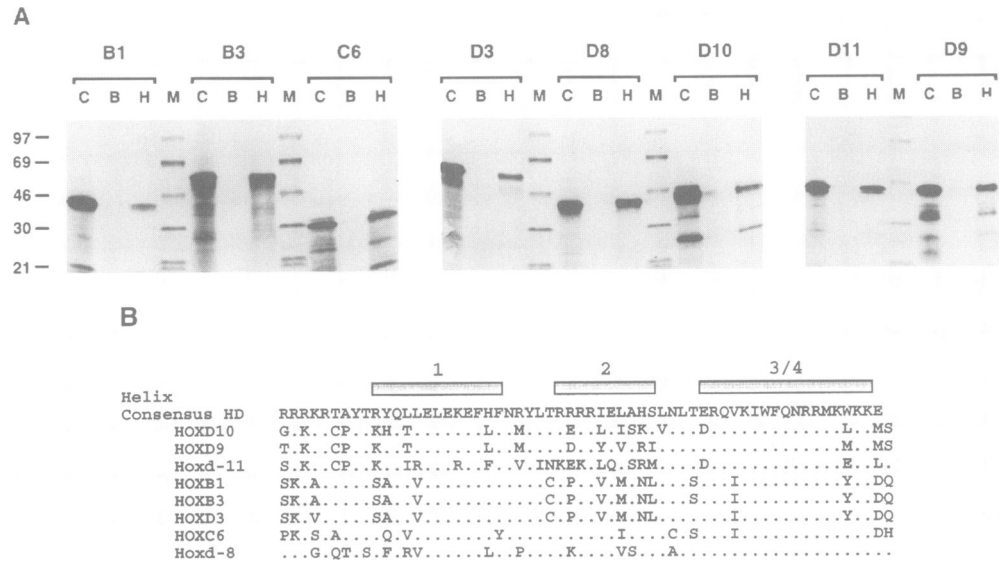

Fig. 1. HOX proteins bind to HMG1. (**A**) Labelled HOXB1, HOXB3, HOXC6, HOXD3, HOXD8, HOXD9, HOXD10 and HOXD11 proteins were synthesized *in vitro* in rabbit reticulocyte lysates. Samples from the lysates (10 μl) were batch-chromatographed with 10 μl of Sepharose beads carrying either BSA or HMG1 (see Materials and methods). The material retained after extensive washing on the HMG1–Sepharose (lanes H) and BSA–Sepharose (lanes B) beads was analysed by SDS–PAGE chromatography and autoradiography, and compared with an equivalent amount of unfractionated reticulocyte lysate (lanes C). Molecular weight standards (lanes M) are indicated in kDa. (**B**) Homeodomain alignment, with respect to a homeodomain consensus (Bürglin, 1994), of the Hox proteins tested for binding to HMG1. Boxes represent the α-helical regions within the homeodomains.

(*exd*) gene and of its vertebrate cognates *pbx1*, *pbx2* and *pbx3* have been proposed recently as co-factors in cooperative DNA binding (Peifer and Wieschaus, 1990; Rauskolb *et al.*, 1993; Chan *et al.*, 1994; Van Dijk and Murre, 1994).

Here we show that the HMG1 protein is capable of establishing protein–protein contacts in the absence of DNA with all tested Hox proteins. We have mapped the contact surfaces between HMG1 and the human HOXD9 protein to the HMG domains and the homeodomain respectively. As a model to study the functional role of these interactions, we utilized the transcriptional activation of HOXD9 on an autoregulatory element. We show that HMG1 and HOXD9 interact functionally, inasmuch as HMG1 stimulates the sequence-specific DNA binding of the HOXD9 protein *in vitro*, and enhances the target-specific transcriptional activation of HOXD9 in transfected cells.

Results

Hox proteins establish protein–protein contacts with HMG1

To test whether homeobox proteins can interact with HMG1, we used an affinity chromatography assay. Recombinant rat HMG1 was produced at high levels in the yeast *Pichia pastoris*, purified to homogeneity and immobilized onto activated Sepharose. A number of different HOX proteins, belonging to three different clusters and seven paralogous groups, were produced by *in vitro* transcription and translation. Unfractionated rabbit reticulocyte extracts were incubated with HMG1–Sepharose beads, or with control beads carrying immobilized bovine serum albumin (BSA). As shown in Figure 1A, labelled HOX proteins associated with HMG1–Sepharose, whereas no association was observed with control BSA–Sepharose beads. Each of the HOX proteins tested bound to HMG1, suggesting that the interaction occurs via the conserved homeodomain (Figure 1B), and may rely on structural characteristics similar for all HOX proteins.

The interaction between HMG1 and HOXD9 occurs via their DNA binding domains

HMG1 consists almost entirely of two DNA binding domains of the HMG box class and a highly acidic C-terminal tail (Figure 2A). To map the surface of interaction with HOX proteins, deletion derivatives of HMG1 were produced in *Escherichia coli*, purified to homogeneity, coupled to activated Sepharose beads and challenged with the *HOXD9* gene product, chosen as a representative HOX protein. Full-length HMG1 (HMG1 in Figure 2B) and three derivatives containing: both HMG boxes (BOX A+B), HMG box A only (BOX A) or HMG box B only (BOX B), all bound labelled HOXD9 protein to similar extents (Figure 2B), indicating that a single HMG box is sufficient for the interaction and that the acidic tail is not required. Both HMG boxes contain a high proportion of lysines and arginines, all of which are accessible at its surface. The amount of HOXD9 associated with the HMG1–Sepharose beads showed no significant variation on changing the ionic strength of the buffer from 50 to 300 mM (Figure 2C), thus ruling out the formation of solely electrostatic interactions between HOXD9 and HMG1. No interaction was detected between HOXD9 and cytochrome c (a basic protein) or polylysine coupled to Sepharose in the presence of 300 mM NaCl (results not shown).

The ability of all HOX proteins to interact with HMG1 suggests that contact occurs via the homeodomain. To test this hypothesis, we fused the homeodomain of HOXD9 to glutathione-*S*-transferase (GST), to generate the GST–D9HD fusion protein (Figure 3A). HMG1 box A was

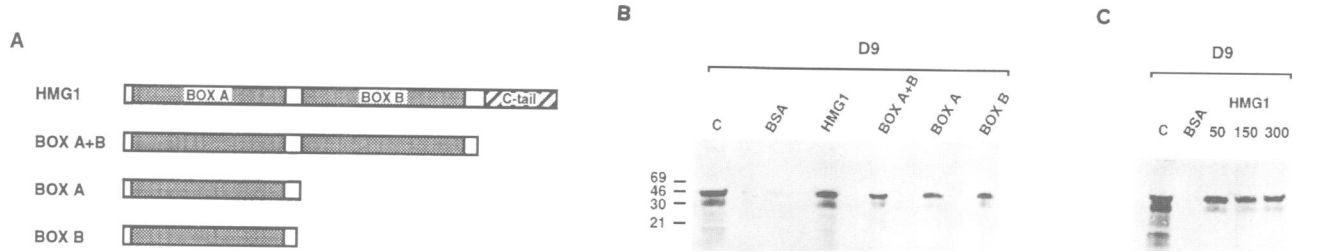

Fig. 2. HMG1 interacts with HOXD9 via the HMG box domains. (**A**) Schematic representation of HMG1 and its derivatives. The DNA binding domains are shown as grey boxes, and the acidic tail as a stippled box. (**B**) Both DNA binding domains of HMG1 interact with HOXD9. Labelled HOXD9 was synthesized *in vitro* and applied directly to the gel (lane C) or batch-chromatographed on Sepharose beads with immobilized proteins (see Materials and methods). Each lane contains the material retained by interaction with the protein indicated at the top. (**C**) The interaction between HOXD9 and HMG1 is insensitive to ionic strength. Labelled HOXD9 was synthesized *in vitro* and applied directly to the gel (lane C) or batch-chromatographed on Sepharose beads with immobilized BSA or full-length HMG1 as indicated. Beads with immobilized HMG1 were washed three times with buffer K containing the amount of NaCl (in mM) indicated at the top of the lanes. Beads with immobilized BSA were washed three times with buffer K containing 50 mM NaCl.

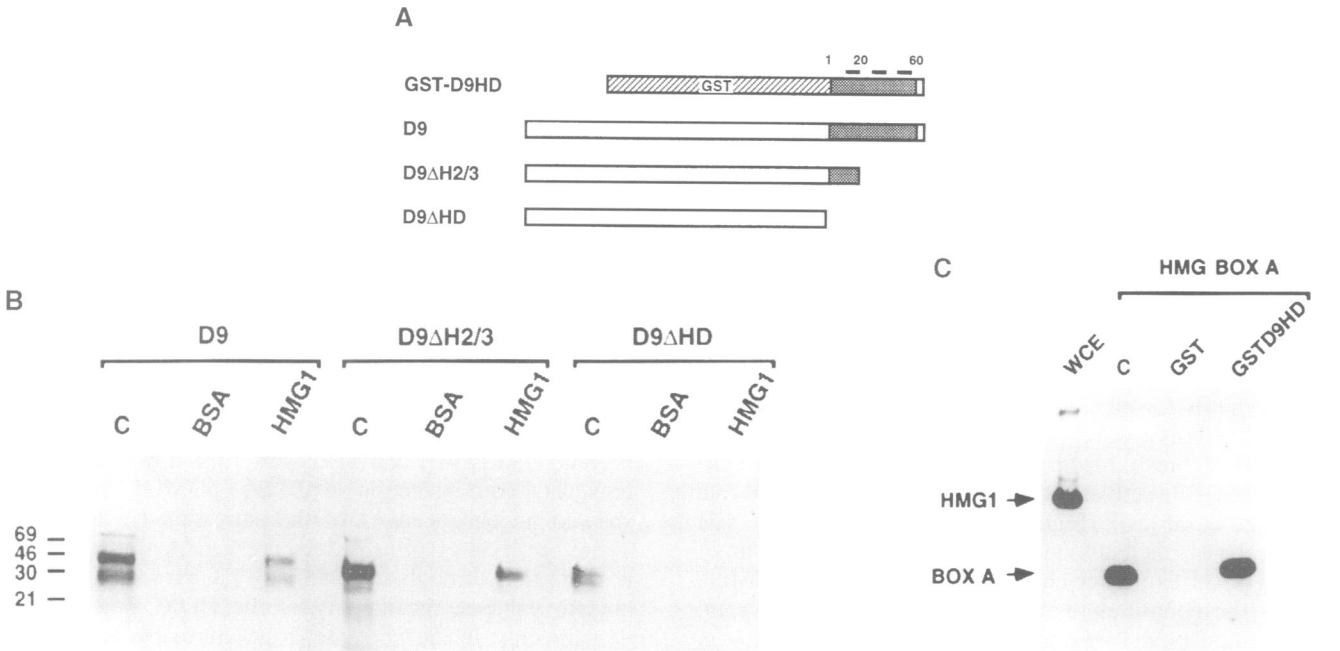

Fig. 3. HOXD9 interacts with HMG1 via the homeodomain. (**A**) Schematic representation of HOXD9 and its derivatives. The DNA binding domain is shown as a grey box. The sizes and location of the three α-helices that make up the homeodomain are indicated by black segments. (**B**) The N-terminus/helix 1 of the homeodomain is sufficient for the interaction between HOXD9 and HMG1. Labelled HOXD9 (D9), HOXD9 lacking helices 2 and 3 of the homeodomain (D9ΔH2/3) and HOXD9 lacking the entire homeodomain (D9ΔHD) were synthesized *in vitro* and applied directly to the gel (lanes C) or batch-chromatographed on Sepharose beads with immobilized BSA (lanes BSA) or full-length HMG1 (lanes HMG1). (**C**) The HOXD9 homeodomain mediates protein–protein contacts with HMG1. GST or the fusion of GST to the HOXD9 homeodomain (GSTD9HD) were immobilized onto glutathione–Sepharose beads and challenged with purified HMG1 box A. Retained material was subjected to SDS–PAGE and revealed by Western blotting with an antibody raised against HMG1. A NIH 3T3 whole cell extract (WCE) was loaded as a control for the antibody. Lane C contains the same amount of HMG1 box A protein as was added to the glutathione–Sepharose beads with immobilized proteins.

retained efficiently by GST–D9HD immobilized on glutathione–Sepharose beads, but did not bind to GST (Figure 3C). The same result was obtained with full-length HMG1 and with the deletion derivative lacking the acidic tail (not shown).

To map the surface of interaction with HMG1 boxes further, full-length HOXD9 (D9 in Figure 3B) and C-terminal deletion derivatives lacking either the whole homeodomain (D9ΔHD) or helices 2 and 3 (D9ΔH2/3) were translated *in vitro* and assayed by affinity chromatography on immobilized HMG1. The mutant lacking the whole homeodomain (D9ΔHD) did not bind at all, whereas the truncated protein still maintaining the N-terminus/helix 1 of the homeodomain (D9ΔH2/3) also retained the ability to bind to HMG1 (Figure 3B).

HMG1 enhances HOXD9-mediated transcriptional activation

The existence of functional interactions between HOXD9 and HMG1 was analysed in cell culture transfection assays. As previously reported (Zappavigna *et al.*, 1991, 1994), the *HOXD9* gene product activates transcription in transient co-transfection assays in NIH 3T3 cells through an evolutionarily conserved, ~100 bp autoregulatory element (HCR). This element derives from the HOXD9 upstream promoter region and includes several binding

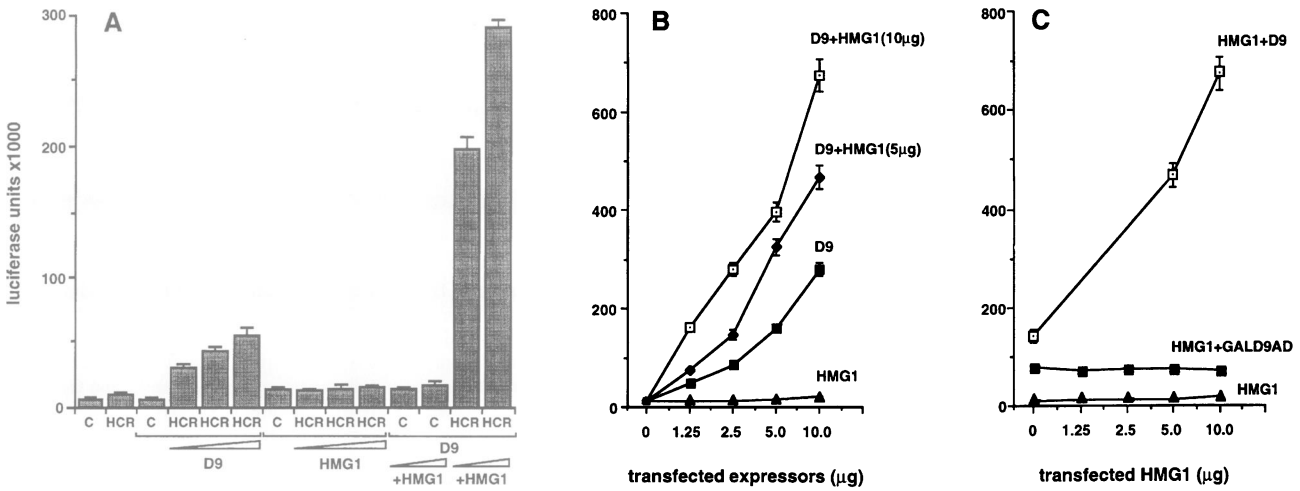

Fig. 4. HMG1 enhances the transcriptional activity of HOXD9 in transient co-transfection assays. (**A**) NIH 3T3 cells were transfected with 10 μg of the pT81luc (C) or the pTHCR (HCR) luciferase reporter constructs, together with increasing amounts (2.5, 5 or 10 μg) of the HOXD9 expressor construct pSGD9 (D9), or with increasing amounts (2.5, 5 or 10 μg) of the pHMG1 expressor construct (HMG1). The same reporter constructs were transfected with a fixed amount (5 μg) of pSGD9 and 5 or 10 μg of pHMG1 (leftmost bars). (**B**) Effect of HMG1 in the presence of various amounts of HOXD9. Various amounts of pHMG1 alone (triangles), of pSGD9 alone (filled squares), of pSGD9 in combination with 5 μg of pHMG1 (diamonds) or of pSGD9 in combination with 10 μg of pHMG1 (open squares) were transfected together with the pTHCR reporter. (**C**) Comparison between the effects of HMG1 on the activity of HOXD9 and on the activity of the GAL4–HOXD9 protein (the activation domain of HOXD9 fused to the DNA binding domain of GAL4). Various amounts of pHMG1 alone (triangles) or in combination with 5 μg of pGALD9AD (filled squares) or 5 μg of pSGD9 (open squares) were co-transfected together with the pTHCR reporter. In all experiments, 1 μg of the pRSVβgal plasmid was co-transfected as an internal standard. The luciferase activity is indicated in arbitrary units. Bars represent the mean ± SEM of at least six independent experiments.

sites for the HOXD9 protein (Zappavigna *et al.*, 1991, 1994; see below). We used a luciferase reporter, pTHCR, where the HCR element is fused to the herpes simplex virus thymidine kinase (TK) promoter. In a typical experiment, HOXD9, expressed from the SV40 promoter-driven construct pSGD9, activates transcription of pTHCR ~8-fold over the basal level (Figure 4A). Co-transfection in NIH 3T3 cells of pSGD9 together with increasing amounts of the pHMG1 expressor construct, which produces HMG1 under the control of its own promoter, led to a strong stimulation of the reporter activity, up to ~30-fold over the basal level (Figure 4A, right) and to ~4- to 5-fold over the maximum level obtained with HOXD9 alone.

As shown in Figure 4B, 5 or 10 μg of HMG1 expressor plasmid enhanced the activity of 1.25–10 μg of HOXD9 expressor linearly. The reverse experiment (Figure 4C) showed that the activity of HOXD9 expressor was enhanced in a dose-dependent fashion by co-transfection of increasing amounts (1.25–10 μg) of HMG1 expressor. Transfection of pHMG1 in the absence of HOXD9 did not significantly increase the basal activity of pTHCR (Figure 4C, and see Figure 4A). The control reporter construct pT81luc (C in Figure 4A), lacking the HCR element, was not activated when co-transfected with the HOXD9 and HMG1 expressors, alone or in combination. Co-transfection of pHMG1 did not affect the production of HOXD9 protein from the pSGD9 construct, as tested by Western blotting (not shown). Identical results were obtained in co-transfection assays utilizing HeLa cells (not shown).

We further tested whether HMG1 could stimulate the transcriptional activation by the HOXD9 activation domain linked to a different DNA binding domain. For this purpose, we generated a construct, pGALD9AD, coding for the DNA binding domain of the yeast GAL4 transcription factor fused to the activation domain of the HOXD9 protein. As reporter we used pTUAS in which the luciferase gene, under the control of the TK promoter, is linked to a multimerized binding site (UAS) for the yeast GAL4 protein. GALD9AD activated transcription from pTUAS; however, co-transfection with the pHMG1 expressor showed no enhancement of the activation (Figure 4C). Therefore, HMG1 protein does not generically stimulate transcription through the minimal TK promoter, and is able to enhance the activity of the HOXD9 activation domain only in the context of the entire homeodomain protein.

As an additional control, we tested if HMG1 could stimulate the activity of a non-homeodomain transcription factor, such as the zinc-finger protein retinoic acid receptor α (RARα). The activity of RARα on its cognate responsive element (RARE) from the RARβ promoter (de Thé *et al.*, 1990) was not enhanced by HMG1 (results not shown).

The possible role of the acidic C-terminus of the HMG1 protein in the enhancement of HOXD9 activity was tested by co-transfecting a HMG1 deletion derivative, lacking the C-terminal 40 amino acids. This construct enhanced HOXD9 activity at the same level as the full-length HMG1 (not shown).

In vivo recognition of target sites by HOX proteins is required for functional interaction with HMG1

We next tested whether DNA binding by the HOXD9 protein was required for stimulation by HMG1. A deletion mutant of HOXD9, lacking the second and third helices of the homeodomain (D9ΔH2/3 in Figure 5), was unable to bind DNA, but was still able to bind HMG1 (see Figure 3C). This truncated protein did not activate transcription of the pTHCR reporter and its activity was not stimulated by co-transfection with the pHMG1 expressor (Figure 5,

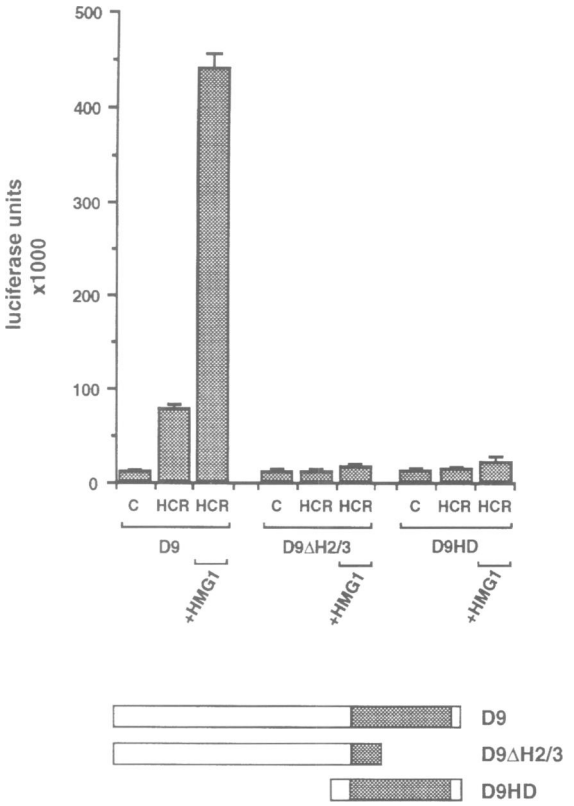

Fig. 5. Analysis of the HOXD9 domains mediating transcriptional enhancement by HMG1. NIH 3T3 cells were transfected with 10 μg of the pT81luc (C), the pTHCR (HCR) or the pTUAS (UAS) luciferase reporter constructs, together with 5 μg of plasmids expressing either HOXD9 (D9), HOXD9ΔH2/3 (D9ΔH2/3) or HOXD9HD (D9HD), and with 5 μg of the pHMG1 expression construct (HMG1) where indicated. One μg of the pRSVβgal plasmid was co-transfected in all experiments as an internal standard. The luciferase activity is indicated in arbitrary units. Bars represent the mean ± SEM of at least three independent experiments. Schematic representations of the proteins expressed in transfections are also shown; the grey box indicates the homeodomain.

left). A construct encoding the HOXD9 homeodomain alone (D9HD in Figure 5) was also unable to activate transcription from the pTHCR reporter, even when co-transfected with pHMG1 (Figure 5, right). These data indicate that transcriptional stimulation by HMG1 requires both the DNA binding and the activation domain of HOXD9. Production and nuclear targeting in transfected cells of the HOXD9 deletion mutants were checked by immunofluorescence (Zappavigna *et al.*, 1994, and results not shown).

We previously reported that the product of the *HOXD8* gene is unable to activate transcription of the pTHCR reporter in a co-transfection assay, due to a lack of recognition of the HCR target element *in vivo* (Zappavigna *et al.*, 1991, 1994). Since we could observe physical interaction *in vitro* between HOXD8 and HMG1 (see Figure 1A), we tested if co-expression with HMG1 could allow HOXD8 to activate the pTHCR reporter. As shown in Figure 6, co-transfection of pSGD8 with pHMG1 did not significantly stimulate the pTHCR reporter activity above the basal level. A chimeric protein, generated by fusing the activation domain of GAL4 to the homeodomain of HOXD8 (GALD8HD in Figure 6), was also unable to activate transcription of pTHCR, even in the presence of co-expressed HMG1 (Figure 6, left). The HOXD8 protein was therefore tested on a different reporter construct, pTCBS, containing a multimerized consensus binding sequence for Hox proteins (Zappavigna *et al.*, 1994). This construct was activated by HOXD8, and this activity could be enhanced by co-expression of HMG1 (Figure 6, right). Similarly, the GALD8HD fusion protein activated the pTCBS reporter, and its activity could also be enhanced by HMG1 (Figure 6, left), indicating that the activation domain of HOXD8 can be replaced by a heterologous one without affecting functional interaction with HMG1.

Finally, we tested if a HOXD8 protein in which the homeodomain was replaced with that of HOXD9 (D8swD9 in Figure 6), thus allowing *in vivo* recognition and activation of the pTHCR reporter (Zappavigna *et al.*, 1994), could also respond to stimulation by HMG1. As shown in Figure 6, the activity of the D8swD9 chimera on the pTHCR reporter was enhanced by co-transfection with the pHMG1 expressor.

Taken together, these data indicate that *in vivo* recognition and activation of the target reporter by a HOX protein is essential for functional interaction with HMG1.

HOXD8 interferes with the HMG1-mediated enhancement of HOXD9 activity

In a previous report, we showed that HOXD8 antagonizes HOXD9 activation on the HCR element (Zappavigna *et al.*, 1994). This repressing activity was shown to be DNA binding independent, and mediated by protein–protein interactions between the two HOX proteins via the N-terminal part of their homeodomains. Since protein–protein interactions between HMG1 and HOXD9 apparently involve the same sub-region of the homeodomain (see Figure 3B), we tested if HOXD8 could exert an antagonistic action on the stimulation by HMG1, through competition for the same protein–protein contact surface.

As shown in Figure 7, co-transfections of the HOXD8 expressor resulted in a 60% repression of HOXD9 activity on pTHCR in the absence of HMG1. Co-transfection of HOXD9 with the HOXD8 expressor in a 1:1 and 2:1 ratio, in the presence of a constant amount of the HMG1 expressor, resulted in an 80% reduction of the HOXD9–HMG1 combined activity (Figure 7, middle). This decrease in activity could be 'rescued' by increasing the amount of co-transfected pHMG1 (Figure 7, right). Thus, HOXD8 is capable of antagonizing HMG1 in the modulation of HOXD9 activity; this antagonism could be due to a competition between HOXD8 and HMG1 for the same protein–protein contact interface on HOXD9, and/or to titration of the amount of HMG1 interacting with HOXD9 in transfected cells.

HMG1 facilitates the binding of HOXD9 to its target sequences

To understand the mechanism of enhancement of HOXD9 activity by HMG1, we tested the influence of HMG1 on *in vitro* binding of HOXD9 to the HCR sequence. The HCR contains at least six binding sites for HOX proteins with an ATTA core consensus sequence (Zappavigna *et al.*, 1991). As shown in Figure 8A, the HOXD9 homeodomain, produced in *E.coli* as a GST fusion protein (D9), bound to the HCR region and assembled non-cooperatively into six electrophoretically resolved complexes, which

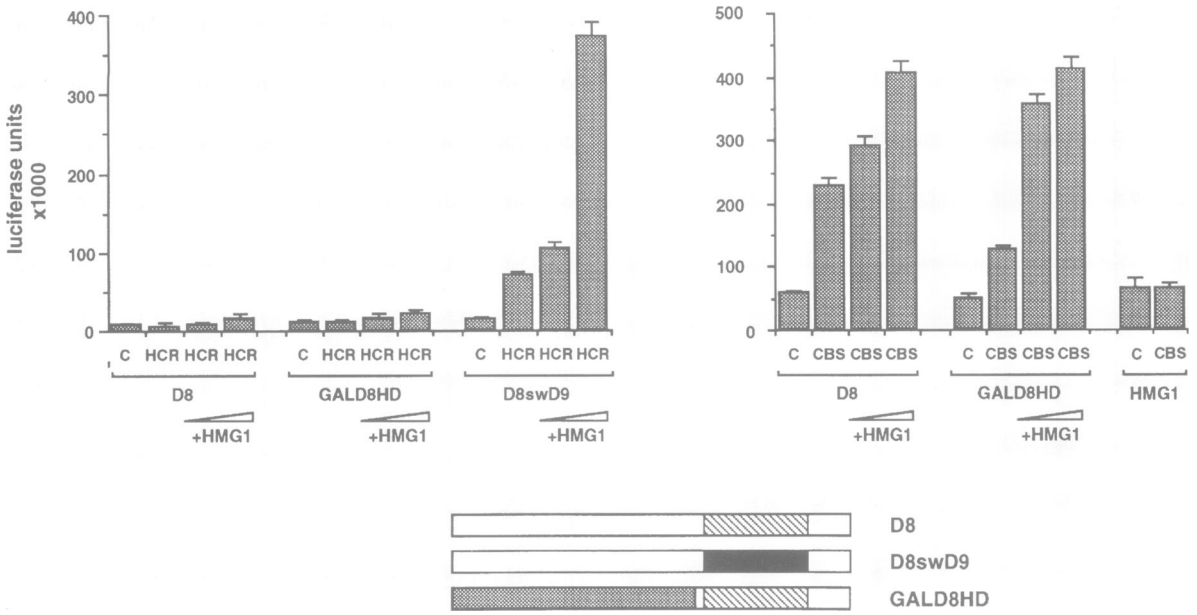

Fig. 6. HOXD8 activity is modulated by HMG1 in a target-specific manner. NIH 3T3 cells were transfected with 10 μg of the pT81luc (C), the pTHCR (HCR), the pTUAS (UAS) or with 5 μg of the pTCBS (CBS) luciferase reporter constructs, together with 5 μg of pSGD8 (D8), pGAL4(149–196)-D8HD (GALD8HD) and pD8swD9 (D8swD9) expressor plasmids and with increasing amounts (5 and 10 μg) of the pHMG1 expression construct where indicated (HMG1). One μg of the pRSVβgal plasmid was co-transfected in all experiments as an internal standard. The luciferase activity is indicated in arbitrary units. Bars represent the mean ± SEM of at least three independent experiments. Schematic representations of the proteins expressed in transfections are also shown: stippled boxes indicate the HOXD8 homeodomain; the black box indicates the HOXD9 homeodomain; the grey box indicates the GAL4 activation domain.

probably correspond to six levels of occupancy of the HOX target sites (A–F in Figure 8A). The addition of increasing amounts of the HMG1 protein (lanes 8–10, Figure 8A) to a fixed amount of HOXD9 (lane 7) led to a significant stimulation of the formation of the slower moving complexes. Derivatives of HMG1, containing the two HMG boxes (BOX A+B, lanes 11–13 in Figure 8A), or HMG box A alone (BOX A, lanes 14–16), were also able to enhance HOXD9 binding to the HCR sites. No alteration of the mobility pattern was observed when BSA was mixed with HOXD9 as a control, in an amount equivalent to the maximal amount of HMG1 added (compare lanes 7 and 17 in Figure 8A). The addition of HMG1 protein to the HCR probe did not result in the formation of any specific retarded complexes (lane 18). These results show that HMG1, as well as one of the HMG boxes alone, enhances the binding of HOXD9 to its target sites. The off-rate of HOXD9 from DNA, as measured by electrophoretic mobility shift assay (EMSA) analysis, was not altered significantly by the addition of HMG1 (not shown), indicating that HMG1 increases the affinity of HOXD9 for its sites by increasing the rate of association of HOXD9 with DNA.

The addition of HMG1 to the HOXD9–DNA binding reaction did not result in the formation of slower migrating complexes in EMSA, indicating that a DNA–HMG1–HOXD9 ternary complex is not formed, or dissociates very rapidly in these conditions. This was more obvious in an EMSA experiment where a single HOX binding site present in the HCR region, HCR-1, was used as probe (Figure 8B). The addition of HMG1 box A+B (BOX A+B in Figure 8B) resulted in an enhancement of the HOXD9 homeodomain (D9) binding without altering the mobility of the complex (lanes 4 and 5 in Figure 8B).

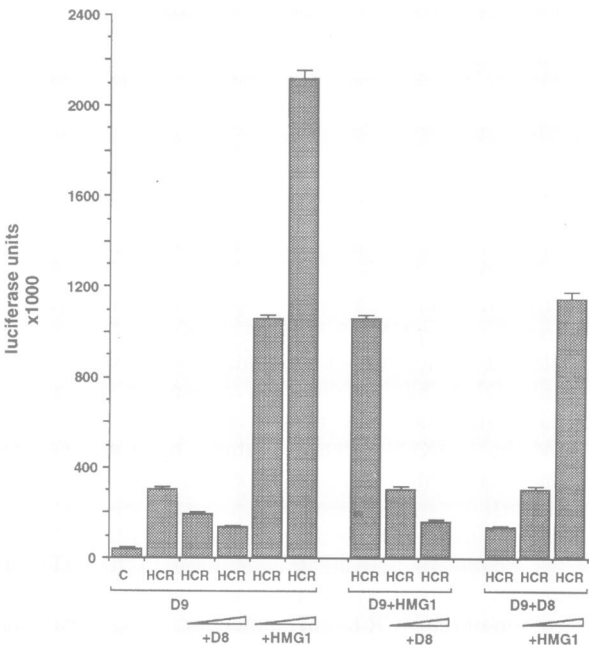

Fig. 7. HOXD8 antagonizes HMG1 in modulating HOXD9 activity. NIH 3T3 cells were transfected with 10 μg of the pT81luc (C) or the pTHCR (HCR) luciferase reporter constructs, together with 5 μg of pSGD9 (D9), with 5 μg of pSGD9 and 5 μg of pHMG1 (D9+HMG1) or with 5 μg of pSGD9 and 5 μg of pSGD8 (D9+D8), and in addition with increasing amounts (5 and 10 μg) of pHMG1(+HMG1), or with increasing amounts (5 and 10 μg) of pSGD8 (+D8), where indicated. One μg of the pRSVβgal plasmid was co-transfected in all experiments as an internal standard. The luciferase activity is indicated in arbitrary units. Bars represent the mean ± SEM of at least three independent experiments.

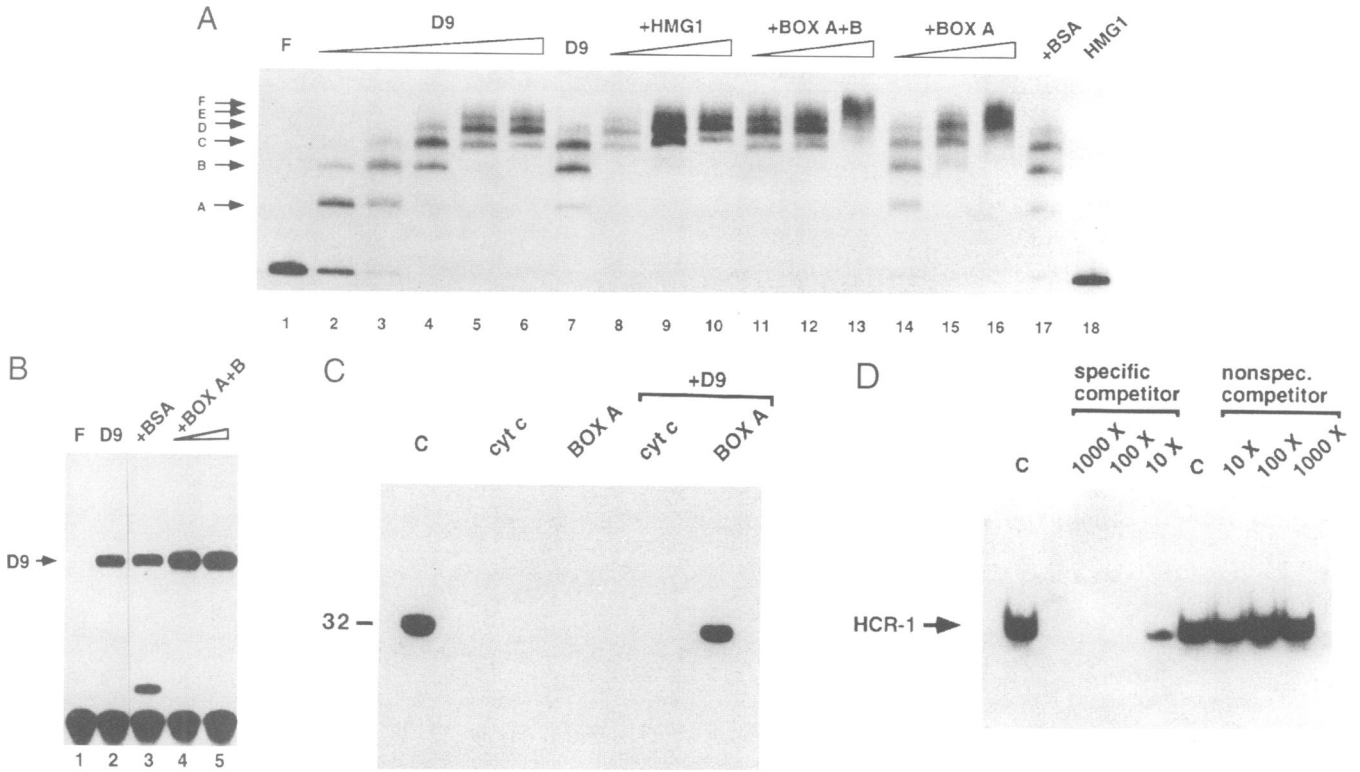

Fig. 8. HMG1 and HOXD9 cooperate in binding DNA. (**A**) HMG1 increases the affinity of HOXD9 for the HCR DNA fragment. Electrophoretic mobility shift assay (EMSA) of an ~100 bp DNA fragment representing the entire HCR region, after binding to purified GST–HOXD9 homeodomain protein (D9). Lane F contains the free probe. Increasing amounts of D9 protein (5–100 ng, lanes 2–6) gave rise to six retarded complexes (A–F, marked on the left by arrows), indicating increasing site occupancy. A fixed amount of D9 protein (lane 7), corresponding to the one used in lane 4, was mixed with increasing amounts of HMG1 (200–800 ng, lanes 8–10), HMG box A+B (25–75 ng, lanes 11–13) or HMG box A (4–16 ng, lanes 14–16). BSA (800 ng) was added to HOXD9 as a control (lane 17). HMG1 alone (800 ng) was incubated with the probe as a control (lane 18). (**B**) HMG1 increases the affinity of HOXD9 for a single HCR site. Ten nanograms of GST–HOXD9 protein (D9) were incubated with a 32 bp duplex DNA, HCR-1, containing a single HOX binding site derived from the HCR region (lane 2). GST–HOXD9 protein (10 ng) was also incubated with 100 ng of BSA (lane 3) or with 20 or 40 ng of HMG1 box A+B (BOX A+B, lanes 4 and 5). Lane 1 contains the free probe (F). (**C**) HMG1–HOXD9 and HOXD9–DNA interactions are not mutually exclusive. Cytochrome c–Sepharose (cyt c) or HMG1 box A–Sepharose (BOXA) beads were incubated with the ^{32}P-labelled HCR-1 duplex DNA, in the absence or in the presence of GST–D9 protein (D9). After extensive washing, resin-bound material was analysed by PAGE (see Materials and methods). Lane C contains the same amount of the labelled 32 bp HCR-1 duplex DNA used for affinity chromatography. (**D**) Specificity of the HOXD9–HMG1–DNA ternary complex. The HCR-1-labelled duplex DNA was bound via the GST–D9 protein to the HMG1 box A–Sepharose beads (lanes C) as indicated in (B). The same amount of labelled HCR-1 was mixed with increasing amounts of cold HCR-1 duplex (specific competitor) or of a cold, non-related 32 bp duplex DNA (specific competitor). The molar excess of competitor DNA over labelled HCR-1 is indicated above the lanes.

The ability of HMG1 to form a ternary complex with HOXD9 and its DNA target was then tested using an alternative method: affinity chromatography on an HMG1-coupled Sepharose matrix. The labelled HCR-1 probe associated almost quantitatively with the HMG1–Sepharose beads in the presence of the HOXD9 homeodomain, while it did not bind HMG1–Sepharose or a control cytochrome c-coupled resin (Figure 8C). The binding of the labelled HCR-1 duplex to HMG1 in the presence of HOXD9 was competed by cold HCR-1, but not by an unrelated duplex DNA. Thus, formation of a specific ternary complex including HMG1, HOXD9 and a Hox binding site can indeed be observed under particular conditions, indicating that HMG1–HOXD9 and HOXD9–DNA interactions are not mutually exclusive.

The effect of HMG1 on the binding of HOXD9 to sequences within the HCR element was analysed also by DNase I footprinting. As shown in Figure 9, increasing amounts of full-length HOXD9 protein produced in *E.coli* (lanes 3–7) protected several sites within the HCR region. The addition of HMG1 protein in two different amounts (lanes 9–13 and 15–19) enhanced HOXD9 binding activity, as revealed by a stronger and more extended footprint over the entire HCR region. No differential stimulation of HOXD9 binding was observed on the multiple sites within the HCR region.

Discussion

Transcriptional regulation in eukaryotes is based on the synergistic action of several interacting DNA binding transcription factors, assembled on a given enhancer/promoter sequence. We have considered the possibility that an 'architectural' component of chromatin like HMG1, which has been hypothesized to play a role in chromatin activation, could interact with classical transcription factors in the generation of transcriptionally active protein complexes. Here we show that protein–protein interactions occur *in vitro* in the absence of DNA between HMG1 and several representative members of the HOX protein family, and that transient expression of HMG1 significantly

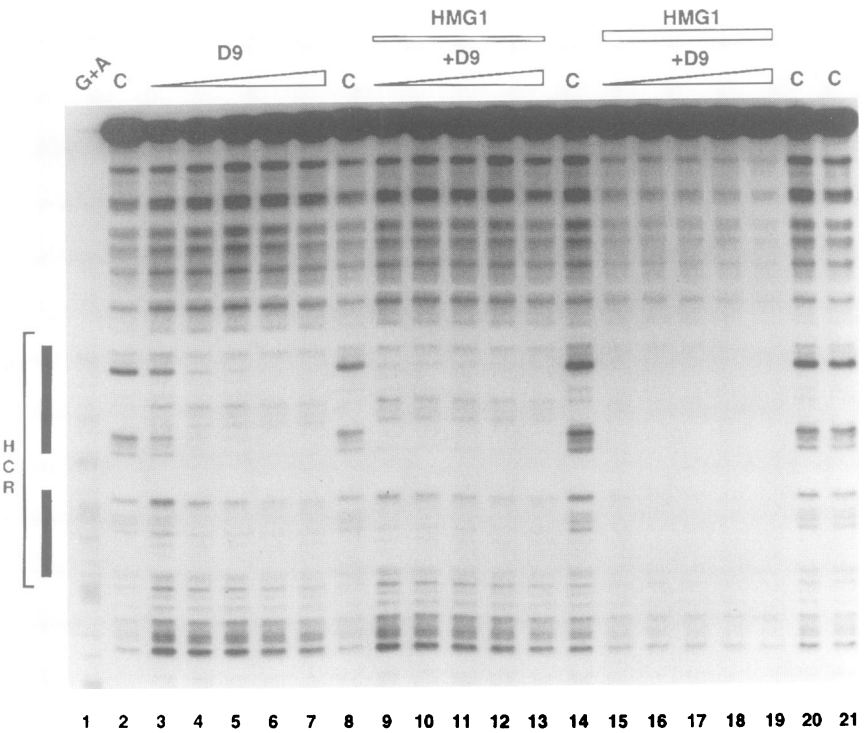

Fig. 9. HMG1 stimulates binding of HOXD9 to all Hox binding sites within the HCR element. DNase I footprinting analysis of a 300 bp region containing the HCR element (square parenthesis). Lane 1 (G+A) contains a Maxam and Gilbert purine ladder; lanes 2, 8, 14, 20 and 21 (C) contain DNase I-digested naked DNA; lanes 3–7 contain increasing amounts of HOXD9 protein (0.0625–1 μg); in lanes 9–13, a fixed amount of HMG1 (2.5 μg) was added to the same amounts of HOXD9 as in lanes 3–7; in lanes 15–19, a greater amount of HMG1 (5 μg) was similarly added to HOXD9. Vertical black bars indicate the footprinted regions, each containing three HOX binding sites.

enhances HOX-controlled transcription in transfected cells.

The protein–protein contacts between HMG1 and the HOX proteins are mediated by their DNA binding domains. Within the homeodomain, the N-terminus/helix 1 region appears to be sufficient for this interaction. The homeodomain is known to be responsible for the interactions between the POU protein Oct1 and the viral transactivator VP16 (Stern *et al.*, 1989), between the *Drosophila* POU proteins I-POU and Cf1-a (Treacy *et al.*, 1992) and between the paired-like protein Phox1 and SRF (Grueneberg *et al.*, 1995). Additionally, the N-terminus/helix 1 region of the homeodomain was shown previously to mediate protein–protein contacts between the human HOXD8 and HOXD9 proteins, and to be required for the repression of HOXD9-mediated transcriptional activation by HOXD8 (Zappavigna *et al.*, 1994). Indeed, HMG1-mediated enhancement of HOXD9 activity can be antagonized by HOXD8 *in vivo*, as would be expected assuming that HOXD8 and HMG1 compete for the same interaction surface on HOXD9.

Our results underscore the double nature of the HOX homeodomain as both a DNA binding module and as a surface mediating contacts with other transcription factors. The primary requirements for the contact with the HMG domain are apparently associated with the homeodomain structural features, since interactions occur equally well with homeodomains whose primary sequences are relatively divergent (see Figure 1B). No additional conserved sequence motif outside the homeodomain is required for the interaction: in particular, the YPWM motif (Mavilio *et al.*, 1986), present upstream from the homeodomain in a subset of HOX proteins, is not involved, since HOXD9, HOXD10 and HOXD11 do not contain it, and yet readily interact with HMG1. In this context, the HMG domains of HMG1 also behave as both a DNA binding module and a protein–protein interaction surface.

The transcriptional activity of the HOXD9 protein on the evolutionarily conserved HOXD9 autoregulatory element (HCR) was chosen as a model system to study the interactions of HOX proteins with HMG1 at the functional level. The transient expression of HMG1 in transfected cells significantly enhances HOXD9-mediated transcription on an HCR-containing reporter. The enhancing effect of HMG1 on transactivation by HOXD9 requires the binding of HOXD9 to DNA, since a mutant carrying a deletion of helices 2 and 3 of the homeodomain does not activate transcription either in the presence or in the absence of transiently expressed HMG1 (Figure 5) though it is still able to interact with HMG1 *in vitro* (Figure 3B). HMG1 enhances activation of HOXD9, but not of HOXD8, on the HOXD9-responsive element. Homeodomain swapping between HOXD9 and HOXD8 changes the target activation specificity and restores the ability to functionally interact with HMG1. Therefore, the HOX–HMG1 functional interaction appears to require the *in vivo* recognition of the target by the homeodomain. In this context, the homeodomain cannot be substituted by another DNA binding domain, at least not by that of GAL4, since the activity of a HOXD9–GAL4DBD chimera on a promoter containing GAL4 binding sites is not enhanced by HMG1. This shows that HMG1 does not generically stimulate the activity of any reporter construct in the presence of a *trans*-activating factor, for example by varying the

accessibility to the transfected reporter DNA, or by interacting with the minimal promoter. This is supported further by the observation that the activity of the retinoic acid receptor α on its cognate responsive element (RARE) from the RARβ promoter was not enhanced by HMG1 (results not shown). A specific interaction with HOX proteins does not necessarily rule out the possibility that HMG1 or HMG1-like proteins could enhance the transcriptional activity of other DNA binding proteins. In fact, the closely related HMG2 protein has been shown to enhance the transcription of an octamer-containing reporter by Oct proteins (Zwilling *et al.*, 1995).

The HMG1-enhanced transcriptional activity of HOX proteins relies on the presence of an activation domain on the *HOX* gene product: a HOXD9 mutant lacking the activation domain, although able to establish protein–protein interactions with HMG1 in solution (Figure 3), is unable to activate transcription even in the presence of HMG1 (Figure 5). The activation domain can be provided also by HOXD8, both in the context of the HOXD8 protein or as a fusion with the HOXD9 homeodomain. The activation domain can even be replaced by a heterologous one such as that of GAL4 (Figure 6), and therefore appears to provide only an effector function.

What is the possible rationale for the HMG1–HOX functional interaction? HMG1 has been shown to facilitate binding of the progesterone receptor to its DNA target (Oñate *et al.*, 1994), and the related protein HMG2 facilitates the binding of the POU domain proteins Oct1, Oct2 and Oct6 to the octamer sequence (Zwilling *et al.*, 1995). The role of HMG1 and 2 as 'binding facilitators' may be more general, as their intervention has been suggested to stimulate the binding of MLTF/USF to the adenovirus major late promoter (Watt and Molloy, 1988), to activate the TFIID–TFIIA complex on several promoters (Shykind *et al.*, 1995) and even to facilitate the assembly of core histone octamers onto DNA (Ner *et al.*, 1994). Our results show that interaction with HMG1 enhances the DNA binding affinity of the homeodomain for its target sites. It has been suggested that HMG1-like proteins may exert a 'DNA chaperone' action by binding transiently to DNA, bending it into a thermodynamically unfavourable conformation, and then exchanging with the protein that has eventually to form a stable complex with its DNA target (Ner *et al.*, 1994). This scenario is indeed attractive also in the context of HOX-mediated transcription. While the DNA is not notably bent when complexed to engrailed and Antennapedia homeodomains (Kissinger *et al.*, 1990; Otting *et al.*, 1990), the yeast MATa1 and MATα2 homeodomain proteins do indeed bend the DNA to some extent (Li *et al.*, 1995), and the homeodomain–DNA interaction involves close contact between the homeodomain N-terminal arm and the minor groove. Thus, one may speculate that the binding of HMG1 to DNA pries open the minor groove in preparation for the loading of the homeodomain. However, this model does not necessarily predict any form of direct protein–protein interaction, and certainly does not require that the interaction surface on the partner proteins should be the DNA binding domain itself.

To account for the specific complementarity of homeodomains and HMG boxes, we favour an alternative interpretation, though not mutually exclusive with the one described above. HMG1 might be recruited by HOX proteins because it can contribute stereochemical information through its DNA-bending properties to nucleoprotein complexes. We propose that the physical contact between the homeodomain and one HMG box directs these two DNA binding domains to adjacent or overlapping DNA segments, generating a complex which is endowed with both sequence and geometrical specificity. Geometrical constraints might be as important for the biological action of HOX proteins as they are for SRY: alterations of the architecture of SRY–DNA complexes are associated with sex-reversal (Pontiggia *et al.*, 1994).

A complex containing DNA, HMG1 and HOXD9 could not be identified by EMSA, as already reported in the case of the interactions between HMG1 and the progesterone receptor, and between HMG2 and the Oct proteins (Oñate *et al.*, 1994; Zwilling *et al.*, 1995). However, we could assemble such a complex on an HMG1-coupled Sepharose matrix, showing that HOX–HMG1 and HOX–DNA interactions are not mutually exclusive, and that a stable HMG1–HOX–DNA ternary complex can form. Computer simulations indicate that box A of HMG1, the homeodomain and DNA (modelled after the DNA molecule complexed with the HMG box protein SRY, Werner *et al.*, 1995) potentially can form a ternary complex where all three participants establish favourable contacts with each other. A similar situation has been described for the enhancer/promoter of the β-interferon gene, where NF-κB and HMG-I/Y bind in the major groove and the minor groove of overlapping sequences (Thanos and Maniatis, 1992), and in addition interact with each other (Du *et al.*, 1993).

Considering the ubiquitous expression of HMG1, and the fact that potentially it can establish contacts with all *HOX* gene products, the interaction with HMG1 or HMG-like proteins might be a common event in HOX-controlled gene regulation. HMG1 is endowed with the biochemical properties to act as a component of HOX-containing transcriptional complexes, and can, therefore, be viewed as a general co-factor in HOX-mediated transcription.

Materials and methods

Plasmids

The expression constructs producing HOXD9, HOXD8 and their mutant derivatives were previously described (Zappavigna *et al.*, 1991, 1994). The pHMG1 plasmid was constructed by cloning a 12.5 kb *Eco*RI fragment containing the whole mouse *Hmg1* gene (Ferrari *et al.*, 1994) into the *Eco*RI site of the pBlueScript KS(+) vector. The pGALD9AD SV40 promoter-driven expressor was constructed by fusing the N-terminal 295 amino acids of HOXD9 to the GAL4 (1–147) DNA binding domain. The pT81HCR reporter construct contains a single copy of the HOXD9 ~100 bp autoregulatory element HCR, cloned into the polylinker of the pT81luc luciferase reporter vector (Zappavigna *et al.*, 1991). The pT109CBS reporter contains an 8mer of the *Hox* consensus binding site 5′-TCGAGTCATCTCAATTAGCGCAGTCGA-3′ (CBS) cloned into the pT109luc vector (Zappavigna *et al.*, 1994).

Production of HOX- and HMG1-derived proteins

Full-length HMG1 was produced in *P.pastoris* as described in Mistry *et al.* (in preparation). Proteins HMG1 box A, HMG1 box B and HMG1 box A+B (an alternative name for HMG1/M1-V176) were produced in *E.coli* as described (Bianchi *et al.*, 1992). HMG1, its derivatives and BSA were immobilized as indicated by the manufacturer on activated CH–Sepharose CL4B (Pharmacia) at an approximate concentration of 1 mg/ml, and stored at 4°C in phosphate-buffered saline (PBS) with 0.1% NP-40. GST and GST–HOX fusions were produced in *E.coli* as

previously described (Zappavigna *et al.*, 1994) and were immobilized as indicated by the manufacturer onto glutathione–Sepharose CL4B (Pharmacia) at ~200 μg/ml.

Protein–protein affinity chromatography

Labelled HOX proteins were synthesized in rabbit reticulocyte lysates (Promega) with [^{35}S]methionine following the manufacturer's instructions. The reaction products were adjusted with rabbit reticulocyte extract programmed with no RNA, to obtain similar HOX protein concentrations in each sample. All subsequent operations were carried out at 4°C. Samples (10 μl) were transferred in 0.5 ml tubes with 300 μl of buffer K [20 mM HEPES pH 7.9, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulphonyl fluoride (PMSF)] and 10 μl of packed beads carrying immobilized protein. Components were mixed on a rotating wheel for 1 h. The beads were centrifuged at 800 *g*, washed twice in buffer K plus 1% BSA, washed once more in buffer K, dried in a Savant lyophilization system and finally resuspended in 20 μl of SDS–PAGE loading buffer. The samples were analysed on 10% SDS–polyacrylamide gels run at 10 V/cm. The proteins were electroblotted onto an Immobilon filter (Millipore), which was exposed for 16–48 h with Hyperfilm-MP (Amersham) or directly quantitated with a Molecular Dynamics PhosphorImager.

The binding of purified HMG1 box A (200 ng per sample) to GST and GST–HOX fusions immobilized onto glutathione–Sepharose CL4B beads (10 μl) was tested in a similar way. However, the soluble proteins were unlabelled and were therefore detected by Western blotting. The filter was blocked by incubation in TBST (50 mM Tris–HCl pH 8.0, 150 mM NaCl and 0.2 % Triton X-100) containing 4% skimmed milk for 1 h. The HMG1 box A epitope was detected with a chicken antibody raised against a deletion mutant of rat HMG1 (HMG1/M1-V176) and affinity purified against the same antigen, a secondary antibody (rabbit anti-chicken IgG, ZYMED), a tertiary antibody (donkey anti-rabbit IgG) coupled to horseradish peroxidase (Amersham) and the ECL Western blotting system (Amersham).

The formation of a HOXD9–HMG1–DNA ternary complex was demonstrated as follows. Ten μl of HMG1–Sepharose beads were incubated on a rotating wheel at 4°C for 30 min with 100 μl of buffer K and 1% BSA in 0.5 ml tubes. ^{32}P-labelled HCR-1 oligonucleotide duplex (5′-GGGACACATTAATCTATAATCAAATACAC-3′), (0.5 ng, 20 000 c.p.m.) and GST–D9HD protein (40 ng), where indicated, were added to the mixture, and the incubation was continued for one more hour. The beads were centrifuged and washed three times with buffer K. DNA was eluted by incubating the beads in 20 mM Tris (pH 8.0), 1 mM EDTA and 1 M NaCl for 30 min, extracted with phenol/chloroform and precipitated with ethanol and 5 μg of tRNA as carrier. The samples were loaded on a 6% non-denaturing polyacrylamide gel and run for 1 h at 5 V/cm in 0.5× TBE. The gel was then dried and exposed for 2–12 h at –80°C with Hyperfilm-MP (Amersham).

Cell culture and transfection

NIH 3T3 cells were maintained in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (GIBCO), 100 IU/ml of penicillin and 100 μg/ml streptomycin and transfected by calcium phosphate precipitation (Di Nocera and Dawid, 1983). In a typical transfection experiment, 10 μg of reporter plasmid, 5 μg of expression construct and 1 μg of pRSV-βgal as an internal control were used per 9 cm dish. Cells were harvested 48–60 h after transfection, lysed and assayed for luciferase and β-galactosidase expression as previously described (Zappavigna *et al.*, 1994).

EMSA and DNase I footprinting

Gel retardation analysis was performed by pre-incubating the purified proteins for 15 min on ice in 18 μl of binding buffer (100 mM KCl, 2 mM $MgCl_2$, 10% glycerol, 4 mM spermidine, 100 μg/ml BSA, 0.1 mM EDTA, 0.25 mM DTT). Two μl of ^{32}P-labelled oligonucleotide probe (0.5 ng, 2×10^4 c.p.m.) were then added and the incubation was continued for an additional 30 min. The incubation mixture was resolved by electrophoresis on a 6% polyacrylamide gel in 0.5× TBE at 10 V/cm. Gels were dried and exposed to a Kodak X-AR film at –70°C. DNase footprinting was performed as previously described (Zappavigna *et al.*, 1991).

Acknowledgements

Thanks are due to Alcide Barberis for continued interest and helpful comments. This work was supported by grant A.07 from Telethon, by the Italian Association for Cancer Research (AIRC), by the European Community BIOTEC Program, and by the CNR (ACRO and Ingegneria Genetica).

References

Baxevanis,A.D. and Landsman,D. (1995) The HMG-1 box protein family, classification and functional relationships. *Nucleic Acids Res.*, **23**, 1604–1613.

Bianchi,M.E. (1995) The HMG-box domain. In Lilley,D.M.J. (ed.), *DNA–Protein: Structural Interactions*. Oxford University Press, Oxford, pp. 177–200.

Bianchi,M.E., Beltrame,M. and Paonessa,G. (1989) Specific recognition of cruciform DNA by nuclear protein HMG1. *Science*, **243**, 1056–1059.

Bianchi,M.E., Falciola,L., Ferrari,S. and Lilley,D.M.J. (1992) The DNA binding site of HMG1 protein is composed of two similar segments (HMG boxes), both of which have counterparts in other eukaryotic regulatory proteins. *EMBO J.*, **11**, 1055–1063.

Bürglin,T.R. (1994) A comprehensive classification of homeobox genes. In Duboule,D. (ed.), *Guidebook to the Homeobox Genes*. Oxford University Press, Oxford, pp. 25–72.

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.

Chan,S.-K., Jaffe,L., Capovilla,M., Botas,J. and Mann,R.S. (1994) The DNA binding specificity of ultrabithorax is modulated by cooperative interactions with extradenticle, another homeoprotein. *Cell*, **78**, 603–615.

de Thé,H., del Mar Vivanco-Ruiz,M., Tiollais,P., Stunnenberg,H. and Dejean,A. (1990) Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. *Nature*, **343**, 177–180.

Di Nocera,P.P. and Dawid,I.B. (1983) Transient expression of genes introduced into cultured cells of *Drosophila*. *Proc. Natl Acad. Sci. USA*, **80**, 7095–7098.

Du,W., Thanos,D. and Maniatis,T. (1993) Mechanisms of transcriptional synergism between distinct virus-inducible enhancer elements. *Cell*, **74**, 887–898.

Duboule,D. and Morata,G. (1994) Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends Genet.*, **10**, 358–364.

Ferrari,S., Ronfani,L., Calogero,S. and Bianchi,M.E. (1994) The mouse gene coding for high mobility group 1 protein (HMG1). *J. Biol. Chem.*, **269**, 28803–28808.

Ge,H. and Roeder,R.G. (1994) The high mobility group protein HMG1 can reversibly inhibit class II gene transcription by interaction with the TATA-binding protein. *J. Biol. Chem.*, **269**, 17136–17140.

Goodfellow,P.N. and Lovell-Badge,R. (1993) SRY and sex determination in mammals. *Annu. Rev. Genet.*, **27**, 71–92.

Grosschedl,R., Giese, K. and Pagel,J. (1994) HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Trends Genet.*, **10**, 94–100.

Grueneberg,D.A., Simon,K.J., Brennan,K. and Gilman,M. (1995) Sequence-specific targeting of nuclear signal transduction pathways by homeodomain proteins. *Mol. Cell. Biol.*, **15**, 3318–3326.

Hayashi,S. and Scott,M. (1990) What determines the specificity of action of *Drosophila* homeodomain proteins? *Cell*, **63**, 883–894.

Jantzen,H.-M., Admon,A., Bell,S.P. and Tjian,R. (1990) Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. *Nature*, **344**, 830–836.

Kissinger,C.R., Liu,B., Martin-Blanco,E., Kornberg,T.B. and Pabo,C.O. (1990) Crystal structure of an engrailed homeodomain–DNA complex at 2.8 Å resolution: a framework for understanding homeodomain–DNA interactions. *Cell*, **63**, 579–590.

Krumlauf,R. (1994) *Hox* genes in vertebrate development. *Cell*, **78**, 191–201.

Li,T., Stark,M.R., Johnson,A.D. and Wolberger,C. (1995) Crystal structure of the MATa1/MATα2 homeodomain heterodimer bound to DNA. *Science*, **270**, 262–269.

Manak,J.R. and Scott,M.P. (1993) Able assistants for homeodomain proteins. *Curr. Biol.*, **3**, 318–320.

Mavilio,F., Simeone,A., Giampaolo,A., Faiella,A., Zappavigna,V., Acampora,D., Poiana,G., Russo,G., Peschle,C. and Boncinelli,E. (1986) Differential and stage-related expression in embryonic tissues of a new human homeobox gene. *Nature*, **324**, 664–666.

Ner,S.S., Travers,A.A. and Churchill,M.E.A. (1994) Harnessing the writhe: a role for DNA chaperones. *Trends Biochem. Sci.*, **19**, 185–187.

Oñate,S.A., Prendergast,P., Wagner,J.P., Nissen,M., Reeves,R., Pettijohn, D.E. and Edwards,D.P. (1994) The DNA bending protein HMG-1 enhances progesterone receptor binding to its target DNA sequences. *Mol. Cell. Biol.*, **14**, 3376–3391.

Otting,G., Qian, Y.Q., Billeter,M., Müller,M., Affolter,M., Gehring,W.J. and Wüthrich,K. (1990) Protein–DNA contacts in the structure of a homeodomain–DNA complex determined by a nuclear magnetic resonance spectroscopy in solution. *EMBO J.*, **9**, 3085–3092.

Paull,T.T., Haykinson,M.J. and Johnson,R.C. (1993) The nonspecific DNA-binding and -bending proteins HMG1 and HMG2 promote the assembly of complex nucleoprotein structures. *Genes Dev.*, **7**, 1521–1534.

Peifer,M. and Wieschaus,E. (1990) Mutation in the *Drosophila* gene *extradenticle* affects the way specific homeo domain proteins regulate segmental identity. *Genes Dev.*, **4**, 1209–1223.

Pil,P.M. and Lippard,S.J. (1992) Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin. *Science*, **256**, 234–236.

Pil,P.M., Chow,C.S. and Lippard,S.J. (1993) High-mobility-group 1 protein mediates DNA bending as determined by ring closures. *Proc. Natl Acad. Sci. USA*, **90**, 9465–9469.

Pontiggia,A., Rimini,R., Harley,V.R., Goodfellow,P.N., Lovell-Badge,R. and Bianchi,M.E. (1994) Sex-reversing mutations affect the architecture of SRY–DNA complexes. *EMBO J.*, **13**, 6115–6124.

Rauskolb,C., Peifer,M. and Wieschaus,E. (1993) *extradenticle*, a regulator of homeotic gene activity, is a homolog of the homeobox-containing human proto-oncogene *pbx1*. *Cell*, **74**, 1101–1112.

Shykind,B.M., Kim,J. and Sharp,P.A. (1995) Activation of the TFIID–TFIIA complex with HMG-2. *Genes Dev.*, **9**, 1354–1365.

Singh,J. and Dixon,G.H. (1990) High mobility group proteins 1 and 2 function as general class II transcription factors. *Biochemistry*, **29**, 6295–6302.

Stelzer,G., Goppelt,A., Lottspeich,F. and Meisterernst,M. (1994) Repression of basal transcription by HMG2 is counteracted by TFIIH-associated factors in an ATP-dependent process. *Mol. Cell. Biol.*, **14**, 4712–4721.

Stern,S., Tanaka, M. and Herr,W. (1989) The Oct-1 homeodomain directs formation of a multiprotein–DNA complex with the HSV transactivator VP16. *Nature*, **341**, 624.

Thanos,D. and Maniatis,T. (1992) The high mobility group protein HMGI(Y) is required for NF-κB-dependent virus induction of the human IFN-β gene. *Cell*, **71**, 777–789.

Treacy,M.N., Neilson,L.I., Turner,E.E., He,Z. and Rosenfeld,M.G. (1992) Twin of I-POU: a two amino acid difference in the I-pou homeodomain distinguishes an activator from an inhibitor of transcription. *Cell*, **68**, 491–505.

Tremethick,D.J. and Molloy,P.L. (1986) High mobility group proteins 1 and 2 stimulate transcription *in vitro* by RNA polymerase II and III. *J. Biol. Chem.*, **261**, 6986–6992.

Tremethick,D.J. and Molloy,P.L. (1988) Effects of high mobility group proteins 1 and 2 on initiation and elongation of specific transcription by RNA polymerase II *in vitro*. *Nucleic Acids Res.*, **16**, 11107–11123.

Van Dijk,M.A. and Murre,C. (1994) *extradenticle* raises the DNA binding specificity of homeotic selector gene products. *Cell*, **78**, 617–624.

van Holde,K. (1988) *Chromatin*. Springer-Verlag, New York.

Verbeek,S. *et al.* (1995) An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature*, **374**, 70–74.

Watt,F. and Molloy,P.L. (1988) High mobility group protein 1 and 2 stimulate binding of a specific transcription factor to the adenovirus major late promoter. *Nucleic Acids Res.*, **16**, 1471–1486.

Werner,M.H., Huth,J.R., Gronenborn,A. and Clore,G.M. (1995) Molecular basis of human 46X,Y sex reversal revealed from the three-dimensional solution structure of the human SRY–DNA complex. *Cell*, **81**, 705–714.

Zappavigna,V., Renucci,A., Izpisua-Belmonte,J.C., Urier,G., Peschle,C. and Duboule,D. (1991) HOX4 genes encode transcription factors with potential auto- and cross-regulatory capacities. *EMBO J.*, **10**, 4177–4187.

Zappavigna,V., Sartori,D. and Mavilio,F. (1994) Specificity of HOX protein function depends on DNA–protein and protein–protein interactions, both mediated by the homeo domain. *Genes Dev.*, **8**, 732–744.

Zwilling,S., König,H. and Wirth,T. (1995) High mobility group protein 2 functionally interacts with the POU domains of octamer transcription factors. *EMBO J.*, **14**, 1198–1208.

Received on April 2, 1996; revised on May 30, 1996