

## HMG-1 as Regulatory *Trans*-Acting Protein in the Acute Phase-Induced Expression of the Rat Liver Haptoglobin Gene

I. GRIGOROV, T. MILOSAVLJEVIĆ, I. CVETKOVIĆ AND M. PETROVIĆ

*Molecular Biology Laboratory, Institute for Biological Research,  
Belgrade, Federal Republic of Yugoslavia*

**Abstract.** Expression of the haptoglobin (Hp) gene is liver specific and acute phase (AP) responsive. It was previously shown that transcriptional induction process of the rat Hp gene during turpentine induced AP response has been mediated by the liver nucleoprotein p29 which was shown to be homologous to the HMG-1 chromatin-associated protein. The results presented in this report offered further evidence for the existence of structural and functional similarities between these two proteins implicating an involvement of HMG-1 in the regulation of the rat Hp gene transcription. By DNA binding assays we found the HMG-1 binding sites in the rat Hp gene *cis*-regulatory subelements A and C and revealed an increase in its DNA-binding after induction of AP response. In view of our previous and here shown data we assume that this increase could be a consequence of AP-induced release of HMG-1 from the chromatin and subsequent increase in its nuclear amount.

**Key words:** Haptoglobin gene — HMG-1 — Protein-DNA interaction — Transcriptional regulation

**Abbreviations:** AP, acute phase; Hp, haptoglobin; HMG-1, high mobility group 1; NE, nuclear extract; NPs, nucleoproteins; C/EBP, CAAT-enhancer binding protein; STAT, signal transducer and activator of transcription.

### Introduction

The hepatic acute phase (AP) response is defined as a metabolic reaction of the liver against a variety of stress stimuli ranging from inflammation and infection to tissue injury (Mackiewicz 1997). It is characterized by an initiation of a signal transduction process that causes a remarkable increase in the transcriptional activity of genes encoding the AP proteins and subsequent increase in their plasma

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Correspondence to: Dr Ilyana Grigorov, Molecular Biology Laboratory, Institute for Biological Research, 29 November 142, 11060, Belgrade, Federal Republic of Yugoslavia  
E-mail [iligr1@ibiss.bg.ac.yu](mailto:iligr1@ibiss.bg.ac.yu)

concentration (Baumman and Gauldie 1994). Since AP proteins exert a broad array of critical functions related to homeostasis and survival of injured organisms, identification of factors involved in their genes expression is critical for understanding the mechanisms that regulate this process. Recent information indicates that expression of these genes arises from specific combinations of liver-specific and liver enriched *trans*-acting regulatory proteins bound to genetic control sequences (Lai and Darnell Jr 1991). Despite an extensive progress in identifying some of these regulatory proteins as typical transcription factors such as C/EBPs (Wedel and Loms Ziegler Heitbrock 1995) or STATs (Ihle 1996), it is an opinion that some of the already well characterized nonhistone proteins, like HMG-1, could be recruited for transcriptional regulation as well (Petrovic et al 1996, Bianchi and Beltrame 1998). HMG-1 belongs to the "high mobility group" class of nonhistone chromatin-associated proteins whose sequence conservation, ubiquity and abundance suggest its important functions. It appears to be involved in a number of biologically fundamental processes including cellular differentiation, chromosomal replication, nucleosomal assembly and transcription (Einck and Bustin 1985, Bustin and Reeves 1996).

HMG-1 has no known specific DNA recognition sequence, but it is capable of recognizing different DNA structures. It prefers binding to DNA containing sharp bends or kinks (Bianchi et al 1989, Pil and Lippard 1992) and has an ability to induce transient bends into DNA molecules and mediate DNA looping and compaction (Pil et al 1993, Štros et al 1994, Štros 1998). This ability to recognize and manipulate DNA structure led to the idea that HMG-1 works as an architectural factor in processes requiring transient manipulation of DNA structure such as gene transcription. This was supported by *in vitro* studies which showed that HMG 1 stimulated transcription in the cell lines overexpressing HMG 1 by modulating the structure of chromatin (Aizawa et al 1994, Ogawa et al 1995). In some cases HMG 1 would be able to interact physically and functionally with other transcription factors. As an architectural transcriptional cofactor, HMG-1 has been reported to facilitate the binding of steroid hormone receptors (Boonyaratanakornkit et al 1998, Melvin and Edwards 1999) and several sequence specific transcription factors, including the adenovirus major late promoter transcription factors (Watt and Molloy 1988), the homeotic proteins HOXD9 and D8 (Zappavigna et al 1996) and p53 (Jayaraman et al 1997), to their target DNA sites.

In this report we demonstrated that HMG-1 is one of the rat liver *trans*-acting nucleoproteins (NPs) involved in the regulation of the rat haptoglobin (Hp) gene expression. Haptoglobin is an AP protein synthesized in the liver whose concentration increases three- to sixfold during the AP response as a result of increased gene transcription (Baumann and Gauldie 1994). Multiple roles have been ascribed to this protein including binding and clearance of hemoglobin, inhibition of superoxide production and stimulation of angiogenesis (Mackiewicz 1997). Our previous studies have shown that transcriptional regulation of the rat Hp gene is based on synergistic action of several interacting DNA-binding NPs assembled on the hormone responsive promoter sequence at position -170 to -56. This sequence, termed

ABC element, consists of the three cooperatively interacting *cis*-acting elements A, B and C which bind a distinct set of liver NPs under basal and AP conditions (Ševaljević et al. 1995, Grigorov et al. 1998). Two of these elements (A and C) interacted in the AP-dependent manner with 29 kDa NP (p29) whose size, charge and epitopes implicated its structural homology with rat liver HMG-1 protein (Petrović et al. 1996). Here, we further corroborate this homology and determine the binding ability of HMG-1 to the regulatory elements of the rat Hp gene. Correlation between its DNA-binding and transcriptional status of the Hp gene has led us to an assumption that HMG-1 might be an important factor in the regulatory mechanism responsible for the rat Hp gene expression during AP response.

## Materials and Methods

### *Induction of the AP-response and nuclear protein extraction*

The AP-response in 2-month-old male Wistar rats was elicited by a single injection of turpentine oil (1  $\mu$ l/g of body weight) in the lumbar region. Liver nuclear protein extracts (NEs) were isolated from the untreated and the 12 h turpentine treated rats following the procedure elaborated by Gorski et al. (1986).

### *Isolation of HMG proteins*

Whole HMG protein fraction were obtained by extracting the liver from the control and the turpentine treated rats with 5% perchloric acid followed by precipitation of the acid-soluble proteins with 6 volumes of acetone to give a fraction containing histone H1 and HMG proteins (Sanders 1977). Histone H1 was removed in part by fractional acetone precipitation.

### *Limited proteolytic cleavage of p29 and HMG-1 proteins*

Limited proteolysis of p29 and HMG-1 by *Staphylococcus aureus* V8 protease were performed following the procedure based on that of Cleveland et al. (1977). The NPs and HMG proteins from the control and the turpentine treated rat livers were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and after Coomassie blue staining, the band corresponding to the p29 or HMG-1 was removed from the gel and the protein was eluted according to Berezney (1991). Eluted protein was dissolved in the sample buffer containing 125 mmol/l Tris-HCl (pH 6.8), 0.5% SDS and 10% glycerol. The samples were then heated to 100°C for 2 min. Proteolytic digestion was carried out at 37°C for 90 min by addition of 2  $\mu$ g of *Staphylococcus aureus* V8 protease per 20  $\mu$ g of protein. Following the addition of  $\beta$ -mercaptoethanol and SDS to final concentrations of 10% and 2% respectively, proteolysis was stopped by boiling the samples for 2 min. Peptide fragments were separated using 15% SDS-PAGE and visualized by silver staining.

*Isolation and labelling of DNA probes*

Nucleotide sequences A (-165/-146), C (-97/-49) and ABC (-170/-56) of the promoter region of the rat Hp gene were used as DNA probes. The fragments of Hp gene subcloned into *Hinc II* site of pUC 13 were obtained from Dr Heinz Baumann (Roswell Park Memorial Institute, Buffalo, NY). Preparation of DNA, radioactive labelling and DNA electrophoresis were performed according to the standard procedures described by Sambrook et al (1989). For South-Western analysis, trimer of fragment A and dimer of fragment C were labelled by a random priming technique using [ $\alpha^{32}\text{P}$ ]dCTP (Amersham Inc).

*DNA affinity chromatography*

DNA affinity column with Hp gene ABC fragment linked to a CNBr-activated Sepharose 4B (Pharmacia) was prepared according to Kadonaga and Tjian (1986). ABC affinity column was equilibrated in Bio-Rad-Econo-Column with the dialysis buffer (25 mmol/l HEPES, pH 7.9, 12.5 mmol/l  $\text{MgCl}_2$ , 1 mmol/l dithiothreitol, 20% glycerol) containing 0.1 mol/l KCl. NEs or whole HMG proteins, resolved in the dialysis buffer containing 0.1 mol/l KCl were mixed with denaturated salmon sperm DNA and allowed to incubate for 10 min at room temperature. The protein-DNA mixture was then applied to the affinity column and incubated for 30 min at 4°C. ABC-bearing nucleo- and HMG proteins were eluted with dialysis buffer containing 1.0 mol/l KCl. Whole eluates were analysed by 11% SDS-PAGE and silver staining or by Western immunoblotting.

*Western immunoblot analysis*

Preparation of anti-HMG antibodies was carried out following the procedure as described previously (Ševaljević et al 1994). The same volumes of HMG protein solution and Freund's complete adjuvant were mixed and injected subcutaneously into rabbits. Over a period of four weeks 3–5 more injections were given with incomplete adjuvant. Antisera were prepared one week after the last immunization. For Western immunoblot analysis, ABC-bearing NPs were subjected to 11% SDS-PAGE and electrophoretically transferred to Hybond P membranes (Amersham Inc) overnight at a constant current of 40 mA. Membranes were blocked for 1 h at room temperature with 5% non fat dry milk in blotting base buffer (0.1% Tween 20, 20 mmol/l Tris-HCl, pH 7.6, 137 mmol/l NaCl) and then incubated for additional 2 h at room temperature in the same buffer containing rabbit anti-HMG antibodies. After washing three times with blotting base buffer containing 1% non fat dry milk, secondary antibody was applied for 1 h at room temperature. Membranes were washed extensively in blotting base buffer and antibody binding was detected on Hyper ECL film by enhanced chemiluminescence using the ECL detection system (Amersham Inc).

*South-Western blot analysis of HMG-1*

South-Western blot analysis of HMG-1 protein was performed according to the method of Bowen et al (1980). After 11% SDS-PAGE of total HMG proteins in

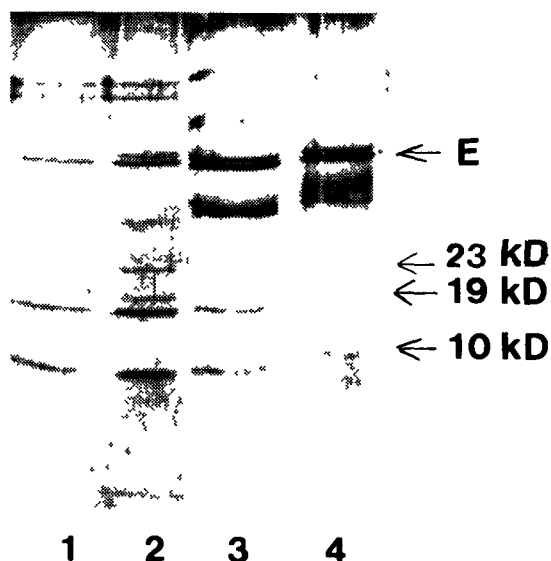
the first dimension, the gels were electroblotted onto Hybond<sup>TM</sup>-C nitrocellulose filters (45  $\mu$ m, Amersham Inc.) in 20 mmol/l Tris-base, 150 mmol/l glycine and 20% methanol for 1 h at 135 mA. Following transfer, the filters were pretreated for 1 h to block nonspecific binding with the binding buffer containing 1 mmol/l EDTA; 10 mmol/l Tris-HCl, pH 7.0; 0.02% bovine serum albumine; 0.02% Ficoll (Pharmacia Inc.); 0.02% polyvinylpyrrolidone and 50 mmol/l NaCl. The filters were subsequently incubated in a binding buffer containing  $10^6$  cpm of  $^{32}$ P-labelled DNA probes per cellulose strip for 1 h at room temperature. In order to remove non-specifically incorporated radioactivity, filters were washed twice with the binding buffer containing 200 mmol/l NaCl. Dried filters were exposed to X-ray film (Kodak) for 1–4 days.

## Results and discussion

HMG-1 protein contains tripartite domain structure, the basic N-terminal (A) and central (B) domains that interact *in vitro* with DNA and an acidic C-terminal (C) domain that may be involved in interactions with histones and in regulating DNA-binding affinity of the HMG-1 protein (Tsuda et al. 1988; Grosschedl et al. 1994; Bustin and Reeves 1996). DNA-binding regions with a homology to HMG-1 domains A and B have been identified in a number of DNA-binding proteins or transcription factors (Baxeavanis and Landsman 1995). The C domain of HMG-1 is polyanionic, containing in the case of rat HMG-1, an unbroken run of 30 glutamic and aspartic residues (Paonessa et al. 1987). Štros et al. (1990) demonstrated that, when HMG-1 from calf thymus was degraded with *Staphylococcus aureus* V8 protease, specific protein fragments were produced. This protease cleaves aspartic/glutamic derived peptide bonds that are in the primary structure of the rat HMG-1 at 56 sites (Chow et al. 1995). Therefore, to confirm previously implicated structural and thereby functional similarities between rat liver p29 and HMG-1 (Petrović et al. 1996), a comparison of the digestion patterns of these two proteins obtained by *Staphylococcus aureus* V8 protease was made.

Limited cleavage of p29 from the nuclear protein extracts of the control (Fig. 1, lane 1) and the liver of 12 h turpentine treated rats (lane 3) produced three specific fragments having molecular masses of 23, 19 and 10 kDa. The cleavage of HMG-1 from the control (lane 2) and the liver of turpentine treated rats (lane 4), resulted in peptide profiles that were identical to those obtained for p29. These results indicate that p29, like HMG-1 (Chow et al. 1995) contains preferential cleavage sites for the V8 protease, which presumably arise because of the folded protein structure. According to Štros et al. (1990) and Chow et al. (1995) peptide of 10 kDa could represent N-terminal domain A of HMG-1 (residues 1–74), peptide of 19 kDa central domain B (residues 80–185) and peptide of 23 kDa B plus C-terminal domains (residues 74–221). On the basis of these cleavage data it was concluded that p29 has similar primary structure as HMG-1 protein.

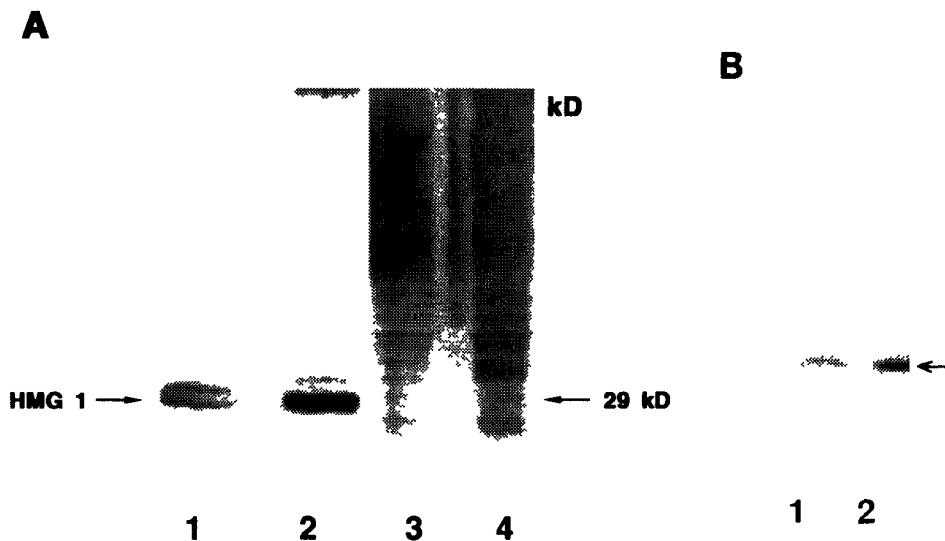
Whether HMG-1 could be involved in an AP-induced expression of the rat Hp gene or not was assessed by DNA affinity chromatography which enables charac-



**Figure 1.** Electrophoretic patterns obtained by 15% SDS-PAGE after limited proteolysis of p29 (lanes 1 and 3) and HMG-1 protein (lanes 2 and 4) from the control (lanes 1 and 2) and the turpentine treated (lanes 3 and 4) rat livers by *Staphylococcus* V8 protease. The resulting peptide fragments were visualised by silver staining, E, position of *Staphylococcus* V8 protease

terization of the protein molecular weight, expression and activity. Equal amounts of the whole HMG fractions from the control (Fig. 2A, lane 1) and the liver of turpentine treated rats (lane 2) were applied to ABC-affinity column. After elution, the whole samples of ABC-bearing HMG proteins were analyzed by SDS-PAGE. In this way it was established that only HMG-1 protein has the ability to bind the ABC element of Hp gene and that its affinity towards it increases during AP response. Lanes 3 and 4 represent silver stained ABC-binding pattern of p29/HMG-1 within the whole NEs of control and turpentine treated rats after DNA affinity chromatography. In spite of low visible AP-related changes in p29/HMG-1 binding for ABC element, Western immunoblot analysis of ABC-bearing NPs (Fig. 2B) clearly showed that anti-HMG antibody detected higher amount of antigen-antibody complexes at the position of 29 kDa under AP conditions (lane 2). These results are in an accordance with the previously obtained South-Western ABC-binding pattern of p29 within the whole NEs (Petrović et al. 1996) suggesting the existence of functional besides structural homology between p29 and HMG-1.

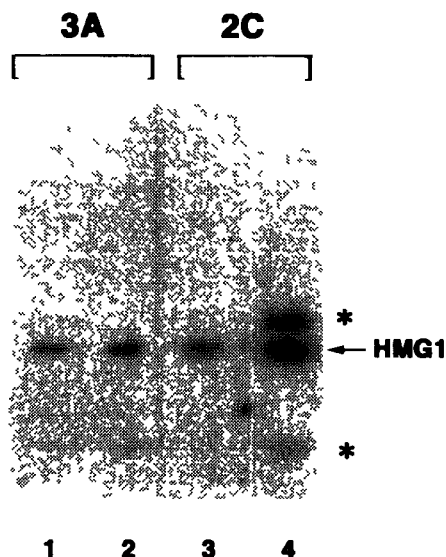
The AP-related affinity of HMG-1 protein to bind ABC subelements was obtained by South-Western analysis. The results revealed that HMG-1 bound subelements A and C (Fig. 3), with higher affinity during AP response (lanes 2 and 4). Considering that the amount of HMG-1 protein in the hepatocyte nuclei is



**Figure 2. A.** Electrophoretic patterns of ABC-bearing rat liver HMG and nucleoproteins obtained after DNA affinity chromatography. HMG and nucleoproteins isolated from the livers of control (lanes 1 and 3) and the turpentine treated (lanes 2 and 4) rats were applied to DNA-affinity column with ABC regulatory element of Hp gene linked to a resin and after elution the whole samples were analysed by 11 % SDS-PAGE and silver staining. Arrow on the left indicates position of HMG-1 protein in gel. Position of 29 kDa within the whole NEs is indicated on the right.

**B.** ECL-Western immunoblot analysis of ABC-bearing nucleoproteins from the liver nuclear extracts of control (lane 1) and turpentine treated (lane 2) rats obtained by DNA-affinity chromatography. The position of complex formed between 29 kDa/HMG-1 and anti-HMG antibody is indicated by an arrow.

significantly higher after turpentine treatment (Petrović et al. 1996), it appeared that the increase in the binding of HMG-1 to A or C subelements arose from its nuclear accumulation. This is supported by the findings that the intranuclear organization of HMG proteins is dynamic rather than static and that these proteins are not always associated with chromatin (Bustin 1999). It seems that in response to specific nuclear localization signal some HMG proteins, including HMG-1, transiently dissociate from chromatin and enter into the nucleus (Falciola et al. 1997; Bustin 1999). Several authors reported phosphorylation of HMG-1 *in vitro* suggesting that this modification may regulate its distribution between the cytoplasm and the nucleus (Wisniewski et al. 1994) and its DNA-binding properties (Watanabe et al. 1994; Alami-Ouahabi et al. 1996; Wisniewski et al. 1999). Previously, we have found that the binding of p29 to the ABC element of Hp gene depended on the extent of its phosphorylation (Petrović et al. 1996). However, the mechanism by which phosphorylation influences specific functions of HMG-1 is still obscure.

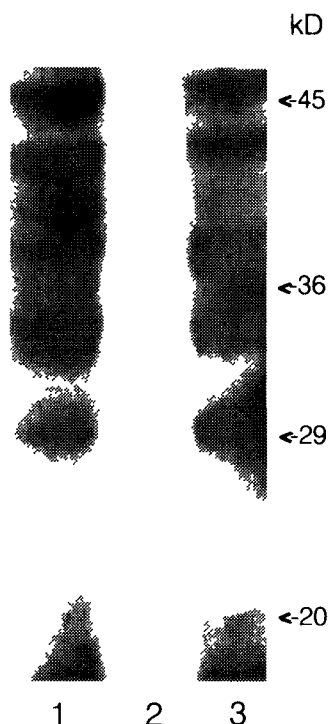


**Figure 3.** South-Western blot analysis of HMG-1 binding to the elements A and C of the rat Hp gene. Whole HMG fraction from the livers of control (lanes 1 and 3) and the 12 h-turpentine treated (lanes 2 and 4) rats were subjected to 11% SDS-PAGE, blotted and probed with [ $\alpha^{32}$ P]dCTP labelled trimer of sequence A and dimer of sequence C. The radioactive spot appearing at the position of HMG-1 is indicated by an arrow, unidentified spots are indicated by an asterix.

The implicated influence of the amount of HMG-1 protein on its DNA-binding properties was additionally confirmed by mixing the control NE and the control HMG fraction *prior to* SDS-PAGE and South-Western blotting with trimer of sequence A from the rat Hp gene. The basis for this experiment was the result of 2D SDS-PAGE which showed presence of only one protein spot at the position of 29 kDa in both the whole NEs and HMG fraction (Petrovic et al 1996). Results in Fig. 4 revealed that HMG-1 protein (lane 2) is located at the same position in gel as p29 from NE (lane 1) and that an addition of HMG proteins to NE resulted in increased DNA-binding of protein at the position of 29 kDa (lane 3) compared to that obtained for p29 from NE only (lane 1).

Correlation between Hp gene transcriptional activation and increased binding of HMG-1 to the structure(s) in the A and C regions of the rat Hp gene regulatory element under AP conditions implicated an involvement of HMG-1 as regulatory *trans*-acting proteins. Recent studies on the characterization of HMG binding sites in chromatin demonstrated that HMG-1, like histone H1, bind to the linker DNA between adjacent nucleosomes (Bustin and Reeves 1996, Zlatanova and van Holde 1998). It has been reported that nucleosomes isolated from active chromatin are often depleted of histone H1 but contain HMG-1 instead. This suggests an interplay between HMG-1 and histone H1 in organizing chromatin (Nightingale et al 1996, Zlatanova and van Holde 1998). Furthermore, HMG-1 competes with H1 for certain distorted DNA structures (Hill and Reeves 1997). Since isolation of HMG fraction from the liver of turpentine treated rats was followed by extraction of significantly higher amount of histone H1 than from the controls (our unpublished data), it is possible that HMG-1 replaces H1 in restricted linker regions to promote the acces-





**Figure 4** South Western blot analysis of influence of the amount of HMG 1 on its binding with trimer of sequence A. Lane 1 DNA-binding of the p29 from the control rat liver nuclear extracts. lane 2 DNA binding of the HMG 1 from the control rat liver HMG fraction. lane 3 DNA binding of p29/HMG-1 obtained after mixing of the control nuclear extracts and HMG proteins. Positions of relative molecular mass values (kDa) are indicated on the right.

sibility of local DNA domains involved in transcription. Therefore, we propose that HMG 1 could influence rat Hp gene activity either through modulating the structure of Hp gene or through positive or negative effects on binding of transcription factors. Whether it is indeed so is a matter of further investigation.

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