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The methylated DNA binding protein-2-H1 (MDBP-2-H1) consists of histone H1 subtypes which are truncated at the C-terminus

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ABSTRACT

The methylated DNA binding protein-2-H1 (MDBP-2-H1), present in rooster liver, is a member of the histone H1 family which inhibits transcription by binding selectively to methylated promoters. Here we have determined the primary structure of MDBP-2-H1. A comparison between histone H1 and MDBP-2-H1 was achieved by analyzing reversed phase HPLC-purified and V8-digested proteins by mass spectrometry and/or microsequencing. In rooster liver the most abundant histone H1 subtypes are H1 01 and H1 11L. Similarly, MDBP-2-H1 contains the same subtypes of histone H1. The histone H1 subtype H1 01 in MDBP-2-H1 has 150 amino acids, whereas the full-size histone H1 01 is 218 amino acids. The difference in mass between the two proteins is explained by C-terminal truncation of histone H1 01.

INTRODUCTION

In recent years the role histone H1 as a repressor has been well documented (reviewed in 1). However, in specific cases the presence of histone H1 on DNA enhances binding of specific transcription factors, thus acting as an activator of transcription (2,3). The question as to whether or not histones H1 bind selectively to methylated DNA has remained controversial. For example, it has been shown that methylation of CpG does not influence the total amount of histone H1 bound to a nucleosome present on the *Xenopus borealis* 5S RNA gene (4). Similarly, no preferential binding of total chicken histone H1 to methylated DNA has been observed (5). In sharp contrast, Ball *et al*. (6) found that ~80% of the methylated CpGs were located in nucleosomes that contained histone H1. More recently McArthur and Thomas (7), using a sensitive assay, showed preferential binding of histone H1 to methylated DNA. Similarly, MDBP-2-H1, which is a member of the histone H1 family (8), was shown to bind selectively to methylated DNA and this preferential binding was only observed for the phosphorylated isoform (9). Since the complete identity of MDBP-2-H1 has remained obscure it was necessary to fully characterize the primary structure of this protein. Here we show that MDBP-2-H1 consists mainly of histone H1 subtypes 01 and 11L truncated at their C-terminal ends.

MATERIALS AND METHODS

Isolation of total histone H1 from rooster liver

Two different methods were used to isolate truncated and full-length histones H1.

In the first procedure nuclear extracts were prepared as described by Sierra (10) with some modifications. The sucrose buffer, nuclear lysis buffer and dialysis buffer were supplemented with 50 mM β-glycerophosphate (Sigma) and 50 mM NaF to inhibit dephosphorylation of histone H1. Low and high salt buffers for chromatography on heparin–Sepharose (FPLC) also contained the same amounts of phosphatase inhibitors. Non-specific proteolytic degradation of proteins was inhibited by adding appropriate amounts of protease inhibitors (0.1 mM PMSF in the sucrose and nuclear lysis buffers and 2 mM benzamidine in the dialysis buffer). The 0.5 M KCl fraction containing MDBP-2-H1 was further purified by precipitating the non-histone proteins with 5% (v/v) $HClO_4$. The histones were subsequently precipitated with 120% (w/v) (1.2 g/ml) trichloracetic acid (TCA).

In the second procedure nuclei were obtained as described above and histones were isolated according to Dingman and Sporn (11). The nuclei were resuspended in 0.3 mM $MgCl_2$ and 0.3 mM K_2HPO_4/KH_2PO_4, pH 6.7, and incubated for 10 min at room temperature to lyse the contaminating erythrocytes. After centrifugation the sediment was washed three times in 0.08 M NaCl and 0.02 M EDTA, pH 6.7. Nuclear lysis was carried out as described above. The supernatant fraction was mixed with an equal volume of 10% $HClO_4$ and stirred on ice for 10 min. The precipitated proteins were sedimented by centrifugation and the histones precipitated by adding 1/5 vol 120% (w/v) TCA to the supernatant. The sediment was washed once with acidified acetone (0.3% HCl) and twice with ice-cold acetone. Histones H1 obtained by this procedure were separated on a strong cation exchange column (Mono S HR5/5; Pharmacia; 12) with a linear gradient of 1–14% guanidine hydrochloride in 50 mM potassium phosphate, pH 6.7, at a flow rate of 1 ml/min. Each fraction containing histones H1 was dialyzed extensively against water in the cold and vacuum dried. Further purification was achieved by reversed phase HPLC on a C4 column (2.1 × 250 mm; Vydac) with a linear gradient of 25–90% buffer B in buffer A [B, 70% acetonitrile, 0.085% trifluoracetic acid (TFA) in H_2O; buffer A, 0.1% TFA in H_2O] over 90 min and the highest peaks were collected. The purity of the proteins was determined by 15% SDS–PAGE using silver staining to visualize the bands.

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In order to show that the proteolytic cleavage of histones H1 occurs intracellularly and not during the preparation procedure two other methods (29) were used. In the first method livers were blended with 5% $HClO_4$ (v/v). After centrifugation histones H1 were precipitated with 1/5 vol 120% TCA (w/v). In the second method nuclei were prepared as described above and then extracted with 5% $HClO_4$ (v/v) followed by acetone precipitation (6 vol).

Preparation of the histone H1 subtypes for peptide mass fingerprinting

Endoprotease Glu-C and Lys-C digestion of histones H1 was done overnight in 100 µl 50 mM NH_4HCO_3, pH 7.8, at 37°C. A negative control, without H1, was included in each set of experiments. The generated peptides were analyzed by liquid chromatography–mass spectrometry (LC-MS) using an ABI 140B separation system equipped with a Vydac C8 column (1 × 250 mm). Peptides were eluted in a linear gradient of 5–50% buffer B in buffer A (B, 80% acetonitrile in H_2O) over 20 min at a flow rate of 50 µl/min. Solvent A was 0.05% TFA, 2% acetonitrile in H_2O. An aliquot of 10% of the effluent was directed to an API III or an API 300 triple quadrupole mass spectrometer (PE Sciex, Concord, Canada) and 90% was collected according to the UV absorbance signal at 214 nm. The ion spray voltage of the mass spectrometer was set to 5000 V and a mass range of 200–2400 was scanned with a step size of 0.5 Da and dwell time of 0.75 ms/mass.

N-Terminal sequence analysis

Sequence analysis was carried out on a model 477A protein sequencer (Applied Biosystems, Foster City, CA) according to the recommendations of the manufacturer.

Chemicals and enzymes

Heparin–Sepharose was purchased from Pharmacia. Sequencing grade endoproteases Glu-C and Lys-C were obtained from Promega.

RESULTS

MDBP-2-H1-like histone H1 can be separated from full-length H1 by cation exchange chromatography

The TCA-precipitated histones H1 were further separated on a Mono-S column. Figure 1A and B shows that histone H1, with a similar size to MDBP-2-H1, was mainly eluted at 7 min elution (F7) with the linear gradient. Further fractions eluting at 8–10 min (F8–F10) contained full-size H1. As revealed later by mass spectrometry, the difference in the chromatographic behavior of histone H1 shown in Figure 1B, lanes F8–F10, was probably due to secondary modifications rather than differences in primary sequence. In the total histone H1 fraction obtained by the second procedure described in Materials and Methods MDBP-2-H1 represented ~1.5% of total histone H1.

The presence of MDBP-2-H1 in our preparations is most probably not due to non-specific proteolytic degradation of histone H1 occurring during nuclei preparation, since a direct extraction of rooster liver with cold 5% $HClO_4$ gave identical results (data not shown).

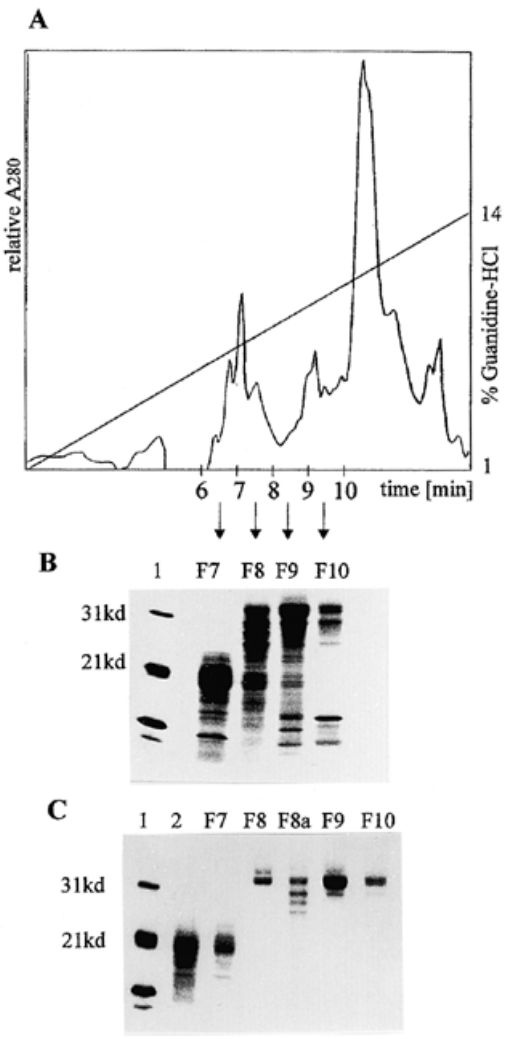

Figure 1. Fractionation of histones H1 by cation exchange chromatography and reversed phase HPLC. (**A**) Elution profiles of histones H1 (TCA precipitated) on a Mono-S column. The fractions eluted at the indicated time were further analyzed on a 15% SDS–polyacrylamide gel (2 µg protein loaded/lane; silver stained). (**B** and **C**) Lane 1, the size standards, fractions F7–F10 correspond to the different elution times shown in (A). (C) Fractions F7–F10 and MDBP-2-H1 (TCA precipitated, lane 2) were further fractionated by reversed phase HPLC (C_4 column). Fractionation was carried out as outlined in Materials and Methods. The main peaks (2 µg) of each fraction were than analyzed on a 15% analytical SDS–polyacrylamide gel (silver stained).

Purification and fractionation of histones H1 by reverse phase HPLC

Fractions from Mono-S columns containing histone H1 and MDBP-2-H1 were further purified by reversed phase HPLC on a C4 column as indicated in Materials and Methods. Figure 1C shows an SDS–PAGE analysis of the HPLC-purified fractions F7–F10 obtained from the Mono-S column (see Fig. 1B) and the fraction obtained by TCA precipitation of the 0.5 M KCl eluate from the heparin–Sepharose column (Fig. 1C, lane 2). Figure 1C shows silver staining of a 15% SDS–polyacrylamide analytical gel. It shows that

Table 1. Sequence alignment of the six chicken histone H1 subtypes including MDBP-2-H1 and their specific peptides (in bold) derived from endoprotease Glu-C digestion [Swissprot accession nos P09987 (26) P08284 (27) P08285, P08286, P08287, P08288 (28]

NH_2-terminus

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                                                                    35
H1 01        218 aa  21,913 MW      SE/TAPA----AAP-DAPA-PG-AKAA-AKKPKKAAGGAKARKP
H1 02        217 aa  21,750 MW      **/***V----***-AVS*-**-****-************P***
H1 10        219 aa  21,982 MW      **/****-----***-AVAX-*A-****-***************** X= A
H1 11L       224 aa  22,397 MW      **/****PAAE/***AA***-*--****-***************
H1 11R       218 aa  21,672 MW      A*/****-----***AA***-*A ****-***************
H1 03        223 aa  22,395 MW      A*/***V----***-*VA*A*TP****P***************
MDBP-2-H1    150 aa  15,078 MW      SE/TAPA----AAP-DAPA-PG-AKAA-AKKPKKAAGGAKARKP
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Globular Domain

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                                                                    109
AGPSVTE/LITKAVSASKE/RKGLSLAE/RKGLSLAALKKALAAGGYDVE/KNNSRIKLGLKSLVSKGTLVQTKGTGASGSFRLNK
*******/*******************/*******************/*********************************K***
*******/*******************/*******************/*********************************S*
*******/*******************/*******************/*********************************S*
*******/*******************/*******************/*********************************S*
*******/*******************/*******************/*********************************S*
AGPSVTE/LITKAVSASKE/RKGLSLAE/RKGLSLAALKKALAAGGYDVE/KNNSRIKLGLKSLVSKGTLVQTKGTGASGSFRLNK
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C-terminus

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                                                                    150
KPGE/VKE/KAPRKRATAAKPKKPAAKKPAAAAK*KPKKAAAVKKSPKKAKKPAAAATKKAAKSPKKAAKAGRPKKA--AKSPAKAKAVKPKAAKPKATKPKAAKAKKTAAKKK
****/T**/**TK*K-P*****************************************************T*******T--****************S**A*********A*T****
****/***/******TP*************S**********A*************************T**AK****AT*******************A*********A****-
****/***/**K*K*S*************************V**************S****S****** VT**VK****VA*******************A*********A*****
****/GL*/*/**K*K*S******A***************V**************S****SV*********---****VA*******************A*********A*****
*S*D****/**K*KTP************************V************************** VT**AK****VAV*****************************A*P***
KPGE/VKE/KAPRKRATAAKPKKPAAKKPAAAAK
```

the main peaks of MDBP-2-H1 are basically free of other contaminating proteins. However, separation of histone H1 into subtypes could not be achieved due to their almost identical retention times on the column. Only the proteins in fractions F8 and F8a gave discrete bands of histone H1 on SDS–polyacrylamide gels.

Identification of MDBP-2-H1 as truncated H1 subtypes

Proteins present in fractions F7–F10 of Figure 1C and TCA-precipitated MDBP-2-H1 were subjected to digestion with endoprotease Glu-C V8 from *Staphylococcus aureus* and Lys-C endopeptidase. The digests were further analyzed by LC-MS. By using flow splitting, peptide masses could be confirmed by microsequencing. Fractions containing peptides were collected according to their UV (214 nm) absorbtion signals. The spectra of fractions F7–F10 were very similar, indicating that each fraction contained the same linker histone subtypes. The peptide masses revealed that all six of the chicken histone H1 subtypes were present in nearly every fraction, but in different quantities. All fractions were enriched in H1 01, whereas H1 11L was under-represented in fraction F10. Fractions F8a and F9 contained more of the subtype H1 11R than did fractions F8 and F10. Figure 2 shows the elution profile of peptides from fraction F7 (see also Fig. 1C). The peaks containing H1 subtype-specific and non-specific peptides are labeled. The main differences in the amino acid sequences of these subtypes are found in the N-terminal domain. Digestion with V8 generated peptides which were characteristic for each subtype (Table 1). Two peptides from MDBP-2-H1 have already been sequenced (8): KPAGPSVTELITK and ALAAGGYDVEK. Both are part of the globular domain, which is the most conserved region of histone H1. Our analysis showed that peptides shared by all subtypes represent the majority of the material. The most abundant histone H1

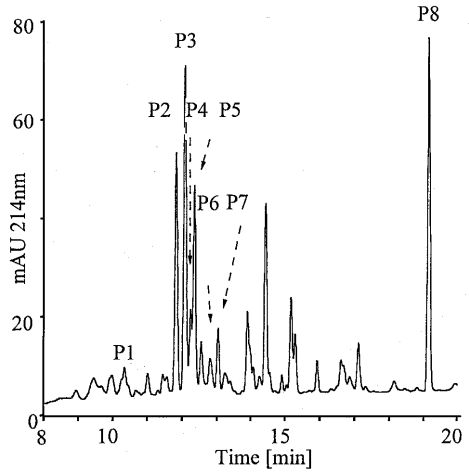

Figure 2. Reversed phase HPLC elution profile of the histone H1 peptides derived from endoprotease Glu-C digestion of fraction F7. The UV spectra show the retention times of specific and non-specific histone H1 peptides present in fraction F7. Labeled peaks contain peptides shared by all of the six subtypes (P5 and P8) and specific peptides (P1, P2, P3, P4, P6 and P7). Amino acid sequences and masses are indicated in Table 2.

subtype in rooster liver is H1 01. In fraction F7, containing MDBP-2 H1, variant H1 01 was truncated at the C-terminus and had only 150 amino acids as determined by mass spectrometric analysis and was confirmed by partial sequencing of the C-terminal peptide. The peptide derived from the V8 digest had a mass of 3.478 kDa. It extended from amino acid 117 to 150. A mass spectrometric analysis of the undigested proteins revealed a mass of 15.078 kDa (Table 1).

Table 2. Characterization of peptides derived from endoprotease Glu-C digestion found in the HPLC-purified fraction F7

Fragment	Calculated mass (av)	Sequence	H1 subtype						
			H1 02	H1 11R	H1 10	H1 01	H1 11L	MDBP-2-H1	Peak in Fig. 2
43–53	1146.0	**LITKAVSASKE**	+	+	+	+	+	+	P5
54–74	2131.3	**RKGLSLAALKKALAAGGYD**VE	+	+	+	+	+	+	P8
3–42	3677.9	**TAPAAAPDAPAPGAKAAAKKPKK AAGG**AKARKPPAGPSVTE				+		+	P3
54–116	6545.5	**RKGLSLAALKKALAAGGYD**VEKN NSRIKLGLKSLVSKGTLVQTKGTGA SGSFRLNKKPGE/VKE				+		+	
75–116	4458.2	KNNSRIKLGLKSLVSKGTLVQTKGT GASGSFRLNKKPGE/VKE				+		+	
117–150	3478.5	**KAPRKRATAA**KPKKPAAKKPAAAA KKPKKAAAVK				+		+	P1
3–10	726.5	TAPAPAAE				+	+		
11–46	3307.8	**AAPAAAPAPAKAAAK**KPKKAAGG AKARKPAGPSVTE					+	+	P2
3–42	3649.8	**TAPAAAPAVAAPAAK**AAAKKPKKA AGGAKARKPAGPSVTE			+			+	P6
54–116	6545.5	**RKGLSLAALKKALAAGGYD**VEKN NSRIKLGLKSLVSKGTLVQTKGTGA SGSFRLSKKPGE/VKE			+			+	
3–43	3718.0	TAPAAAPAAAPAPAAKAAAKKPKKA AGGAKARKPAGPSVTE		+				+	P4
76–117	4373.4	KNNSRIKLGLKSLVSKGTLVQTKGT GASGSFRLSKKPGEGLE			+			+	
3–42	3679.9	**TAPVAAPAVSAPGAKAAAK**KPKKA AGGAKPRKPAGPSVTE	+					+	P7

+, presence of the peptide in the indicated subtype of histone H1; underlined, identified by mass; bold, identified by sequencing.

This finding is consistent with the calculated mass of a C-terminal truncated H1 01 subtype at amino acid position 150 by taking into consideration that the N-terminus is acetylated (data not shown). Acetylation of the N-terminus of subtypes H1 01, H1 11L and H1 02 was determined by Lys-C digestion, which generates longer N-terminal peptides than Glu-C. These peptides were also identified by mass similarity and sequencing. The second most prominent subtype was H1 11L. Peptides from the other subtypes were detectable but only in trace amounts. Table 2 summarizes the results obtained from peptide mass fingerprinting and microsequencing. These data show that MDBP-2-H1 is a fraction consisting of histones H1 that are truncated at their C-termini. At least for subtype H1 01 we show the complete primary sequence from amino acid 1 to 150 (Table 1) and no further peptides covering the C-terminal domain of histone H1 01 (wild-type) could be detected. Therefore, one can conclude that one of the main forms of MDBP-2-H1 is the truncated H1 subtype H1 01. Moreover, an MS-MS analysis of MDBP-2-H1 revealed the same mass as calculated for amino acids 1–150 of H1 01. During extensive analysis of histone H1 we identified the unknown amino acid in position 13 of H1 10 as an alanine (A).

DISCUSSION

It has been shown that MDBP-2-H1, which is a member of the histone H1 family (8), is a protein which acts as a transcriptional repressor and binds preferentially to a promoter sequence containing one single pair of methylated CpGs (9,13). Similarly, several reports have established a positive correlation between DNA methylation, binding of histone H1 and transcriptional repression (24,25). For calf thymus histone H1 it was shown that the histone variant H1c inhibits transcription from a methylated template more efficiently than other histone H1 subtypes (25). Further investigations revealed that MDBP-2-H1 was only active as a repressor when serine residues were phosphorylated and *in vivo* repressor binding activity was down-regulated by estradiol (9,14). Here we show that MDBP-2-H1 is a subset of histone H1 subtypes which are all truncated in their C-terminal domains. It was shown that the histone variant H1 01 had only the first 150 amino acids. One explanation for the fact that MDBP-2-H1 never gave a sharp band on SDS–polyacrylamide gels is the presence of other subtypes, like H1 11R and H1 11L. They are also most probably truncated in the C-terminal domain because after extensive investigation we could

only detect peptides belonging to the amino and globular domains. The C-terminal peptides of other subtypes than H1 01 could not be found either. This might be due to quantitative under-representation of these minor variants. While the C-terminal part of histone H1 did not seem to be involved in DNA binding, the globular domain has two DNA binding sites (15). MDBP-2-H1 could be a proteolytic degradation product that was generated by the purification procedure. However, although we cannot completely exclude this possibility, the data presented here and in previous publications (8,9,13,14,20,23) support the hypothesis that MDBP-2-H1 has a functional role *in vivo*. Four different isolation methods were applied to separate the truncated histone H1 from the full-size histone H1. Care was taken to minimize non-specific degradation during the purification steps by addition of high concentrations of protease and phosphatase inhibitors. Even a direct extraction of liver with 5% (v/v) $HClO_4$ yielded the same amount of MDBP-2-H1 as the more elaborate procedures. Another argument in favor of a biologically relevant role for MDBP-2-H1 is its presence in hen liver in an inactive form (loss of preferential binding to methylated DNA; 8). It was also demonstrated that the globular domains of H1 and H5 bind highly cooperatively to DNA to form complexes (16). MDBP-2-H1 binding to methylated synthetic oligonucleotides was first identified by UV crosslinking experiments as a 40 kDa nucleoprotein, while the purified unbound protein had an apparent molecular mass of ~20 kDa (13). Furthermore, the footprint of the 40 kDa nucleoprotein on the synthetic oligonucleotide extended over 30 bp, with an axis of symmetry in the middle (13). These results indicate that MDBP-2-H1 might bind to methylated DNA as a dimer of subunits of the same molecular weight. The main property of MDBP-2-H1 (or C-terminal truncated histones H1) is its preferential affinity for methylated DNA sequences. In this case the DNA sequence tested was the promoter region of the avian vitellogenin gene. The gene is silenced in rooster when the promoter is methylated (17,18) and its expression is regulated by hormones. Thus the truncated histones H1 seem to be involved in hormone-dependent transcriptional control. It is known that H1 variants isolated from transcriptionally inactive chromatin bind with higher affinity to DNA than do H1 subtypes isolated from nuclei in which transcription occurs (19). Additionally, histones H1 are often secondarily modified, by phosphorylation for example, which modulates their biological function. MDBP-2-H1 functions only as a transcriptional repressor when its serine residues are phosphorylated (20). Similarly, sea urchin sperm-specific histones H1 are phosphorylated during condensation of bulk chromatin (21). The site where phosphorylation occurs is also important. Phosphorylation at sites in the C-terminal domain had no effect on DNA binding, whereas phosphorylation of an isolated peptide from the N-terminal part reduced its affinity for DNA (22). So far we have focused on the identification of MDBP-2-H1 and have not addressed the question of which serine residues are phosphorylated in the truncated histones. This secondary modification plays a crucial role not only in MDBP-2-H1 action but also in other linker histones. Furthermore, an unidentified protein present in rooster liver and not in egg-laying hens (Bruhat and Jost, unpublished results) is known to enhance the binding kinetics of MDBP-2-H1 to methylated DNA (8,23). These results indicate that the mechanism of histone H1 as a repressor is complex and that other proteins in the nuclear compartment are important for fine tuning of histone H1 function. The C-terminal truncated histone H1 known as MDBP-2-H1 represents another linker histone variant which may influence transcription of specific genes.

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