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Regulation of Rat Haptoglobin Gene Expression Is Coordinated by the Nuclear Matrix

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ABSTRACT

Using computer stress-induced duplex destabilization (SIDD) analysis and binding experiments, we identified a S/MAR element (−599/−200 bp) (Hp-S/MAR) adjacent to the *cis*-element (−165/−56 bp) in the rat haptoglobin gene. We examined its functional interactions with the lamins and lamin-associated proteins in the basal state and during acute-phase (AP) response-induced increased transcription. Colocalization, electrophoretic mobility shift assay (EMSA), and re-electrophoresis of nucleoprotein complexes, South-Western and Western blot analysis and coimunoprecipitation experiments revealed that the lamins, PARP-1, C/EBPβ, and Hp-S/MAR assembled higher order complexes through direct lamin-Hp-S/MAR and probably PARP-1–Hp-S/MAR interactions although C/EBPβ did not bind to the Hp-S/MAR but established direct interaction with PARP-1. The transition from constitutive to increased haptoglobin gene transcription during the AP response was associated with quantitative and qualitative changes in Hp-S/MAR–protein interactions, respectively, observed as increased association of the lamin(s) with the Hp-S/MAR and as the appearance of a 90 kDa Hp-S/MAR-binding protein. Also, during the AP response the contact between C/EBPβ and PARP-1 established in the basal state was lost. DNA chromatography with the haptoglobin *cis*-element and Western blot analysis suggests that PARP-1 was a coactivator during constitutive and elevated transcription. The results show that the lamin components of the nuclear matrix form a network of functional, dynamic protein–protein and protein–Hp-S/MAR associations with multiple partners, and underline the involvement of PARP-1 in the regulation of haptoglobin gene transcription. We concluded that the interplay of these interactions fine tunes haptoglobin gene expression to meet the changing requirements of liver cells. J. Cell. Biochem. 107: 1205–1221, 2009.

KEY WORDS: S/MAR; NUCLEAR MATRIX; LAMINS; HAPTOGLOBIN; PARP-1; C/EBPβ; ACUTE PHASE

Systemic inflammation is a complex defense mechanism that is activated in response to different types of trauma or infections with the aim of restoring an organism's normal functioning [Ruminy et al., 2001]. An early event of the inflammatory reaction is the acute-phase (AP) response that is characterized by increased synthesis in the liver of a group of plasma proteins referred to as the AP proteins (or AP reactants). The AP proteins assist the injured organism in its attempt to restore homeostasis [Poznanović et al., 1997]. Haptoglobin is one of the several major AP proteins in mammals [Kushner and Mackiewicz, 1987]. Its central role is the binding of hemoglobin that is released by hemolysis [Bowmann and Kurosky, 1982]. In rats, the stimulation of haptoglobin synthesis during the AP response is the result of increased haptoglobin gene transcription [Ševaljević et al., 1989]. Increased haptoglobin synthesis is incorporated in the overall endocrine response to trauma. It undergoes temporal regulation resulting from the complex interplay of interleukins (ILs) IL-1, IL-6, and glucocorticoids [Baumann et al., 1990]. The transcription activity of the haptoglobin gene reaches a maximum between 12 and 48 h after induction of acute inflammation [Ševaljević et al., 1988]. The essential haptoglobin gene *cis*-element is defined by the proximal promoter hormone responsive element (HRE) that contains three *cis*-acting "sub"-elements: A (−165/−146 bp), B (−146/−88 bp), and C (−97/−49 bp), with each binding a distinct set of transcription factors. The regulation of haptoglobin gene transcription has been exclusively studied by examination of *cis–trans* interactions

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between the HRE and gene regulatory proteins. The dynamic binding profiles of gene regulatory proteins to the haptoglobin *cis*-element in the course of the AP response have been characterized, that is, of C/EBPα and C/EBPβ [Ramji et al., 1993; Pajović et al., 1994; Lee et al., 2002; Milosavljević et al., 2003; Dinić et al., 2005], p53 [Mihailović et al., 2005], STAT3, and STAT5b [Zauberman et al., 2001; Grigorov et al., 2002], as well as of HMG-1 [Grigorov et al., 2001].

The term S/MAR designates sections in the DNA sequence of differing lengths that function as association points with the nuclear matrix. Although the overall base composition is not the primary determinant whether a DNA region can be considered a S/MAR, their functioning does require an ''AT-patch pattern'' with a proclivity for local DNA strand unpairing under torsional strain [Bode et al., 2006]. The potential for strand separation enables the establishment of attachments between the chromatin and nuclear matrix proteins with an affinity for binding to single-stranded DNA [Ludérus et al., 1994]. The strand separation potential of S/MARs is commonly expressed as a stress-induced duplex destabilization (SIDD) profile which predicts the free energy G(x) needed to effect the separation of a base pair at each position x along the DNA sequence at certain level of torsional tension [Benham, 1993]. The results are displayed as a SIDD profile in which sites of strong destabilization appear as deep minima.

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme that participates in several nuclear processes that involve DNA. One important PARP-1 function is its participation in the repair of single- and double-stranded DNA nicks [Schreiber et al., 2006]. In addition, PARP-1 also plays a role in chromatin remodeling [Tulin and Spradling, 2003; Kim et al., 2004], which is not only integrated in the repair process but most likely assists in transcription initiation [Kraus and Lis, 2003]. Furthermore, PARP-1 has a defining role in programmed cell death [Virag and Szabo, 2002]. PARP-1 performs multiple functions primarily through its enzymatic ability to polymerize ADP-ribose from NAD^+ and post-translationally modify itself and other target proteins. Its function is also achieved through different types of PARP-1–DNA interactions as well as specific PARP-1–protein associations. Aside from the PARP-1 binding to breaks in the DNA strand during DNA repair that was observed first [Malanga and Althaus, 2005], PARP-1 also has an affinity towards DNA secondary structures that characterize S/MARs [Sastry and Kun, 1990; Galande and Kohwi-Shigematsu, 1999; Soldatenkov et al., 2002; Lonskaya et al., 2005], reflecting its presumed involvement in chromatin remodeling. Furthermore, an increasing number of reports indicate that PARP-1 can also bind to DNA in a sequence-specific manner. Recently, we characterized a novel PARP-1 consensus DNA-binding motif (AGGCC) in the PARP-1 promoter [Vidaković et al., 2009].

Examinations of PARP-1 interactions with nuclear matrix proteins have described its direct interaction with lamin B [Vidaković et al., 2004, 2005], the consequence of which is the association of the largest part of nuclear PARP-1 with the peripheral nuclear lamina in rat liver cells. PARP-1 interactions with transcription factors [Oei et al., 1997; Nie et al., 1998; Hassa et al., 2003; Wesierska-Gadek et al., 2003] suggest that PARP-1 plays a role in the regulation of gene expression as a cofactor [Cervellera and Sala, 2000; Martin-Oliva et al., 2004; Lis and Kraus, 2006]. Also, the direct involvement of PARP-1 in transcriptional regulation was recently demonstrated by Vidaković et al. [2009] who described the suppression of the PARP-1 promoter by PARP-1 protein.

There is growing evidence that the temporal and spatial organization of nucleic acids and regulatory proteins in the nucleus provides a structural and functional infrastructure for transcriptional activation and suppression [DeFranco, 2002]. One aspect of transcriptional gene regulation involves the formation of dynamic chromatin loops through S/MAR attachments with the nuclear matrix [Bode et al., 2006]. The aim of the present study was to examine in more detail the involvement of nuclear matrix proteins in the regulation of rat haptoglobin gene expression through functional interactions with a S/MAR that was identified 5' upstream from the coding region. We examined using the SIDD algorithm the haptoglobin gene 5′ region surrounding a 28 bp A-box (−314/−342 bp) previously shown to establish dynamic associations with the A/C-type lamins [Poznanović et al., 1994], for the presence of a potential S/MAR element, as well as S/MAR DNA-nuclear matrix-associated protein binding in the context of increased haptoglobin gene transcriptional activity. We have identified a S/MAR region and examined its dynamic binding to three different classes of nuclear matrix-related proteins: (i) the lamins, the fundamental architectural components of the peripheral nuclear lamina and the nuclear matrix; (ii) the nuclear matrix-associated component of the liver-enriched transcription factor C/EBPβ that participates in the regulation of haptoglobin gene transcription; and (iii) the multifunctional protein PARP-1 which in its non-modified state directly interacts with lamin B and establishes functional associations with different regulatory complexes. The observed dynamic changes of the protein–DNA-binding profile reflect the involvement of individual molecular components of the complex assembled at the point of chromatin attachment to the nuclear matrix in the regulation of haptoglobin gene transcription. The obtained results support the concept that the nuclear matrix spatially organizes and coordinates the functional interactions of proteins with S/MAR elements during transcription.

MATERIALS AND METHODS

ANIMALS AND TREATMENTS

All the animal procedures were approved by the Committee for Ethical Animal Care and Use of the Institute for Biological Research, Belgrade, which acts in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication No. 85/23, revised in 1986). Experiments were performed on 2.5-month-old adult albino Wistar rats weighting 220–250 g. The animals were kept under constant temperature, humidity, and a controlled daily lighting schedule (12 h light/dark intervals). Food and water were freely available. Before the experiments the rats fasted for 24 h. The AP reaction was induced according to the standard accepted procedure of induction of the inflammatory response in the rat, that is, by a subcutaneous injection of 1 μl turpentine oil/g body weight in the lumbal region of 2.5-month-old male rats [Ševaljević et al., 1994]. Control animals

were injected with pyrogen-free saline. The animals were killed 4 and 24 h after the initial treatment, as will be indicated.

ISOLATION OF RAT LIVER NUCLEI

Nuclei were isolated from the livers of control and turpentine-treated rats according to two procedures. For Southern dot-blot analysis of nuclear matrix-associated DNA, nuclei were isolated in the presence of polyamines and metal chelators [Gorski et al., 1986]. For all other purposes, nuclei were isolated and purified as described by Kaufmann and Shaper [1984]. Unless otherwise indicated, all the steps were carried out on ice. Minced rat livers (0.5 g/ml) in 0.25 M sucrose, 50 mM Tris–HCl (pH 7.4), 5 mM $MgSO_4$ (STM) were homogenized with several strokes of a Potter-Elvehjem Teflon-glass homogenizer, centrifuged at 1,000*g* for 15 min, and washed once with STM. The crude nuclear pellet was resuspended in 2.2 M sucrose, 50 mM Tris–HCl (pH 7.4), 5 mM $MgSO_4$ (DS), layered over a 5-ml cushion of the same buffer, and centrifuged at 72,000*g* in a Beckman SW-28 rotor for 60 min at 4°C. The pellet was resuspended in STM buffer, layered over a 5-ml cushion of DS buffer, and centrifuged for 30 min at 72,000*g* at 4°C. All buffers contained 1 mM of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF).

NUCLEAR MATRIX ISOLATION

The nuclear matrix was isolated essentially as described [Belgrader et al., 1991]. Freshly isolated purified nuclei were heat-stabilized by incubating at 42°C for 20 min [Ludérus et al., 1992]. The nuclei (10^8 nuclei/ml) were incubated with 2 mM Na-tetrathionate in 0.25 M sucrose, 50 mM Tris–HCl (pH 7.4), 5 mM $MgSO_4$ for 1 h at 4°C, washed twice with the same buffer without Na-tetrathionate and incubated with 100 μg/ml of RNase-free DNase I overnight at 4°C. The nuclei were then subjected to consecutive extraction/centrifugation steps: twice with high salt buffer: 0.25 M $(NH_4)_2SO_4$, 10 mM Tris–HCl (pH 7.4), 0.2 mM $MgSO_4$, once with freshly prepared 1% Triton X-100 in low salt (LS) buffer: 10 mM Tris–HCl (pH 7.4), 0.2 mM $MgSO_4$, followed by two washes with LS buffer. Nuclear matrices were resuspended in 0.5 M sucrose, 10 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.4), 1 mM PMSF to which an equal volume of sterile glycerol was added, and the matrices were kept at −20°C.

ISOLATION OF THE PERIPHERAL NUCLEAR LAMINA

The peripheral nuclear lamina structures were isolated as described by Stuurman et al. [1990]. In order to prevent the formation of disulfide bonds, the nuclei were isolated in the presence of 10 mM iodoacetate instead of Na-tetrathionate. After the first ultracentrifugation step, the nuclei were resuspended and incubated in STM containing iodoacetate for 1 h on ice. After several washes, the purified nuclei were digested with 100 μg/ml DNase I and 250 μg/ml RNase A overnight on ice. After three washes with STM without iodoacetate, the nuclei were resuspended in LS buffer supplemented with 20 mM dithiothreitol (DTT). And an equal volume of 3.2 M NaCl containing 20 mM DTT was then slowly added with stirring. After 15 min, the peripheral lamina fraction was pelleted by centrifugation at 7,000*g*. The extraction was repeated once more. The peripheral nuclear lamina was subsequently extracted with 1% Triton X-100 in LS buffer and washed once more with LS buffer.

SOLUBILIZATION OF THE ISOLATED PERIPHERAL NUCLEAR LAMINA (PREPARATION OF THE L-EP1 FRACTION)

The isolated peripheral nuclear lamina was solubilized according to Aebi et al. [1986] in several steps as follows: (i) pre-solubilization wash—the freshly isolated peripheral nuclear lamina was incubated for 30 min (0.25 mg protein/ml) in 10% sucrose, 2% Triton X-100, 20 mM MES-KOH (pH 6.0), 300 mM KCl, 2 mM EDTA, 1 mM DTT and centrifuged at 6,000*g* for 20 min to yield a supernatant and lamina-enriched pellet; (ii) solubilization—the pellet was resuspended (0.3 mg protein/ml) in 2% Triton X-100, 20 mM Tris–HCl (pH 9.0), 500 mM KCl, 2 mM EDTA, 1 mM DTT and after 30 min centrifuged at 200,000*g* for 40 min to yield a supernatant consisting of solubilized lamins and an insoluble pellet. The solubilized nuclear lamina proteins were dialyzed against 1,000 volumes of buffer containing 10 mM Tris–HCl (pH 7.4), 50 mM NaCl, 2 mM EDTA, and 1 mM DTT. Dialysis brings about lamin reassembly after 4 h. The lamina filaments were pelleted by centrifugation at 10,000*g* at 4°C for 15 min. This protein fraction was referred to as LEP-1.

PREPARATION OF SOLUBLE NUCLEAR MATRIX PROTEINS (SNMPs) AND A LAMIN-ENRICHED PROTEIN FRACTION (L-EP2)

Soluble nuclear matrix proteins (SNMPs) and a second lamin-enriched protein fraction (L-EP2) were prepared as follows: 3–5 mg of nuclear matrix proteins were incubated in deionized 8 M urea, 20 mM 4-morpholineethanesulfonic acid (MES) (pH 6.6), 1 mM EGTA, 1 mM PMSF, 0.1 mM $MgCl_2$, 1% β-mercaptoethanol at 30°C [Zackroff et al., 1982]. Centrifugation at 10,000*g* (room temperature, 30 min) removed the proteins that resisted solubilization. The obtained supernatant was dialyzed overnight at 37°C against 1,000 volumes of assembly buffer: 0.15 mM KCl, 25 mM imidazole hydrochloride (pH 7.1), 5 mM $MgCl_2$, 0.125 mM EGTA, 2 mM DTT, and 0.2 mM PMSF. The reassembled filaments (prepared from control and AP L-EP2 fractions) were pelleted by centrifugation at 10,000*g* at room temperature for 15 min and stored at −80°C, as were also the supernatants (SNMP).

ISOLATION OF THE RAT LIVER NUCLEAR EXTRACT

Nuclear extracts were prepared from livers of control and turpentine-treated rats according to Gorski et al. [1986]. Tissues were excised and homogenized in 10 mM HEPES (pH 7.6), 25 mM KCl, 1 mM spermidine, 1 mM (DTT), 1 mM EDTA, 1 mM PMSF, 2 M sucrose, and 10% glycerol. The nuclei were pelleted by centrifugation through a cushion of the same solution at 72,000*g* in a Beckman SW 28 rotor for 30 min at 40°C. The nuclei were resuspended in lysis buffer (10 mM HEPES (pH 7.6), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol), and the chromatin was pelleted by centrifugation at 82,000*g* in a Ti 50 rotor for 60 min at 4°C. Solid $(NH_4)_2$ SO_4 was added to the supernatant and the precipitated proteins were sedimented at 82,000*g* in a Beckman Ti 50 rotor for 30 min at 4°C. Nuclear extracts were dialyzed against 25 mM HEPES (pH 7.6), 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol, resuspended in dialysis buffer and frozen in small aliquots at −80°C.

ANALYSIS OF NUCLEAR MATRIX-ASSOCIATED DNA

For the analysis of nuclear matrix-associated DNA, nuclei obtained in the presence of polyamines and metal chelators were extracted by gently mixing with 2 M NaCl in 5 mM HEPES/NaOH (pH 7.4), 0.25 mM spermidine, 0.1 mM spermine, 2 mM EDTA/KOH (pH 7.4), 2 mM KCl, and 0.2 mM PMSF. After incubation on ice for 15 min, the histone-depleted nuclei were centrifuged at 60,000*g* for 15 min at 25°C. The precipitate was washed in restriction nuclease digestion buffer and digested with a total of 50 U of *Eco*RI, *Hin*dIII, and *Pst*I per 1 A_{260nm} unit of extracted nuclei. The nuclear matrices with attached DNA were collected by centrifugation at 10,000*g* at 4°C.

SDS–PAGE

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), 20 μg of proteins was loaded onto a 1-mm thick 4% stacking/12% separating slab gels [Laemmli, 1970] in a Bio-Rad Mini-PROTEAN II electrophoresis cell. The gels were stained using Coomassie brilliant blue R-250. Protein concentrations were determined according to the method of Lowry et al. [1951].

WESTERN BLOT ANALYSIS

Twenty micrograms of proteins separated by SDS–PAGE was electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Western blot analysis was performed using rabbit polyclonal antibodies to rat PARP-1, C/EBPβ, NF-kB, goat polyclonal antibodies to rat A/C, and B lamins (obtained from Santa Cruz Biotechnology) and mouse monoclonal antibodies to poly(ADP-ribosyl) residues (PAR) (obtained from Alexis). Staining was performed by the chemiluminescent technique according to the manufacturer's instructions.

SOUTH-WESTERN BLOT ANALYSIS

After SDS–PAGE, proteins were transferred to nitrocellulose filters by electroblotting. Following transfer, the filters were soaked in the binding buffer (BB) containing 50 mM NaCl, 1 mM Na-EDTA, 10 mM Tris–HCl (pH 7.0), 0.02% bovine serum albumin (BSA), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and competitor DNA (20 μg of salmon sperm DNA) for 1 h as described [Bowen et al., 1980]. The filters were probed with 100 cpm/lane of the ^{32}P-labeled rat haptoglobin gene Hp-S/MAR (−541/−165 bp) DNA in BB for 1 h at room temperature. After binding, the filters were washed four times for 15 min with BB containing 200 mM NaCl. After washing the membrane was subjected to autoradiography. After autoradiography, the membrane was stripped and subjected to Western blot analysis with anti-CEBPβ, anti-lamins A/C and B and anti-PARP-1 antibodies.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The EMSA was performed according to Fried and Crothers [1981]. SNMPs (5 μg) were preincubated in 1 mM $MgCl_2$, 10% glycerol, 2.5 mM EDTA, 2.5 mM DTT, 0.25 M NaCl, 0.05 M Tris–HCl (pH 7.5) at 30°C for 10 min. Protein–DNA binding (25 min at 20°C) was carried out with 100 ng of the purified DNA insert of the rat haptoglobin gene S/MAR (−541/−146 bp) and 2.5 μg of linearized vector as competitor DNA [Urh et al., 1995]. After pre-electrophoresis, the samples were loaded onto 1 mm thick 5% polyacrylamide gels and separated at 20 V per gel by standard Tris–borate–EDTA non-denaturing (ND) PAGE. The gels were stained with ethidium bromide and further used for re-electrophoresis, as well as with silver [Rabilloud et al., 1994] for more precise presentation of the obtained results.

RE-ELECTROPHORETIC ANALYSIS OF THE NUCLEOPROTEIN COMPLEXES OBTAINED AFTER EMSA

Re-electrophoresis was performed to separate and identify the proteins in the nucleoprotein complexes according to their molecular masses by SDS–PAGE [Poznanović et al., 1999]. Two gels were always run simultaneously during EMSA. Both gels were stained with ethidium bromide. After the position of the Hp-S/MAR insert and pUC13 vector were established, one gel was stained with silver and after development of the stain, gel pieces (5 mm × 5 mm) were cut from the ethidium bromide-stained gel from the positions corresponding to nucleoprotein complexes I and II observed on the silver stained gels. The positions of the bands on the different gels were monitored by the distance between Hp-S/MAR and pUC13. The gel pieces were equilibrated in 0.0625 M Tris–HCl (pH 6.8), 2.3% SDS, 10% glycerol for a total of 1 h. After two more changes of buffer, the slices were placed on top of a standard polyacrylamide gels and SDS–PAGE was carried out as described above.

IMMUNOPRECIPITATION EXPERIMENTS

Immunoprecipitation was carried out with nuclear extracts according to Cella et al. [1998]. Nuclear extracts (500 μg) were incubated with 1 μg of C/EBPβ (Santa Cruz Biotechnology) antibody over night on ice. Protein A-Sepharose-coupled beads (Santa Cruz Biotechnology) were added for 2 h at 4°C under constant agitation. The beads were pelleted and washed three times with extraction buffer (10 mM HEPES [pH 7.6], 400 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol) and once with TNE buffer (140 mM NaCl, 50 mM Tris–HCl [pH 7–8], 5 mM EDTA). Immunoprecipitated proteins were boiled in sample buffer and analyzed by SDS–PAGE. Immunoblot analysis was performed with anti-C/EBPβ, anti-PARP-1, and anti-NF-κB (p65) antibodies (Santa Cruz Biotechnology).

POLY(ADP-RIBOSE)POLYMERASE-1 (PARP-1) ASSAY

The PARP activity assay was carried out according to Quesada et al. [2000] with minor modifications. The reaction mixture (final volume 50 μl) contained 100 mM Tris–HCl (pH 8.0), 14 mM β-mercaptoethanol, 10 mM $MgCl_2$, 400 μM [^{14}C]NAD^+ (10,000 cpm/nmol) and as an enzyme source, an amount of nuclei, nuclear matrices and different nuclear protein fractions that corresponded to 100 μg protein. After 15 min of incubation at 30°C, the reaction was stopped by the addition of ice-cold trichloroacetic acid 40% (v/v) and the radioactivity in the acid-insoluble material was counted on a Beckman LS8100 liquid scintillation spectrometer. One milliunit is defined as the amount of enzyme activity catalyzing the incorporation per minute of 1 nmol of ADP ribose into acid-insoluble material.

DNA PROCEDURES

The rat haptoglobin gene *cis*-element (−165/−56 bp) subcloned into the *Eco*RI and *Hin*dIII site of pUC13, the Hp-S/MAR (−541/−165 bp)

subcloned into the *Eco*R1 and *Pst*I site of pUC13 and the haptoglobin 5′ region (−705/+159 bp) subcloned into the *Eco*R1 and *Pst*I site of pUC13 were obtained from Dr. Heinz Baumann from the Department of Molecular Cell Biology, Roswell Park Institute, Buffalo, NY, USA. DNA procedures, Southern analysis, and 5′ end-labeling were essentially performed as described [Sambrook et al., 1989].

DNA AFFINITY CHROMATOGRAPHY

A DNA affinity column with the rat haptoglobin gene *cis*-element (−165/−56 bp) linked to CNBr-activated Sepharose CL-4B (Pharmacia) was prepared according to Kadonaga and Tijan [1986]. The DNA column was equilibrated with Z buffer: 25 mM HEPES (pH 7.6), 12.5 mM $MgCl_2$, 10% glycerol, 0.1% IGEPAL, 10 μM $ZnSO_4$, 1 mM DTT containing 0.1 M KCl. Two milligrams of lyophilized SNMP was dissolved in Z buffer containing 0.1 M KCl and denatured salmon sperm DNA (100 μg/1 mg SNMP), and incubated at room temperature for 10 min. The protein–DNA competitor mixture was applied to the DNA column and incubated at 4°C for 1 h. Proteins in the SNMP fractions prepared from control and rats undergoing the AP response that bound to the DNA column were subsequently eluted with Z buffer containing 1 M KCl. The eluted proteins were separated by SDS–PAGE, electroblotted onto nitrocellulose, and analyzed by Western blotting with anti-CEBPβ and anti-PARP-1 antibodies.

S/MAR-BINDING PROCEDURE

S/MAR binding was performed essentially as described [Ludérus et al., 1994]. Restriction fragments of a pUC13 plasmid containing the rat Hp-S/MAR (−541/−146 bp) were amplified and end labeled with [γ-^{32}P] dATP by the Klenow fragment of *E. coli* polymerase I. Unincorporated nucleotides were removed by spun-column chromatography with Sephadex G-50. From 0.5 to 1 mg of L-EP1 proteins were dialyzed for 2 h at room temperature against BB (10 mM Tris–HCl [pH 7.4], 50 mM NaCl, 2 mM EDTA). Under these conditions aggregation of lamins takes place. BB containing radioactively labeled Hp-S/MAR (15,000 cpm in 5 ng of DNA) and increasing concentrations of competitor DNA (20–200 μg of poly dIdC) were added in the dialysis bag, and binding was carried out for 4 h at room temperature. After the binding reaction, lamin aggregates were sedimented in a microcentrifuge for 30 min at 10,000*g*. Radioactive DNA fragments in the pellet and supernatant were purified, size separated on a 1% agarose gel, and visualized by autoradiography.

SIDD ANALYSIS

S/MAR prediction was performed by the SIDD algorithm, a powerful predictive tool for localizing in genomic sequences sites with a secondary structure-forming potential. The sites in question are base-unpairing regions (BURs), the hallmark of S/MARs. SIDD profiles were calculated according to the algorithm developed by Benham et al. [1997]. Essentially, the SIDD algorithm predicts the propensity of a DNA base pair to undergo strand separation under superhelical tension. The incremental free energy [G(x)] needed to separate the base pair at each position (x) is computed in the context of a defined stretch of DNA. A value of G(x) near or below zero indicates an essentially completely destabilized base pair with a high probability to denature at equilibrium. SIDD profiles, that is, plots of G(x) versus (x), visualize regions of the sequence where superhelical stress will destabilize the duplex. The WebSIDD [available on web page: www.genomecenter.ucdavis.edu/benham/sidd, Benham and Bi, 2004] algorithm was used to search for potential S/MAR sequences in the rat haptoglobin gene 5′-flanking region (−1031/+60 bp). The sequence for analysis was obtained from the Gene bank database (NCBI Sequence Viewer, Access No.: NW 047536).

THE ANALYZED NUCLEOTIDE SEQUENCE OF THE 5′ END OF THE HAPTOGLOBIN GENE

The 5′ end (−1031/+60 bp) of the haptoglobin gene [Marinković and Baumann, 1990] used for the computer-based SIDD analysis for the potential S/MARs is shown. Bold underlined letters indicate the predicted S/MAR element (−599/−200 bp), the *cis*-element (−165/−56 bp) is plain underlined, and the polyA tract is double underlined.

DENSITOMETRY

Quantification of protein bands was performed with TotalLab (Phoretix) electrophoresis software (v. 1.10). Quantification was performed from bands obtained from at least three separate experiments. The values are expressed as a percent change ± SEM with respect to the "control" band corresponding to the sample chosen to be 100% in the same experiment, as will be indicated.

RESULTS

S/MAR IDENTIFICATION IN THE 5′ REGION OF THE HAPTOGLOBIN GENE

A close approximation of native nuclear matrix–DNA interactions can be obtained in the nuclear matrix prepared in the presence of polyamines and metal chelators, followed by DNA digestion with restriction nucleases (as described in the Materials and Methods Section). Southern dot-blot analysis of the isolated nuclear matrix-associated DNA with haptoglobin cDNA and the 5′ region (−705/+159 bp) of the haptoglobin gene (Fig. 1A) revealed that during intensive haptoglobin gene transcription, the isolated nuclear matrix was 4.41 ± 0.2-fold enriched in DNA sequences contained in the 5′ region of the haptoglobin gene rather than its coding sequence. This result pointed to the existence of a S/MAR or a S/MAR-like sequence in the 5′ region of the haptoglobin gene. Using SIDD analysis we searched for a potential S/MAR element in the extended 5′ region (−1031/+60 bp) of the haptoglobin gene. The region included a part of the coding sequence that was preceded by the haptoglobin gene *cis*-element (−165/−56 bp) and a 28 bp A-box further upstream (−314/−342 bp) that establishes dynamic associations with the A/C-type lamins [Poznanović et al., 1994]. SIDD analysis revealed the presence of a potential S/MAR element as an upstream extension of the *cis*-element (Fig. 1B). The S/MAR was recognized as an unpairing element (UE) encompassing one dominant G(x) minimum at −599/−200 bp. SIDD profiles can also assist in a predictive functional classification of S/MARs [Winkelmann et al., 2006]. Further inspection of the Hp-S/MAR region revealed the presence of other sequences also found in S/MARs, such

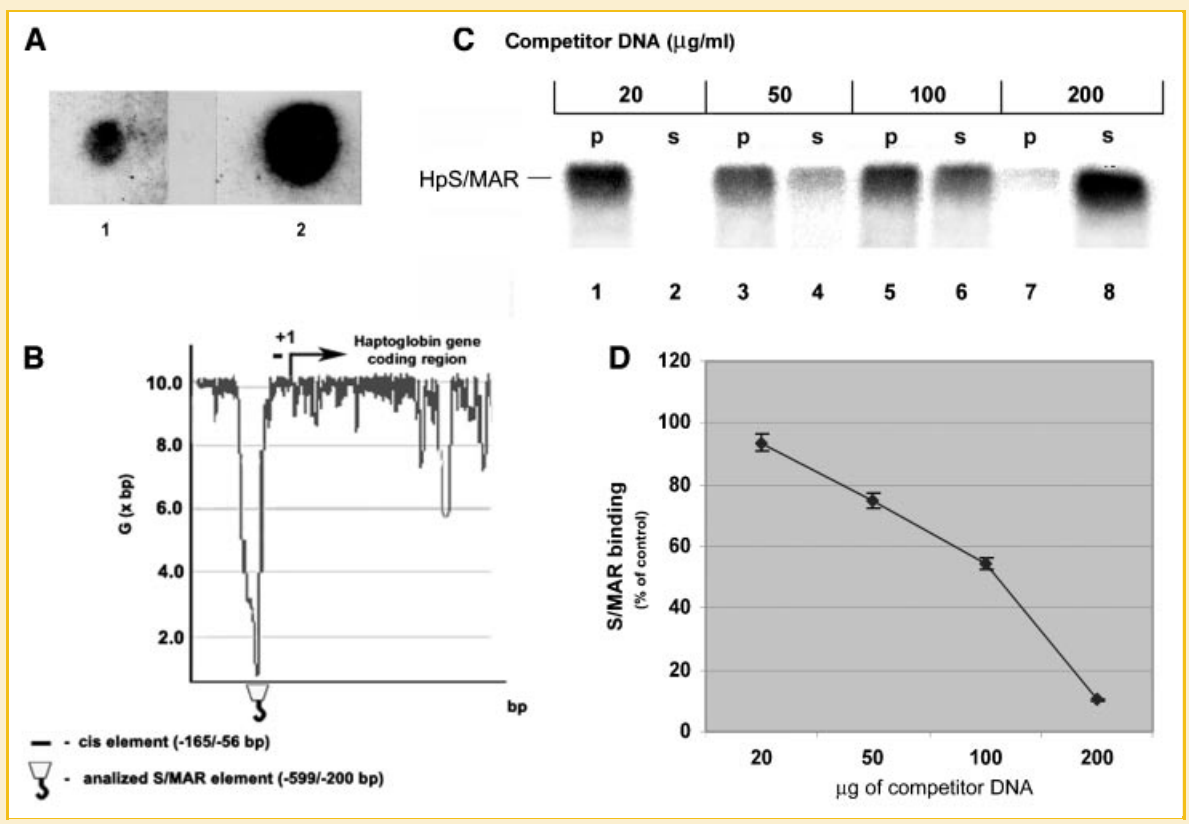

Fig. 1. Prediction and characterization of the S/MAR element (Hp-S/MAR) close to the haptoglobin gene *cis*-element. A: Assessment of the preferential, in vivo association of the 5′ region of the haptoglobin gene with the nuclear matrix. Southern dot-blot analysis with haptoglobin cDNA (1) and the 5′ region (−705/+159 bp) of the haptoglobin gene (2) of the nuclear matrix-associated DNA prepared from nuclei isolated in the presence of poyamines-metal chelators and subjected to digestion with restriction endonucleases. B: SIDD profile of the Hp-S/MAR, that is, plots of G(x) versus x which visualize the region in the sequence where superhelical stress destabilizes the duplex. G(x) — the free energy needed to separate the base pair at each position x. C: DNA gel electrophoresis of the samples obtained after the S/MAR-binding procedure performed with the L-EP1 fraction induced to aggregate after the removal of urea by dialysis in the presence of the radioactively labeled Hp-S/MAR DNA (50 ng) and increasing concentrations of competitor DNA (20–200 µg poly dIdC/ml). After binding, the lamin aggregates were sedimented and the radioactive DNA in the pellet (p) and supernatant (s) was purified, size separated on 1% agarose gels by electrophoresis, and visualized by autoradiography. D: Specific Hp-S/MAR binding to L-EP1 was quantified according to the operational definition that S/MARs possess ≥50% protein-binding activity in the presence of 10^3-fold higher amounts of competitor DNA (i.e., 50 µg poly dIdC/ml). The protein-bound radioactivity was measured in a scintillation counter and expressed as S/MAR binding, that is, the percentage of bound cpm relative to the input radioactivity (100%).

as AT-rich territories that characterize the origin of replication (ATTA, ATTTA) and TG-rich fragments generally associated with copies of TG, CA, or TA that are responsible for DNA kinking [McNamara et al., 1990; Boulikas, 1993].

In order to obtain experimental proof that the identified potential S/MAR region possesses the hallmark of S/MAR elements, that is, a pronounced affinity for binding to the nuclear matrix or its essential protein components, the lamins, we performed well-established S/MAR-binding analysis [Ludérus et al., 1994]. Using this procedure, the authors showed that interactions of the nuclear lamina with S/MARs are specific and saturable as well as evolutionarily conserved. We prepared the peripheral nuclear lamina Stuurman et al. [1990] and purified it by two solubilization cycles with Triton X-100 at different pH values [Aebi et al., 1986; Ludérus et al., 1994]. The obtained protein fraction, referred to as L-EP1, mostly consisted of the A- and B-type lamins as it was depleted of lamina-associated proteins (Fig. 2A, lane 2). S/MAR-binding analysis of the predicted S/MAR was performed as follows: the protein constituents of L-EP1 were completely solubilized in a buffer containing 500 mM KCl and 6 M urea after which they were dialyzed against a low ionic strength buffer without urea which induced the insoluble hydrophobic lamins to polymerize. The reassociated lamina polymers were incubated with 50 ng/ml of the radioactively end-labeled predicted S/MAR restriction fragments in the presence of increasing amounts of poly(dIdC) competitor DNA. The amount of competitor DNA required to displace the S/MAR fragment from the lamina aggregates served as a measure of the relative strength of the interaction [Ludérus et al., 1994]. The lamina-bound predicted S/MAR DNA was separated from the unbound DNA by centrifugation and analyzed by electrophoresis and radiography (Fig. 1C). Specific Hp-S/MAR binding to L-EP1 was quantified according to the operational definition that S/MARs possess $\geq$50% protein-binding activity in the presence of 10^3-fold higher amounts of competitor DNA (i.e., 50 μg poly dIdC/ml). The S/MAR-binding analysis revealed the binding of almost 75% and 55% of the total S/MAR-contained input radioactivity (Fig. 1D) to the repolymerized lamina proteins in the presence of 50 and 100 μg/ml competitor DNA, respectively. These results provided experimental support for the SIDD data, and it was concluded that the analyzed sequence (−599/−200 bp) was a S/MAR and will henceforth be referred to as Hp-S/MAR.

EXAMINATION OF INTERACTIONS BETWEEN NUCLEAR MATRIX AND ASSOCIATED PROTEINS BY COLOCALIZATION EXPERIMENTS

Our working hypothesis was based on the assumption that protein–DNA interactions between Hp-S/MAR and proteins associated with

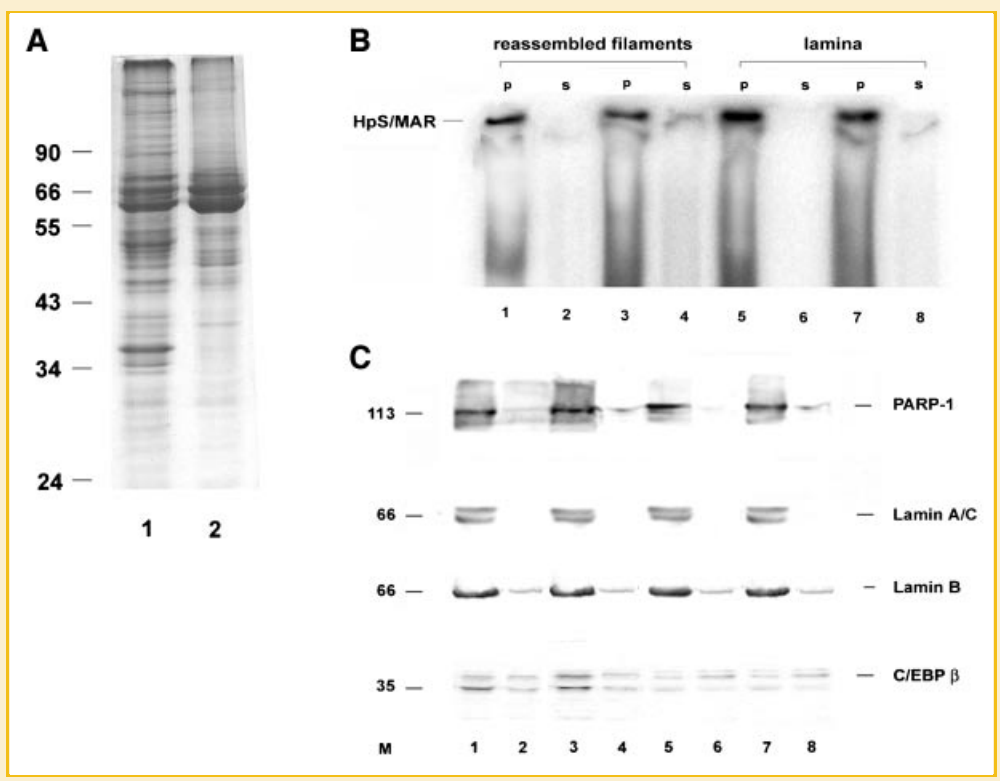

Fig. 2. Assessment of protein–protein and protein–DNA interactions of nuclear matrix and associated proteins by colocalization experiments. A: Coomassie-stained profile of L-EP1 (lane 2) and L-EP2 (lane 1) protein fractions. B: DNA gel electrophoresis after the S/MAR-binding procedure. Proteins (L-EP2 fraction: lanes 1–4; L-EP1 fraction: lanes 5–8) aggregated after urea removal by dialysis in the presence of the radioactively labeled Hp-S/MAR DNA (50 ng) and competitor DNA (50 μg poly dIdC/ml). After binding, lamin aggregates were sedimented and radioactive DNA present in the pellet (p) and supernatant (s) was purified, size separated by in 1% agarose gels by electrophoresis, and visualized by autoradiography. The position of S/MAR element is indicated. C: In a parallel S/MAR-binding procedure, L-EP2 (lanes 1–4) and L-EP1 (lanes 5–8) aggregates were sedimented and the proteins in the pellet (lanes 1, 3, 5, and 7) and supernatant (lanes 2, 4, 6, and 8) were subjected to Western blot analysis with anti-CEBPβ, anti-lamin A/C and B, and anti-PARP-1 antibodies. Control samples: lanes 1, 2, 5, and 6; AP samples: lanes 3, 4, 7, and 8.

the peripheral nuclear lamina part of the nuclear matrix reflect the involvement of the individual molecular constituents of the assembled complex in fine-tuning haptoglobin gene transcription. The presence of the A-box that establishes dynamic associations with the A-type lamins in the identified Hp-S/MAR lent additional support to the supposition that the lamins, as S/MAR-binding proteins, assume the role of moderators of haptoglobin gene expression. We next examined dynamic interactions between three different classes of nuclear matrix-related proteins: (i) the lamins, (ii) the nuclear matrix-associated component of the liver-enriched transcription factor C/EBPβ that participates in the regulation of haptoglobin gene transcription, and (iii) PARP-1, which in its non-modified state directly interacts with lamin B from where it is recruited upon demand as a result of appropriate modifications.

In order to characterize the protein–protein interactions of the lamin-based structural backbone of the peripheral nuclear lamina/nuclear matrix, parallel colocalization experiments were performed with the L-EP1 and an additional lamin-enriched protein fraction L-EP2. Fraction L-EP2 was obtained by a procedure [Zackroff et al., 1982] based on nuclear matrix fractionation by solubilization in urea and subsequent dialysis under low ionic strength in the presence of β-mercaptoethanol. Dialysis restores the native conformation of proteins, thus promoting the reassembly of lamin filaments and the reassociation of non-lamin proteins that possess the capability of reassembling into macromolecular complexes by virtue of their high affinity to the lamins and/or an inherently high hydrophobicity [Gerner et al., 1999]. Compared to L-EP1, the L-EP2 fraction was enriched in non-lamin proteins (Fig. 2A, lane 1). Colocalization experiments were performed by the complete solubilization of the protein constituents of L-EP1 and L-EP2 in a buffer that contained 500 mM KCl and 6 M urea, followed by dialysis against low ionic strength buffer without urea. Dialysis was accompanied by the quantitative reassociation of the lamins into an insoluble precipitate. After dialysis, the reassembled proteins were incubated with Hp-S/MAR DNA in the presence of 20 ng/μl of poly(dIdC) competitor DNA. The DNA and the proteins present in the pellets and supernatants were examined by DNA electrophoresis/autoradiography (Fig. 2B) and Western blot analysis (Fig. 2C), respectively.

The Hp-S/MAR was quantitatively retained in both the L-EP1- and L-EP2-derived reassembled precipitates (Fig. 2B; lanes 1, 3, 5, and 7). The quantitative association of the Hp-S/MAR with reformed A- and B-type lamin polymers in the L-EP2 sample (Fig. 2B, lanes 1 and 3) provided additional support for the conclusion drawn from the S/MAR-binding experiment above. Western blot analysis of the lamin-associated proteins revealed the quantitative precipitation of the A-type lamins (Fig. 2C, lanes 1, 3, 5, and 7) and a nearly quantitative precipitation of lamin B and PARP-1 (Fig. 2C, lanes 1, 3, 5, and 7). The latter finding reflects the association of PARP-1 and lamin B [Vidaković et al., 2004, 2005]. All precipitates obtained from the L-EP1 (Fig. 2C, lanes 5 and 7) and L-EP2 (Fig. 2C, lanes 1 and 3) samples contained PARP-1. Considering that transcription factor C/EBPβ establishes dynamic associations with the nuclear matrix [Dinić et al., 2000], the membrane was probed with anti-C/EBPβ antibody. C/EBPβ is usually represented by 32, 35, and 38 kDa isoforms. Both L-EP1 and L-EP2 contained the 35 and 38 kDa C/EBPβ isoforms (Fig. 2C). The largest portion of the C/EBPβ present in L-EP2 was found in the precipitates obtained after dialysis/centrifugation (Fig. 2C, lanes 1 and 3). The precipitate obtained from the sample at 24 h of the AP response (lane 3) was enriched by 97 ± 4% with the 38 kDa C/EBPβ isoform compared to the control sample (lane 1), whereas the 35 kDa isoform remained unchanged. Smaller amounts of C/EBPβ were found in the reassembled fractions (pellets) that were obtained from the L-EP1 samples (Fig. 2C, lanes 5 and 7). This result reflects the depletion of the L-EP1 fraction with non-lamin proteins when compared to L-EP2. Overall, a seemingly more balanced partitioning of C/EBPβ isoforms into soluble (Fig. 2C, lanes 6 and 8) and insoluble, reassembling species (lanes 5 and 7) was observed in the L-EP1-derived sample.

The nearly quantitative partitioning of PARP-1 with both lamin-enriched protein fractions underlines the importance of hydrophobic bonds in PARP-1–lamin interaction. Comparing the results of colocalization experiments with the respective lamin-enriched protein fractions obtained by similar procedures showed that C/EBPβ–lamin interactions rely on hydrophobic and other types of intermolecular forces of attraction. For the L-EP1 fraction, the peripheral nuclear lamina was isolated under conditions of continuous iodoacetate alkylation of protein sulfhydryl groups, followed by a step-wise solubilization with the non-ionic detergent Triton X-100. Triton forms micelles which trap and solubilize hydrophobic proteins [Bordier, 1981]. Relative to L-EP2, L-EP1 was depleted in C/EBPβ but not in PARP-1. In contrast, L-EP2 was prepared from the nuclear matrix that was isolated under normal oxidizing conditions by atmospheric O_2 and was subsequently solubilized with the chaotropic reagent urea which represents a more rigorous treatment. Whereas urea induced complete protein denaturation, resulting in an open and non-functional protein structure [Bennion and Daggett, 2003], the Triton-solubilization preserved, to some extent, the in vivo interactions since the non-denaturing solubilization allowed the proteins to retain parts of their native state. As a result, adventitious associations were reduced [Aebi et al., 1986]. The colocalization experiments show that the reassembled nuclear protein complexes between the lamins, the examined lamin-associated proteins, and the HpS/MAR did not exhibit quantitative differences between the basal state and AP response.

IDENTIFICATION OF Hp-S/MAR-BINDING PROTEINS

Using South-Western blot analysis we screened the nuclear lamina proteins for interaction with the Hp-S/MAR. To that end, the peripheral nuclear lamina was isolated according to the procedure described by Stuurman et al. [1990] during basal transcription of the haptoglobin gene and in the course of the AP response induced by turpentine injection. Haptoglobin synthesis undergoes temporal regulation as a result of a complex endocrine interplay during the AP response. Between 2 and 4 h after induction of the AP response the corticosterone concentration peaks [Ivanović-Matić and Poznanović, 1996]. During this initial stage of the AP response, glucocorticoids act synergistically with cytokines. As a result, at about 12 h after induction of the AP response the rate of haptoglobin gene transcription begins to

increase, at about 24 h the maximal level of transcription is established [Ševaljević et al., 1988, 1995] and is maintained up to about 48 h after induction of the AP response [reviewed in Poznanović et al., 1997]. In the basal state, DNA binding by distinct 40, 45, 48, 50, 55, 60–68, and 116 kDa (Fig. 3A, lane 1) nuclear lamina-associated proteins was visualized. At 4 h of the AP response, increased DNA binding by 55, 60–68, and 116 kDa proteins was observed (Fig. 3A, lane 2). After this initial increase, at 24 h of the AP response only a 55 kDa protein exhibited a further relative increase in Hp-S/MAR binding whereas the 60–68 and 116 kDa proteins displayed the same increased DNA binding compared to the basal state. Western blot analysis of the stripped South-Western blots (Fig. 3B) was undertaken to tentatively identify potential DNA-binding proteins. Western analysis revealed the enrichment by 21 ± 2.3% of the peripheral nuclear lamina with the 113 kDa full-length PARP-1 at 4 h of the AP response (Fig. 3B, lane 2). As the AP response progressed, the amount of nuclear lamina-associated PARP-1 returned to the control level (lane 3). The proteins in the 60–68 kDa region were identified by Western analysis as the major nuclear lamina/nuclear matrix proteins, that is, the A- and B-type lamins (lamin A 68 kDa; lamin C, 62 kDa; lamin B, 66 kDa). Despite increased Hp-S/MAR binding at 4 and 24 h, the relative abundance of lamin B remained unchanged during the AP response (Fig. 3B, lanes 1–3) which is in agreement with its constitutive, unchanging level of expression in the adult. On the other hand, the A-type lamins, notably lamin C, displayed increased relative concentrations, in agreement with their more dynamic expression patterns [Moir and Goldman, 1993]. Quantification revealed that lamin A was increased by 28 ± 3.7% in both 4 and 24 h AP samples (Fig. 3B, lanes 2 and 3, respectively). In comparison with lamin A, lamin C underwent a disproportionate increase. Thus, at 4 and 24 h of the AP response the levels of lamin C were, respectively, 70 ± 4.2% (lane 2) and 67 ± 3.7% (lane 3) higher than in the control. This overall enrichment with the A/C lamins reflects the previously reported significant enrichment of nuclei and nuclear matrices with protein, RNA, and DNA isolated from rat liver during the AP response [Ševaljević et al., 1984] as a result of a dramatic activation of gene transcription and increase in nuclear ploidy of liver parenchymal cells [Ševaljević et al., 1983]. These results revealed an increased association of PARP-1 with the peripheral nuclear lamina and its enrichment with lamins A and C during the initial stage of the AP response at 4 h after turpentine administration. This was followed by its continuous enrichment with lamin A and notably lamin C throughout the AP response. The results suggest that increased binding of PARP-1 and of the A/C- and B-type lamins to the Hp-S/MAR characterized the initial stage of the AP response and preceded the establishment of increased AP protein gene transcription at 24 h (Fig. 3B, lane 3).

The stripped South-Western membrane was also probed with anti-C/EBPβ antibody. C/EBPβ is represented by 32, 35, and 38 kDa isoforms that were observed on the Western blots (Fig. 3B). In contrast to PARP-1, the development of the AP response was accompanied by a steadily increasing association of the 35 kDa C/EBPβ isoform with the peripheral lamina, that is, 240 ± 19% and 500 ± 31% increases at 4 and 24 h, respectively. No C/EBPβ-Hp-S/MAR binding (Fig. 3A) was observed. Increased AP protein gene transcription at 24 h was also accompanied by increased Hp-S/MAR binding to the 55 kDa protein (Fig. 3A, lane 3). It should be noted

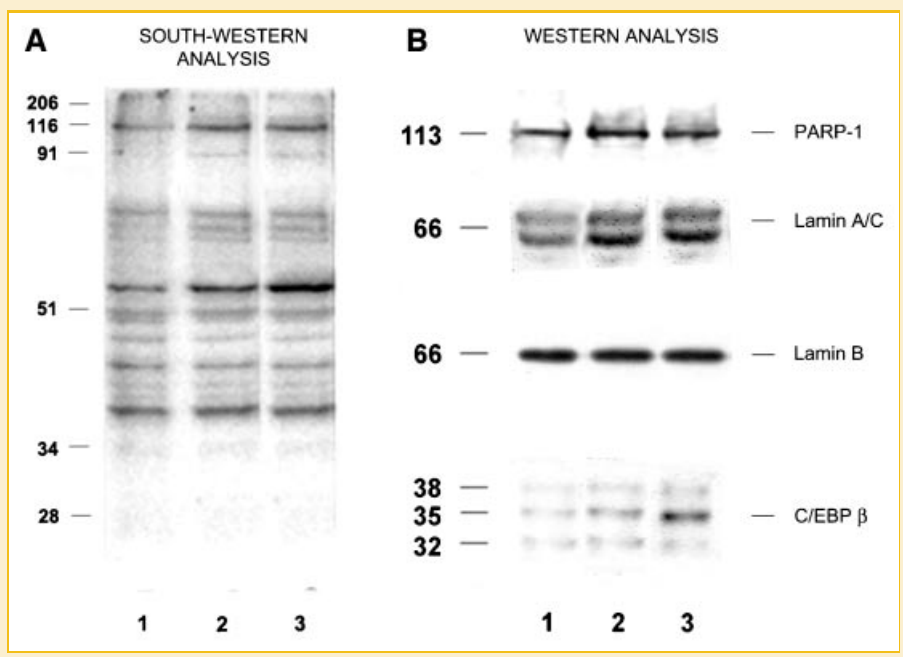

Fig. 3. South-Western blot analysis of nuclear lamina proteins exhibiting binding affinity for the Hp-S/MAR. A: Twenty micrograms of the nuclear lamina proteins was separated by SDS–PAGE, electroblotted, and probed with the ^{32}P-labeled Hp-S/MAR (−541/−146 bp) in the presence of 20 μg of salmon sperm DNA as a competitor (lanes 1–3). B: After autoradiography, the filters were stripped and subjected to Western blot analysis with anti-CEBPβ, anti-lamins A/C and B, and anti-PARP-1 antibodies (lanes 1–3). Lane 1: control nuclear lamina; lane 2: 4 h AP response nuclear lamina; lane 3: 24 h AP response nuclear lamina.

that the 55 kDa protein is a DNA-binding protein with limited sequence specificity [Poznanović et al., 1999] and that it translocates to the nuclear matrix during the AP response [Poznanović et al., 1994].

Potential lamin protein–Hp-S/MAR interactions were examined further under milder conditions that more closely resemble the in vivo state by EMSA, using a SNMP that was obtained by solubilization of the nuclear matrix with urea [Zackroff et al., 1982]. The resulting fraction, although partially lamin-depleted, contains lamin hetero- and homodimers [Aebi et al., 1986]. SNMP fractions were prepared from control rats during basal transcription of the haptoglobin gene (C-SNMP) and rats 24 h after induction of the AP response (AP-SNMP) by turpentine injection. Prior to EMSA, the SNMPs were screened for the presence of solubilized A-type lamins, C/EBPβ isoforms (32, 35, and 38 kDa) and PARP-1 (113 kDa) by Western blot analysis. Compared to C-SNMP (Fig. 4A, lane 3), the AP-SNMP sample (Fig. 4A, lane 4) had 78 ± 6.42%, 192 ± 12.9%, and 162 ± 14.6% higher relative concentrations of 32, 35, and 38 kDa C/EBPβ isoforms, respectively, and slightly elevated concentrations of PARP-1 and the A-type lamins.

After EMSA, the C-SNMP and AP-SNMP fractions formed three different nucleoprotein complexes with the Hp-S/MAR DNA, as judged by their positions in a polyacrylamide gel (Fig. 4B). The nucleoprotein complexes designated as I_C and I_{AP} had the same molecular mass in C- and AP-SNMP, respectively. C-SNMP also generated complex IIc with a high mass (Fig. 4B, lane 2), and AP-SNMP generated complex II_{AP} with a higher mass (Fig. 4B, lane 3). Assessment of the protein components of the nucleoprotein complexes by re-electrophoresis revealed, at the level of detection allowed by the procedure, that complex I_C (Fig. 4C, lane 2) and

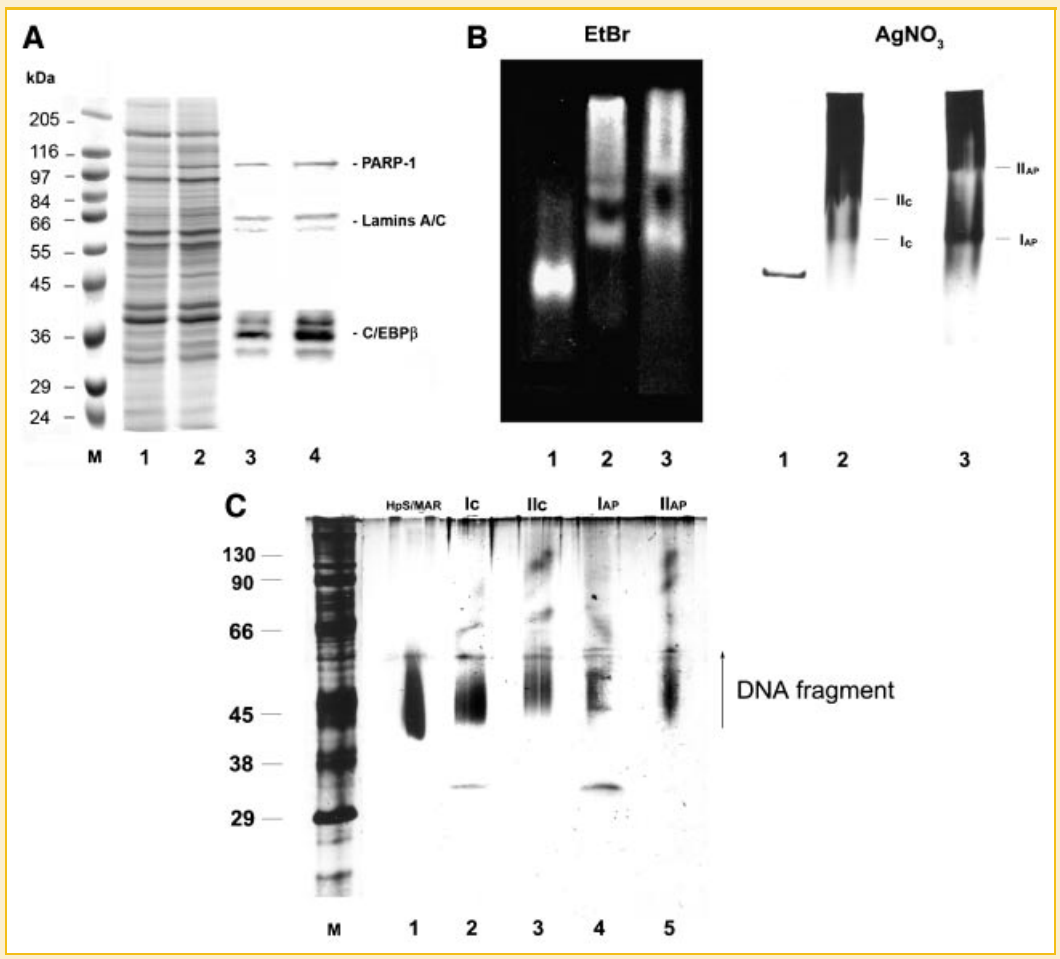

Fig. 4. Protein–Hp-S/MAR interactions examined under conditions that resemble the in vivo state. A: Profiles of SNMPs after SDS–PAGE (lanes 1 and 2) and Western blot analysis (lanes 3 and 4) with antibodies to PARP-1, lamins A/C, and C/EBPβ. Lanes 1 and 3: control SNMP; lanes 2 and 4: 24 h AP response SNMP. B: Detection of nucleoprotein complexes between Hp-S/MAR and SNMP using EMSA. SNMPs (5 μg) were incubated with 100 ng of purified Hp-S/MAR (−541/−146 bp) in the presence of 2.5 μg of linearized vector pUC13 as competitor. The gels were stained with ethidium bromide (5 μg/ml) and silver as indicated. Lane 1: position of the free Hp-S/MAR under non-denaturing conditions (395 bp). Roman numerals indicate the positions of the formed nucleoprotein complexes: I_C and II_C (lanes 2) in the control sample (C-SNMP) and I_{AP} and II_{AP} (lane 3) in the AP response sample (AP-SNMP). C: Separation and identification of the proteins from the nucleoprotein complexes I and II according to their molecular masses by SDS–PAGE, referred to as re-electrophoresis (see Materials and Methods Section). Lane 1: free Hp-S/MAR DNA without the addition of proteins. Lane 2: the components of complexes I_C after re-electrophoresis; lane 3: re-electrophoresis of complexes II_C obtained after EMSA with C-SNMP; lane 4: re-electrophoresis of complex I_{AP}; lane 5: re-electrophoresis of complex II_{AP}, both obtained after EMSA with AP-SNMP.

complex I_{AP} (Fig. 4C, lane 4) consisted of the Hp-S/MAR (located after silver staining between 45 and 66 kDa markers; see Fig. 4C, lane 1), a 35 kDa protein and a relatively abundant protein(s) with a median mass of about 66 kDa. Complex II_C consisted of the DNA, a 66 kDa protein, and a protein with a median mass value of about 120 kDa (Fig. 4C, lane 3). Re-electrophoresis of complex II_{AP} revealed its greater complexity. It was resolved into the 66 and 120 kDa proteins and a protein of about 90 kDa (Fig. 4C, lane 5). These results show that different transcriptional activities of the haptoglobin gene were accompanied by specific DNA–protein interactions between the Hp-S/MAR sequence and the nuclear lamina and associated proteins. Based on the same estimated masses after re-electrophoresis and Western blot analysis (Fig. 4A, lanes 2 and 4), we assumed that the 35 and 120 kDa Hp-S/MAR element-binding proteins were C/EBPβ and PARP-1, respectively, and the protein with a median mass value of about 66 kDa lamin. Since the lamins which are highly insoluble proteins are present in the SNMP fraction in the soluble, dimerized form, these results provide additional evidence that the formation of multiprotein complexes with Hp-S/MAR DNA is not the result of non-specific protein aggregation on lamin polymers. The presence of lamin protein(s) in every complex indicates that they possess a very pronounced Hp-S/MAR-binding affinity. This finding points to the possibility that protein–protein interactions with the lamin(s) were responsible for the association of the other protein(s) in the nucleoprotein complexes obtained after EMSA rather than their direct interaction with the Hp-S/MAR, although, we cannot rule out PARP-1 affinity towards the Hp-S/MAR that has been reported by several laboratories [Sastry and Kun, 1990; Galande and Kohwi-Shigematsu, 1999; Soldatenkov et al., 2002]. This result also stresses that of the three C/EBPβ isoforms, the 35 kDa protein has a major influence on haptoglobin gene transcription regulation since it is more abundant in the AP sample after re-electrophoresis (Fig. 4C, lane 4).

CHARACTERIZATION OF PROTEIN–PROTEIN INTERACTIONS

The colocalization of the lamins, PARP-1, the 35 kDa C/EBPβ isoform, and the Hp-S/MAR-containing DNA suggests that these molecules assemble higher order complexes by means of mutual dynamic molecular interactions through: (i) protein–DNA interactions of the Hp-S/MAR with the lamins and PARP-1 and (ii) protein–protein interactions between the proteins of the nuclear matrix backbone, the lamins, PARP-1, and gene regulatory proteins, the 35 kDa C/EBPβ isoform. Next we examined whether protein–protein contacts that do not involve the lamins also influenced the associations of the macromolecular components in close proximity to the upstream Hp-S/MAR. The potential of PARP-1 and C/EBPβ to establish protein–protein interactions was examined by coimmunoprecipitation experiments. To that end, nuclear extracts were prepared from control rats and rats at 24 h of the AP response. Protein precipitation was induced by the addition of C/EBPβ antibody (Fig. 5). Screening of the precipitate with a PARP-1 antibody revealed the presence of a potential C/EBPβ–PARP-1 association during basal transcription (Fig. 5, lane 2). However, at 24 h of the AP response PARP-1 was not present in the precipitated

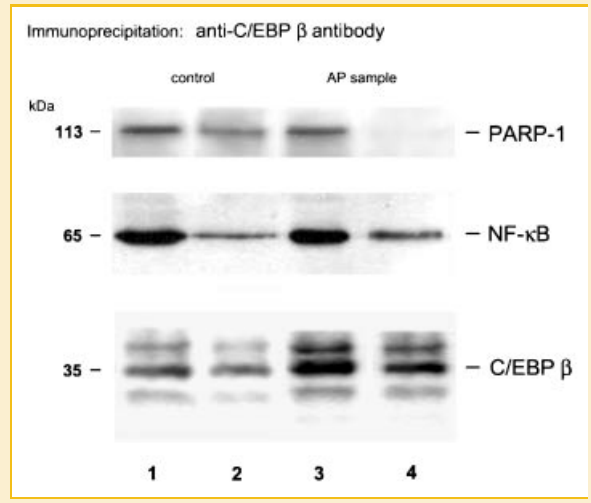

Fig. 5. Coimmunoprecipitation of C/EBPβ and PARP-1. Immunoprecipitation was performed with nuclear extracts isolated from control (lanes 1 and 2) and AP response (24 h) samples (lanes 3 and 4) using anti-C/EBPβ antibody. Lane 1: input of the control sample; lane 2: control sample after immunoprecipitation with C/EBPβ antibody; lane 3: input of the AP sample; lane 4: AP sample after immunoprecipitation with C/EBPβ antibody. The obtained precipitates were probed with anti-C/EBPβ, -PARP-1, and -NF-κB (p65) antibodies. NF-κB as a pronounced C/EBPβ-binding partner was used as a positive control.

protein complex (Fig. 5, lane 4), despite the slightly increased relative concentration of C/EBPβ in the AP nuclear extract (Fig. 5, lane 3) compared to the control (Fig. 5, lane 1). Since cooperative association of C/EBPβ and NF-κB results in the synergistic transcriptional activation of the major AP reactant SAA2 [Schrem et al., 2004], we performed a control experiment in which the precipitate was screened with an antibody to the p65-subunit of NF-κB transcription factor. Association of the p65-subunit with C/EBPβ was observed in the basal state (Fig. 5, lane 2). In contrast to the loss of C/EBPβ–PARP-1 contact, increased AP gene transcription was not accompanied by the release of the p65-subunit from its association with C/EBPβ (Fig. 5, lane 4). These results lend support to the conclusion that the association of C/EBPβ with PARP-1 changed during the AP response. The physical dissociation of PARP-1–C/EBPβ contacts is a contributing factor towards the observed increased DNA-binding activity of C/EBPβ for the *cis*-element that promotes increased AP gene transcription.

PARP-1 ENZYMATIC ACTIVITY

Given that PARP-1 and C/EBPβ establish dynamic interactions, changes in the enzymatic activity of PARP-1 and/or post-translational modifications of C/EBPβ by the addition of PAR residues could potentially affect the activity of C/EBPβ in the transcriptional regulation of the haptoglobin gene. We examined the changes in endogenous PARP-1 enzymatic activity in the isolated nuclear matrices (Fig. 6A). Total basal PARP-1 activity did not change in the control and nuclear matrix isolated during the AP response. In order to visualize the proteins post-translationally modified by the addition of PAR at basal PARP-1 enzymatic activity, Western blot analysis with an anti-PAR antibody was performed (Fig. 6B). Nuclear matrix

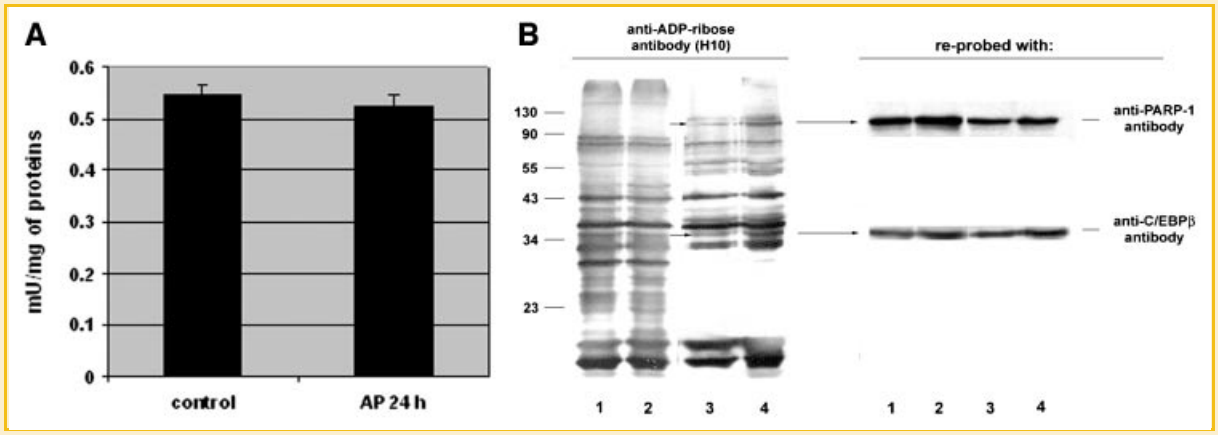

Fig. 6. PARP-1 activity 24 h after induction of the AP response. A: The PARP activity assay was performed with control nuclear matrix samples and samples isolated 24 h after AP response induction. The values of PARP activity are averages from at least three different experiments. B: Western blot analysis of the nuclear matrix and nuclear extracts with anti-PAR, anti-PARP-1, and anti-C/EBPβ antibodies. Lane 1: control nuclear matrix proteins; lane 3: control proteins of the nuclear extract; lane 2: AP response nuclear matrix; lane 4: AP response nuclear extract.

proteins (lanes 1 and 2) and proteins of the nuclear extract that represents a pool of nuclear transcription factors (lanes 3 and 4) were isolated from control rats (lanes 1 and 3) and rats at 24 h after induction of the AP response (lanes 2 and 4), electrophoretically separated and probed with an anti-PAR antibody. Western blot analysis revealed that under basal conditions and during elevated transcriptional activity of liver cells, several well-defined nuclear proteins were PARylated (Fig. 6B). The nuclear matrices (lanes 1 and 2) were enriched with PAR proteins compared to the respective nuclear extracts (lanes 3 and 4). While no qualitative differences in composition of PARylated proteins were observed in either the control or AP samples within the same protein fraction, a marked difference in composition of PARylated proteins was observed between the nuclear matrix and nuclear extract. Whereas the nuclear matrix possessed PARylated proteins in the range from about 10 to 90 kDa and above 150 kDa, the nuclear extract was characterized by the apparently complete absence of PARylated proteins in the 15–30 and >130 kDa regions. Stripping and reprobing the membranes with anti-PARP-1 and anti-C/EBPβ antibodies (Fig. 6B) did not provide evidence for PARP-1 automodification in the nuclear matrix (lanes 1 and 2). Only the most abundant, major 35 kDa C/EBPβ isoform in the nuclear matrices and nuclear extracts appeared to be modified (Fig. 6B). However, since the abundance of the nuclear matrix-associated 35 kDa C/EBPβ isoform is increased during the AP response, it is difficult to distinguish between increased protein content and increased protein PARylation. Overlaying the Western blots showed that the 32 and 38 kDa isoforms were not modified. These results point to the possible control of the interaction between PARP-1 and C/EBPβ by the post-translational PARylation of the 35 kDa C/EBPβ isoform.

PARP-1 BINDING TO THE HAPTOGLOBIN *cis*-ELEMENT

It has been extensively documented that C/EBPβ is an essential haptoglobin gene regulatory protein that interacts with the haptoglobin gene *cis*-element, maintaining both basal and elevated transcription during the AP response. So far our experiments show a direct interaction of C/EBPβ with PARP-1 in the basal state, and point to a possible interaction of Hp-S/MAR with PARP-1 but not with C/EBPβ (Fig. 3). Next we examined whether PARP-1 was present in the protein fraction eluted from a haptoglobin gene *cis*-element coupled DNA affinity column. Western blot analysis of the eluted fraction revealed the presence of PARP-1 in both control and AP samples after elution with 1 M KCl (Fig. 7, lanes 2 and 4, respectively). This result suggests that PARP-1 participates in constitutive and elevated haptoglobin gene transcription. It remains to be examined whether this is achieved through a direct interaction of PARP-1 with the haptoglobin *cis*-element or through PARP-1 association with DNA-interacting regulatory proteins.

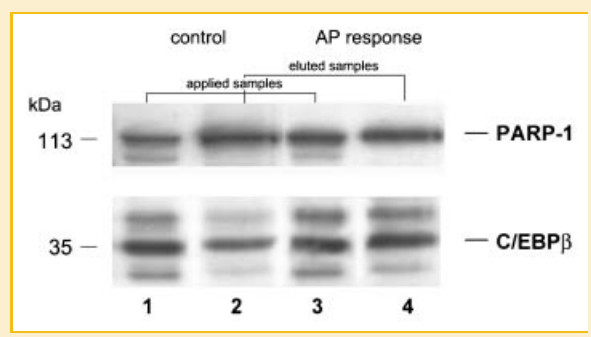

Fig. 7. Examination of PARP-1-binding affinity for the haptoglobin gene *cis*-element. SNMPs isolated from control (lane 1) and rat livers 24 h after the onset of the AP response (lane 3) were applied to a DNA-affinity column coupled with rat haptoglobin gene *cis*-element (−165/−56 bp). The *cis*-element bound proteins were eluted with 1 M KCl (lane 2: control; lane 4: AP response samples) and probed with anti-C/EBPβ and anti-PARP-1 antibodies.

DISCUSSION

Much of the research on transcriptional gene regulation has been devoted to investigations of *cis–trans* interactions between DNA regulatory elements lying immediately adjacent to the transcription start site and specific transcription factors. However, there are aspects of differential gene transcription that involve the activation or repression of chromatin domains through interactions between proteins and DNA regions residing further upstream from the core promoter [Klar and Bode, 2005]. The additional S/MAR–protein interactions that punctuate the domains probably represent further control points that could fine-tune the expression of a given gene. As most S/MAR-binding proteins exist in corepressor/coactivator complexes that bring about gene regulation, they represent essential components of regular cellular functions as well as epigenetic processes.

We examined the functional interaction of the nuclear matrix with the rat haptoglobin gene 5′ upstream region in the course of the AP response-induced increase in transcription. Using computer SIDD analysis of the 5′ haptoglobin gene region (−1031/+60 bp) we identified a potential S/MAR element (−599/−200 bp) essentially adjacent to the *cis*-regulatory element (−165/−56 bp). The in silico result was subsequently confirmed by S/MAR-binding experiments. According to the SIDD profile, the Hp-S/MAR resembles a functional type of S/MAR. Taking into account its proximity to the haptoglobin gene promoter and literature data, we assumed that the Hp-S/MAR is involved in the regulation of haptoglobin gene transcription. In their study of the selective anchoring of chromatin loops by S/MARs, Heng et al. [2004] proposed that, aside from structural S/MAR elements that form stable interactions with nuclear matrix proteins and delineate loop domains, S/MAR elements within the domain form facultative functional interactions with the nuclear matrix during transcription. The latter type of dynamic S/MAR elements mediates between genes and the nuclear matrix-associated molecular assemblies of the transcriptional machinery. As the gene forms an association with the nuclear matrix, transcription is initiated and the transcript is elongated as the gene spools through the transcriptional machinery. The presented model refines earlier proposals for a role of the nuclear matrix in gene expression based on the premise that the chromatin loop domain is an integral component of the transcriptional regulatory mechanism associated with the nuclear matrix [Davie, 1995; Jackson et al., 1996].

Our working hypothesis was based on the assumption that through dynamic protein–protein and protein–S/MAR associations with multiple partners, the components of the nuclear matrix/peripheral lamina form a network of functional interactions in the 5′ region of the haptoglobin gene. The interplay between these factors fine-tunes haptoglobin gene expression in order to meet the changing requirements of liver cells. This is supported by findings showing that the lamins simultaneously establish protein–DNA and protein–protein contacts that reflect changes in rat haptoglobin gene transcription. The colocalization experiments that were performed with two lamin-enriched nuclear protein fractions prepared according to different experimental approaches showed the lamins as dynamic interacting partners for transcription factor C/EBPβ and PARP-1. Therefore, the lamins can be regarded as transcriptional moderators that determine the macromolecular assembly on the Hp-S/MAR element and are involved in haptoglobin gene transcriptrional processes. Interactions with the nuclear matrix proteins/lamins determine the location and actions of their protein partners in haptoglobin gene transcription. In the present work, we describe the combined actions of the lamins, C/EBPβ, and PARP-1 within the nuclear matrix milieu. The lamins are one of the central components of the nuclear matrix and together with other proteins associated with this nuclear structure and examined herein, that is, C/EBPβ, an established upregulator of haptoglobin gene transcription and PARP-1 which has acquired the status of a transcriptional modulator, we propose that the said proteins participate in the transcriptional regulation of the haptoglobin gene in a concerted manner.

Since S/MARs display their structural and regulatory functions as complexes with specific S/MAR-binding proteins, we searched for Hp-S/MAR-binding nuclear lamina-associated protein partners. The results of the EMSA/re-electrophoresis experiments disclosed the potential protein constituents of Hp-S/MAR–protein complexes. Regardless of whether the proteins were prepared from control or AP samples, re-electrophoresis revealed a general feature of the established nucleoprotein complexes, namely the presence of at least two different proteins, one of which belongs to the group of lamin protein(s). C/EBPβ and PARP-1 were present in the lower and higher mass complexes, respectively. In addition, the AP sample generated a nucleoprotein complex consisting of Hp-S/MAR, (a) lamin protein(s) and a 90-kDa protein. The presence of more than one protein in the nucleoprotein complexes does not allow us to draw definite conclusions as to the identity of the DNA's direct protein-binding partner. However, in light of the observed pronounced affinity of the lamins for the Hp-S/MAR and their constant presence in each nucleoprotein complex after EMSA (Fig. 4), we can assume that the lamins established a direct interaction with the DNA. Also, the affinity of PARP-1 towards S/MAR DNA that has been described [Sastry and Kun, 1990; Galande and Kohwi-Shigematsu, 1999; Soldatenkov et al., 2002] and shown here (Figs. 3 and 4) cannot rule out the possibility that PARP-1 established a direct interaction with the Hp-S/MAR. This conclusion is supported by our recently published result describing the direct association of PARP-1 with its promoter-proximal S/MAR element in a process that strongly downregulates muPARP-1 gene transcription. C/EBPβ did not exhibit an affinity for Hp-S/MAR in the South-Western analysis but its affinity for the lamins was reported [Uskoković et al., 2002] and confirmed by the colocalization experiments presented here. The observed presence of C/EBPβ in nucleoprotein complexes during basal and elevated haptoglobin gene transcription is most probably the result of its association with lamin protein(s). This lends additional support to the hypothesis that the lamins, through multiple inter-molecular connections, redirect transcriptional coactivators to specific DNA sequences.

PARP-1 exhibited pronounced binding affinity for the reassembled lamina. Our previous results demonstrated an inherent ability of PARP-1 to reassemble with the lamins after a cycle of solubilization/dialysis using either urea or Triton X-100 and disulfide reduction, indicating that its interaction is dominated by hydrophobic forces. Together with in vivo crosslinking,

coimmunoprecipitation experiments and HALO-FISH and confocal analysis these results show that the lamins are prominent PARP-1-binding partners which could contribute to the functional sequestration of the enzyme on the nuclear matrix [Vidaković et al., 2004, 2005]. Interactions of PARP-1 with the lamina and the Hp-S/MAR could regulate gene expression by influencing chromatin structure. PARP-1 could serve as a chromatin-opening element capable of shifting the equilibrium of the chromatin structure to the unfolded DNA fiber. The PARP-1 function as a chromatin-opening element is related to its activity in post-translational regulation (PARylation) of several histones. This results in their displacement from chromatin, leading to its decompaction and transcriptional activation [Kim et al., 2005; Kraus, 2008].

In the L-EP1 sample that was prepared by solubilization with the non-ionic detergent Triton X-100, smaller amounts of lamin-associated C/EBPβ were observed after colocalization experiments than in the L-EP2 sample prepared by solubilization with urea. We concluded that C/EBPβ preferentially partitioned with a lamin-enriched nuclear matrix fraction through hydrophobic as well as other types of intra-molecular attractive forces, although the direct interaction of C/EBPβ and the lamins has to be confirmed. Based on earlier findings [Uskoković et al., 2002] and the results presented here we conclude that C/EBPβ and the lamins function in the same subnuclear compartment. A possible explanation for lamins as potential targeting sites for C/EBPβ lies in the discovery of the short amino acid sequences in *trans*-regulatory proteins, termed nuclear matrix targeting sequence or signal (NMTS) that mediate the association of their active forms with the nuclear matrix [Zeng et al., 1997; Tang et al., 1998; DeFranco and Guerrero, 2000]. The data provide insight into the mechanisms by which gene-regulatory factors are targeted to the nuclear matrix and argue against the indiscriminate attachment of these proteins to the nuclear matrix during subcellular fractionation. Some of the identified NMTS regions contain hydrophobic segments interspersed between regions of hydrophilic amino acids or form an amphipathic α-helix [Tang et al., 1998]. These findings support our previous results that C/EBPβ, which as a bZIP transcription factor superfamily member possessing a hydrophobic α-helix structure region, is anchored to the nuclear matrix through hydrophobic protein–protein interactions with the lamins. By interacting with the lamins that are in contact with the Hp-S/MAR located in very close proximity to the *cis*-element, C/EBPβ is poised on its target sequence on the haptoglobin gene promoter *cis*-element [Poznanović et al., 1999; Uskoković et al., 2002].

The enzymatic activity of PARP-1 is greatly stimulated by its binding to damaged DNA. Hence, most early studies of PARP-1 have focused on its role in DNA repair and cell death pathways [Schreiber et al., 2006]. Although PARP-1 is activated by DNA breaks, in vitro PARP-1 binds to other DNA structures such as cruciform or curved DNA [Bürkle et al., 2000; Soldatenkov et al., 2002; Lonskaya et al., 2005] either alone or in a heterodimeric complex with Ku protein to specifically base-unpaired regions [Galande, 2002]. Studies have suggested a role for PARP-1 in the regulation of chromatin structure by a post-translational modification of histones [Kim et al., 2004] whereas Wacker et al. [2007] provided direct visual evidence for PARP-1-dependent compaction of chromatin in a manner independent of its enzymatic activity. In addition, Krishnakumar et al. [2008] described PARP-1 binding to 90% of the RNA polymerase II-transcribed promoters in MCF-7 cells, suggesting that PARP-1 exerts some type of effect, either stimulatory or inhibitory, at sites of ongoing transcription. A role for PARP-1 in transcription was proposed to be related to its enhancer/promoter-binding activity where, in conjunction with other transcription-related factors, PARP-1 would act as a promoter-specific exchange factor that alternates between binding and release of components of the transcriptional machinery [Lis and Kraus, 2006]. The observed interactions between C/EBPβ and PARP-1 in basal haptoglobin gene expression but not during the AP response (Fig. 5), the PARP-1/lamina interaction with the Hp-S/MAR (Figs. 2–4) and the presence of PARP-1 on the haptoglobin gene *cis*-element during basal and increased transcription (Fig. 7) stress the importance of the protein–protein and protein–DNA interactions described herein for proper haptoglobin gene regulation. The assumption that PARP-1, together with C/EBPβ, affects the activity of the haptoglobin gene promoter is supported by recent data suggesting that PARP-1 plays a significant role in the regulation of the inflammatory response. PARP-1 can influence the stress/inflammation response through regulation of transcription factors and associated gene transcription. PARP-1 was identified as to be essential for the activity of transcription factors such as NF-κB [Hassa et al., 2003; Martin-Oliva et al., 2004], which is involved in cellular responses to stress stimuli, as well as Sp1 and Oct-1 [Nie et al., 1998]. The functions of these proteins can be modulated either by protein–protein interactions with native PARP-1 or through non-covalent interaction with ADP-ribose residues linked to the auto-modified PARP-1. Remarkably, neither the DNA-binding nor PARP-1 enzymatic activity is required for full activation of NF-κB in response to various stimuli in vivo [Hassa et al., 2001]. Nevertheless, the precise mechanism by which PARP-1 affects transcription lacks clarity and the ambiguity is evident in some cases [Aguilar-Quesada et al., 2007].

Under basal PARP enzymatic activity and during elevated transcription the soluble PARP-1 in the nuclear extract and the major 35 kDa C/EBPβ isoform appeared ADP-ribosylated. Poly(-ADP-ribosyl)ation could be one of the mechanisms governing the association/dissociation between regulatory proteins in the coactivator complex formed at the haptoglobin gene promoter. Ju et al. [2006] described the role of PARP-1 in the recruitment of gene-stimulatory factors, and evidence for PARP-1 promoter-specific coregulator functions was summarized by Kraus [2008]. While poly(ADP-ribosyl)ation targets DNA-binding factors in some cases (HES1, Sp1, NFAT, Elk1), in others it does not accompany PARP-1 coregulator activity (NF-B, B-Myb, HTLV Tax-1). The AP-response-promoted increase in haptoglobin gene transcription coincided with C/EBPβ gene upregulation and its concomitantly increased association with the nuclear matrix and increased binding to the haptoglobin gene promoter [Grigorov et al., 1998], as well as decreased interaction with PARP-1. Aside from poly(ADP-ribosyl)ation, other post-translational modification(s) notably post-translational phosphorylation that was shown to promote increased binding of C/EBPβ to the haptoglobin *cis*-element during the AP

response [Grigorov et al., 1998], could fine-tune the PARP-1/C/EBPβ interaction. Post-translational modification of PARP-1 through auto poly(ADP-ribosyl)ation [Ju et al., 2004], acetylation [Hassa et al., 2005], or phosphorylation [Kauppinen et al., 2006] during cellular signaling change PARP-1 activity. It remains to be established whether in the course of reorganization of the transcription complex required for enhanced haptoglobin gene activity during the AP response, a post-translational modification event(s) initiated the observed separation of C/EBPβ and PARP-1. This result, together with the observed binding of both C/EBPβ and PARP-1 to the haptoglobin *cis*-element during basal and increased transcription observed by DNA-affinity chromatography, suggests that PARP-1 remained on the haptoglobin gene *cis*-element during the AP response in a different protein–protein complex. Alternatively, the latter result is in agreement with the assumption that PARP-1 is capable of directly interacting with the haptoglobin *cis*-element. The presented findings revealed the propensity of PARP-1 to establish multiple contacts, that is, that its functioning at the *cis*- and Hp-S/MAR elements involves interactions with DNA, the nuclear matrix (lamin proteins), and DNA-binding transcriptional regulator proteins such as C/EBPβ described herein.

There is increasing evidence that the nuclear matrix is involved in the regulation of gene expression. The nuclear matrix is seen as a nuclear framework that supports the formation of transcriptionally competent nuclear domains and facilitates the assembly of active transcription complexes [McLarren et al., 2001]. Such a function is compatible with the documented implementation of chromatin higher order organization by the nuclear matrix, which provides anchorage sites for S/MARs. Through interactions with gene regulatory proteins, the nuclear matrix provides a platform for the formation of functional, dynamic protein–protein and protein–S/MAR associations within a given domain [Bode et al., 2003].

To conclude, in rat liver the transition from constitutive to a higher rate of haptoglobin gene transcription during the AP response is associated with quantitative and qualitative changes in Hp-S/MAR–protein interactions, that is, herein observed as increased association of the lamin(s) and PARP-1 (during the early stage of the AP response) and as the appearance of a 90 kDa Hp-S/MAR-binding protein. While PARP-1 interaction with the *cis*-element remained unchanged in the course of the AP response, the association of C/EBPβ with the Hp-S/MAR-bound PARP-1 was lost on account of its accumulation on the haptoglobin *cis* element.

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