Interplay between Human High Mobility Group Protein 1 and Replication Protein A on Psoralen-Cross-linked DNA†

Madhava C. Reddy,‡ Jesper Christensen,§ and Karen M. Vasquez*,‡

Department of Carcinogenesis, The University of Texas M. D. Anderson Cancer Center, Science Park-Research Division, 1808 Park Road 1-C, Smithville, Texas 78957, and Biotech Research & Innovation Centre, Fruebjergvej 3, 2100 Copenhagen ϕ, Denmark

Received September 27, 2004; Revised Manuscript Received December 21, 2004

ABSTRACT: Human high mobility group box (HMGB) 1 and -2 proteins are highly conserved and abundant chromosomal proteins that regulate chromatin structure and DNA metabolism. HMGB proteins bind preferentially to DNA that is bent or underwound and to DNA damaged by agents such as cisplatin, UVC radiation, and benzo[*a*]pyrenediol epoxide (BPDE). Binding of HMGB1 to DNA adducts is thought to inhibit nucleotide excision repair (NER), leading to cell death, but the biological roles of these proteins remain obscure. We have used psoralen-modified triplex-forming oligonucleotides (TFOs) to direct a psoralen−DNA interstrand cross-link (ICL) to a specific site to determine the effect of HMGB proteins on recognition of these lesions. Our results reveal that human HMGB1 (but not HMGB2) binds with high affinity and specificity to psoralen ICLs, and interacts with the essential NER protein, replication protein A (RPA), at these lesions. RPA, shown previously to bind tightly to these lesions, also binds in the presence of HMGB1, without displacing HMGB1. A discrete ternary complex is formed, containing HMGB1, RPA, and psoralen-damaged DNA. Thus, HMGB1 has the ability to recognize ICLs, can cooperate with RPA in doing so, and likely modulates their repair by the NER machinery. The abundance of HMGB1 suggests that it may play an important role in determining the sensitivity of cells to DNA damage under physiological, experimental, and therapeutic conditions.

Human high mobility group box (HMGB[1]) 1 and -2 proteins are the most abundant nonhistone nuclear proteins in mammalian cells (reviewed in refs *1*−*5*). HMGB1 and -2 contain two DNA binding domains called HMG boxes A and B and a carboxyl terminal domain. The functions of these proteins that require such high levels in the nucleus are not known. Both HMGB1 and -2 have been implicated in the regulation of chromatin structure and function as well as in various aspects of DNA metabolism. Beyond this intranuclear role, it has been reported recently that HMGB1 can function as a novel inflammatory cytokine and a late mediator of delayed endotoxin lethality (reviewed in refs *4*−*7*). The lack of chromosomal HMGB1 protein in knockout mice results in death within a few hours after birth due to hypoglycemia, but HMGB2 knockout mice are viable (*8*, *9*). One common feature of these proteins is their ability to bind to bent DNA and, once bound, to induce further bending in the DNA helix (*1*, *10*). HMGB1 and -2 proteins bind in the minor groove of DNA and show a preference for noncanonical or unusual DNA structures, including bulge loops (*11*−*13*), cruciforms or four way junctions (*14*−*16*), B−Z DNA junctions (*17*), cisplatin−DNA adducts (*18*−*20*), BPDE−DNA adducts (*21*), chromium-damaged DNA (*22*), and UV-damaged DNA (*23*). However, the actual biological functions of HMGB1 and -2 remain obscure. It is thought that the recognition of distorted or unusual DNA structures by HMGB proteins may influence DNA repair by inhibiting the NER proteins from binding to and repairing the lesion, a process termed "DNA repair shielding" (*1*, *24*−*29*). Such a role has wide-ranging implications for genomic instability and carcinogenesis.

Psoralen is a naturally occurring, tricyclic intercalating agent that can form both DNA monoadducts and ICLs between thymine bases in opposite strands of duplex DNA upon exposure to UVA irradiation. Psoralen + UVA (PUVA) therapy is used in the treatment of several hyperproliferative skin diseases such as psoriasis, vitiligo, and cancer (*30*). The effectiveness of PUVA treatment is in part due to the formation of DNA ICLs, which can induce cytotoxicity, mutagenesis, and recombination and can block both DNA replication and transcription (*30*, *31*). ICLs are considered to be one of the most lethal of DNA lesions, as a single ICL can kill repair-deficient bacteria and yeast, and it has been estimated that ∼40 ICLs can kill repair-deficient mammalian cells (*31*). Both NER and recombinational repair pathways are involved in the repair of psoralen cross-links in *Escheri-*

† Supported by an NCI grant to K.M.V. (CA93729) and an NIEHS center grant (ES07784).

* To whom correspondence should be addressed. Telephone: (512) 237-9324. Telefax: (512) 237-2475. E-mail: kvasquez@sprd1.mdacc.tmc.edu.

‡ The University of Texas M. D. Anderson Cancer Center.

§ Biotech Research & Innovation Centre.

[1] Abbreviations: HMGB, high mobility group box; BPDE, benzo[*a*]pyrenediol epoxide; NER, nucleotide excision repair; ICL, interstrand cross-link; PUVA, psoralen plus ultraviolet A.; TFO, triple helix-forming oligonucleotide; RPA, replication protein A; XPA, xeroderma pigmentosum complementation group A protein; bp, base pair(s); HMT, [2-[4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen]hexyl-1-*O*-(2-cyanoethyl)(*N*,*N*-diisopropyl)phosphoramidite; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; TBST, Tris-buffered saline plus Tween.

10.1021/bi047902n CCC: $30.25
Published on Web 02/25/2005

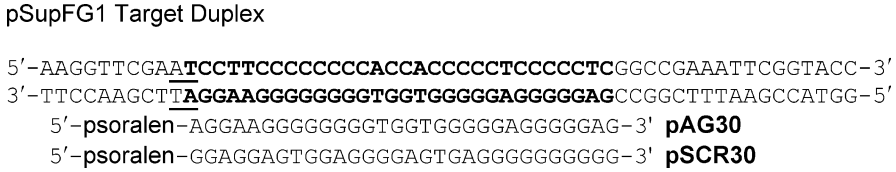

FIGURE 1: Sequence of the pSupFG1 triplex forming target site duplex and corresponding psoralen-conjugated TFOs. pAG30 is a single-stranded oligonucleotide; it binds with high affinity to the 30-bp polypurine target site in the pSupFG1 reporter gene in an antiparallel orientation. pSCR30 is a control oligonucleotide with the same base composition of pAG30 but a scrambled sequence, so that it does not form a triple helix. TFOs were synthesized with a 5′-psoralen derivative and a propanolamine on the 3′-end. Upon activation with UVA (365 nm), cross-links are delivered by psoralen-conjugated TFOs between thymines on the opposite strands in the DNA. The triplex−duplex junction containing the psoralen cross-linking site is underlined.

chia coli, but the involvement of these pathways in the repair of ICLs in mammalian cells is not well understood.

Whereas psoralen itself displays little sequence specificity, we can direct psoralen ICLs to specific sequences using triplex-forming oligonucleotides (TFOs). These are single-stranded oligonucleotides that recognize and bind to specific sequences in duplex DNA, forming triple-stranded DNA helices. TFOs have been used in vast array of applications, sharing the common goal of altering gene structure and/or function both in vitro and in vivo (reviewed in refs *32*−*34*). These structures can enhance the frequencies of mutation and promote recombination in a site-specific manner both in cells and in animals (*33*, *35*−*38*). In addition, psoralen-modified TFOs have been used to direct site-specific ICLs both in vitro and in vivo (*39*−*42*), thus providing a noncanonical, site-specific DNA "lesion" for protein−DNA interaction studies as we have used here and demonstrated previously (*41*).

Recently, we found that the human NER recognition factors, RPA and XPA, bind specifically and with high affinity to psoralen-cross-linked triplex DNA lesions. In addition, NER is involved in the error-generating processing of these lesions (*41*, *43*). We hypothesize that HMGB1 and/or HMGB2 proteins bind these TFO-directed psoralen ICLs and "shield" them from repair or, alternatively, may recruit the NER factors to the lesion. Human NER is a multistep process involving at least 25 proteins, including the damaged DNA recognition factors XPA and RPA (*44*, *45*). Human RPA is a stable heterotrimer containing 70, 34, and 14 kDa subunits. This protein complex is highly conserved in eukaryotes and is indispensable for DNA replication, homologous recombination, and NER, suggesting that it has multiple roles in DNA metabolic processes (*46*, *47*). The single-stranded DNA binding protein, RPA, physically interacts with XPA and enhances its binding to damaged DNA, an interaction that facilitates the recruitment of other NER proteins to the lesion.

In the present work, we sought to study the recognition of psoralen-cross-linked triplex structures by the HMGB1 and HMGB2 proteins and their interactions with the NER damage recognition proteins, XPA and RPA, on these lesions. Our results reveal that the human HMGB1 protein recognizes and binds to psoralen-cross-linked triplex DNA with high affinity and specificity. Competition experiments demonstrate that HMGB1 is capable of binding the ICL in the presence of RPA and the XPA−RPA complex regardless of the order of addition; i.e., even when nearly all the damaged DNA is in a specific complex with RPA. RPA is not able to displace prebound HMGB1 from the ICL lesion, but rather stabilizes the formation of HMGB1−DNA−RPA ternary complexes. We hypothesize that the binding of HMGB1 protein to psoralen-cross-linked triplex DNA may modulate the repair of these mutagenic structures.

EXPERIMENTAL PROCEDURES

Oligonucleotides. The sequences of the 57-bp synthetic duplex target (pSupFG1 triplex site) and corresponding 30 base psoralen-conjugated TFOs used as substrates in this study are shown in Figure 1. pAG30 binds with high affinity to the 30-bp polypurine target site in the target duplex and pSCR30 is a 30-base scrambled control oligonucleotide that does not bind the 57-bp target duplex (*41*). Oligonucleotides were synthesized with a 5′-psoralen derivative [2-[4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen]hexyl-1-*O*-(2-cyanoethyl)(*N*,*N*-diisopropyl)phosphoramidite (HMT)] and a 3′-propanolamine by the Midland Certified Reagent Company, Inc. (Midland, TX). Complementary synthetic single-stranded oligonucleotides corresponding to the pSupFG1 sequence were annealed at a 1:1 molar ratio to form the synthetic target duplex. Duplexes were 5′-end-labeled by transfer of ^{32}P from [γ-^{32}P]ATP with T4 polynucleotide kinase, purified by 12% polyacrylamide gel electrophoresis (PAGE), electroeluted, and concentrated by using Centricon centrifugal filter devices (Millipore, Bedford, MA). The concentration of DNA was determined by UV absorbance at 260 nm.

TFO-Directed Psoralen ICL Formation. Triplex structures were formed by incubating radiolabeled or unlabeled duplexes with psoralen-conjugated TFOs in a triplex binding buffer [10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 10% (vol/vol) glycerol] at 37 °C for 16 h. Samples were irradiated with 1.8 J/cm^2 of UVA light at 365 nm to form psoralen ICLs. Efficiency of cross-linking was determined by denaturing PAGE and quantified using a phosphorimager. ICLs were formed in these substrates at the targeted triplex−duplex junction with up to 90% efficiency (data not shown).

Expression and Purification of Recombinant Proteins. Recombinant baculovirus expressing histidine-tagged full-length human HMGB1 and HMGB2 proteins were expressed in Sf9 cells and purified by phosphocellulose chromatography as previously described (*48*, *49*). To confirm that HMGB1 and -2 proteins were functional, DNA nicking assays were carried out as previously described (*48*, *49*). The three-subunit histidine-tagged RPA complex was expressed by coinfection of Sf9 cells at a multiplicity of infection of 5 for His-RPA1 and RPA2 baculoviruses and of 10 for the RPA3 baculovirus (*50*). The infected cells were harvested, and the expressed complex was purified by Ni^{2+}-chelate chromatography and further purified by salt gradient elution from a Mono-Q FPLC column as previously described (*51*).

The purity of protein preparations was determined by SDS−PAGE and silver staining. The XPA-maltose binding protein was expressed in *E. coli* PR745 from pMAL constructs and purified as previously described (*38*).

Protein−DNA Binding Assays. Protein−DNA binding interactions were examined by electrophoretic mobility shift assays (EMSA) by incubating human recombinant purified proteins ($\sim 1 \times 10^{-8}$ M unless otherwise stated) HMGB1, HMGB2, XPA, and RPA in binding buffer (25 mM Tris-HCl pH 7.6, 100 mM NaCl, 1 mM DTT, 5 mM EDTA, 100 μg/mL BSA, 0.01% Nonidet P-40, and 10% glycerol) in a 20 μL reaction volume for 10 min at 30 °C. Radiolabeled triplex or duplex DNA substrates ($\sim 1 \times 10^{-8}$ M) were then added to the proteins in the same buffer and incubated for an additional 20 min at 30 °C. Competition assays were performed by adding increasing concentrations of unlabeled competitor DNA (either duplex or psoralen-cross-linked triplex DNA) or competitor proteins (RPA or HMGB1) to the reaction and incubating the combined mixture for an additional 20 min at 30 °C. The samples were then electrophoresed through a 6% (29:1 acrylamide:bis) native polyacrylamide gel, buffered in 1× TBE (89 mM Tris-borate, pH 8.0, 2 mM EDTA). Electrophoresis was carried out at 4 °C for ~3 h at 25 mA. The gel was dried, DNA−protein complexes were visualized by autoradiography, and reactions were quantified using a phosphorimager.

Southwestern Blot Analysis. The protein−DNA complexes were separated by native PAGE and electroblotted onto a nitrocellulose membrane using a semidry transfer unit (Hoefer, San Francisco, CA). The membrane was washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) before blocking with TBST containing 5% nonfat dry milk powder. The membrane was probed with HMGB1 antibody (Stressgen, San Diego, CA) (at a 1:1000 dilution) and incubated for 3 h at room temperature. The membrane then was washed with TBST and treated with a horseradish peroxidase-conjugated antibody to mouse IgG for 1 h at room temperature. After successive washes with TBST, the protein bands were visualized by using a chemiluminescence kit according to the manufacturer's instructions (Amersham, Piscataway, NJ). Following HMGB1 detection, the membrane was stripped and reprobed with RPA/p70 antibody (NeoMarkers, Fremont, CA) at a 1:1000 dilution. The detection of RPA protein was accomplished as described above.

RESULTS

Specific Recognition by Human HMGB1 Protein of Psoralen-Cross-Linked Triplex DNA. We tested HMGB1 and HMGB2 for high-affinity binding to either radiolabeled psoralen-cross-linked triplex DNA, undamaged duplex DNA, or duplex DNA incubated with a scrambled control oligonucleotide (that does not form triplex) by EMSA analyses (Figure 2). At 10 nM added protein, most of the psoralen-cross-linked triplex DNA was bound to HMGB1 protein, as indicated by the alteration of the radiolabeled HMGB1−psoralen ICL complex mobility. Under identical conditions, the HMGB1 protein did not bind undamaged duplex DNA or duplex DNA incubated with the control oligonucleotide (Figure 2, compare lanes 2 and 10 with lane 6), indicating a strong specificity for damaged DNA compared to undamaged

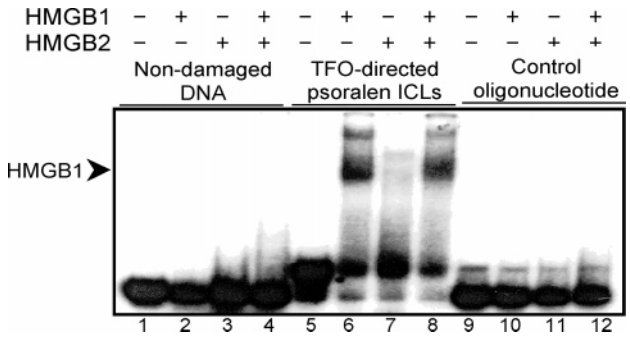

FIGURE 2: Specific recognition by human HMGB1 protein of psoralen-cross-linked triplex DNA. Purified recombinant human HMGB1 and HMGB2 proteins were incubated alone and together with the $\sim 10^{-8}$ M radiolabeled 57-bp synthetic duplex (lanes 1−4), TFO-directed psoralen ICLs formed between the specific TFO and duplex DNA (lanes 5−8) and duplex DNA plus control oligonucleotide (lanes 9−12) in binding buffer for 20 min at 30 °C. After incubation, protein−DNA complexes were separated from free radiolabeled DNA substrate by an EMSA on a 6% native polyacrylamide gel in 1× TBE buffer. The gel was run for 3 h at 25 mA at 4 °C. The gel was dried, and the bands were visualized by autoradiography.

DNA. Although HMGB1 clearly recognizes the synthetic supFG1 psoralen-cross-linked triplex structures, we considered the possibility that these effects were unique to that particular site. To address this question, similar assays were performed with a psoralen-cross-linked triplex formed on a different recognition site, the adenine phosphoribosyl transferase (APRT) gene, which has been well characterized (*39*, *52*). HMGB1 also bound the APRT psoralen-cross-linked substrate with high affinity and specificity under the same binding conditions (data not shown), consistent with our observations with the supFG1 triplex target site. To determine the binding affinity of HMGB1 to psoralen-cross-linked triplexes, the psoralen ICL substrate was incubated with increasing concentrations of HMGB1 (from 0 to 10^{-6} M), and the apparent K_d was determined to be between 10^{-8} and 10^{-7} M. A similar experiment was performed to determine whether HMGB1 could recognize a site-specific psoralen ICL (in the absence of triplex formation). We found that HMGB1 does bind to the psoralen ICL in the absence of triplex formation, but with lower affinity. Under conditions that allow for ~90% binding of HMGB1 to a TFO-directed psoralen ICL, only ~25% binding of the psoralen ICL alone (i.e. without TFO) was observed (data not shown).

Under the same experimental conditions, very little mobility shift was observed for the TFO-directed psoralen cross-linked DNA in the presence of human HMGB2 protein, and none was seen for the undamaged duplex DNA or the undamaged duplex DNA incubated with the control oligonucleotide (Figure 2, compare lanes 3 and 11 with lane 7). Thus, high-affinity recognition of this type of DNA lesion is characteristic of HMGB1 and not of HMGB2.

Specificity of HMGB1 Protein Binding to Psoralen-Cross-Linked Triplex DNA. We compared the abilities of unlabeled duplex DNA or psoralen-cross-linked triplex DNA to compete with radiolabeled psoralen-cross-linked triplex substrate bound to HMGB1 (Figure 3) by adding increasing concentrations of the same unlabeled duplex or psoralen-cross-linked triplex as competitor DNAs. In these experiments, HMGB1 was incubated with radiolabeled TFO-directed psoralen ICL substrates and titrated with increasing

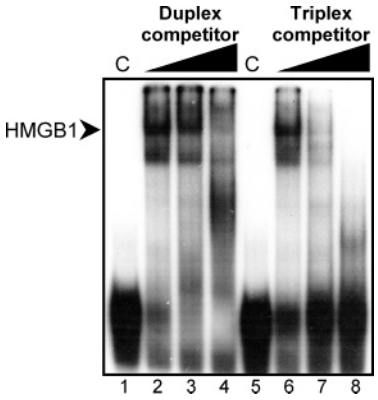

FIGURE 3: Specificity of HMGB1 protein binding to psoralen-cross-linked triplex DNA. Purified recombinant human HMGB1 was incubated with radiolabeled psoralen-cross-linked triplex DNA ($\sim 10^{-8}$ M) and increasing concentrations (lanes 2 and 6, 10^{-8} M; lanes 3 and 7, 10^{-7} M; lanes 4 and 8, 10^{-6} M) of unlabeled duplex (lanes 2−4) or unlabeled psoralen-cross-linked triplex competitor DNA (lanes 6−8) in binding buffer for 20 min at 30 °C. Protein−DNA complexes were separated from free radiolabeled triplex DNA by EMSA on a 6% native polyacrylamide gel in 1× TBE buffer at 4 °C. The gel was dried and the bands were visualized by autoradiography.

concentrations (in 10-fold increments) of unlabeled competitor duplex (Figure 3, lanes 2−4) or psoralen-cross-linked triplex DNA (Figure 3, lanes 6−8). Results indicated that HMGB1 bound tightly to the psoralen-cross-linked triplex DNA, even in the presence of an excess of unlabeled duplex competitor DNA. A shift in mobility of much of the radiolabel suggests that the duplex may actually associate with the complex of HMGB1 and damaged DNA (Figure 3, lane 4). In contrast, addition of unlabeled psoralen-cross-linked triplex DNA displaced the labeled damaged DNA substrate from the HMGB1 complex so that most of the labeled DNA migrates as protein-free DNA. This striking specificity indicates that there is a specific site for the damaged DNA on HMGB1 but that undamaged duplex DNA does not compete for this site. We determined a K_d value of between 10^{-8} and 10^{-7} M for binding of HMGB1 to the psoralen-cross-linked supFG1 triplex site, similar to the affinities determined for both the supFG1 and the APRT substrates estimated from protein titration experiments.

Interaction of HMGB1 and RPA on Psoralen-Cross-Linked Triplex DNA. Previously, we reported that the human recombinant XPA and RPA proteins are involved in the initial recognition of psoralen-cross-linked triplex DNA (*41*). To address whether HMGB1 could modulate the ability of RPA to bind to psoralen-cross-linked triplex DNA, we performed competition experiments between HMGB1 and RPA proteins by using EMSA analyses. Addition of HMGB1 to a preformed complex of RPA and TFO-directed psoralen ICLs (Figure 4) led to complete loss of the band corresponding to the RPA complex (Figure 4A, lane 5). Rather than simply displacing RPA, HMGB1 seems to form complexes with the damaged DNA that include RPA, as suggested by their slower mobilities as compared to the major HMGB1−ICL complex formed in the absence of RPA (Figure 4A, lane 3; Figure 4B, lane 4). When HMGB1 binds to the damaged DNA in the absence of RPA, the radioactivity is distributed among several species with altered mobilities. Addition of RPA to these complexes does not result in formation of the simple RPA−DNA complex seen in lane 4 of Figure 4A and lane 3 of Figure 4B. Rather, it results in an increase of label appearing in a band whose mobility is similar to, or slightly slower than, that of the major HMGB1−DNA complex (Figure 4A,B, bracket labeled HMGB1). Thus, RPA appears to stabilize this HMGB1-damaged DNA complex. The presence of both HMGB1 and RPA in this complex was confirmed by southwestern immunoblotting experiments (Figure 4C). Both HMGB1 and RPA antibodies gave positive signals for the HMGB1-damaged DNA complex whose formation is enhanced by RPA. Because the mobility of this complex (or complexes) is spread over a broader range than that of the RPA−DNA complex (lane 4 vs lane 5, Figure 4C), we cannot say for certain whether all of the RPA initially bound to DNA or only a fraction of it ends up in the HMGB1-damaged DNA complex. Thus, the stoichiometry of this complex and its heterogeneity remain the subject of future studies. It is clear, however, that RPA, HMGB1, and damaged DNA all participate in this complex, in accordance with the thermodynamics of reversible equilibrium. Further confirmation of the presence of RPA in the HMGB1-damaged DNA complex comes from supershifts induced upon anti-RPA p34 antibody addition (Figure 4D, compare lanes 7 and 8).

Because RPA directly interacts with XPA and enhances its interaction with damaged DNA, we examined whether the XPA−RPA protein complex affected the HMGB1-damaged DNA interaction (Figure 5). The results of EMSA experiments were similar in the presence or absence of XPA (compare Figure 4A, lanes 6 and 7 and Figure 4B, lanes 6 and 7 with Figure 5, lanes 9 and 10 and lanes 7 and 8, respectively). In previous experiments (*41*) and in the current studies, binding by XPA alone to the TFO-directed psoralen ICLs was not detectable by EMSA (Figure 5, lane 3), but the presence of XPA was confirmed by antibody supershift analysis (data not shown). This suggests that RPA (or the XPA−RPA complex) can effectively form a ternary complex with HMGB1 on psoralen-cross-linked triplex DNA.

DISCUSSION

HMGB1 is an abundant and highly conserved nonhistone DNA-binding protein that interacts with specific DNA structural motifs such as cisplatin−DNA damage, four-way junctions, and supercoils. Here we have shown that HMGB1 can also bind to psoralen ICLs in the context of three-stranded helical distortions induced by triplex formation (Figure 2). HMGB1 and HMGB2 clearly differ in this regard, under our experimental conditions. Whereas recombinant human HMGB1 protein bound specifically and with high affinity to TFO-directed psoralen ICLs ($K_d \sim 10^{-8}$−10^{-7} M), HMGB2 did not bind to these lesions (neither the supFG1 or the APRT triplex target sites) under the same experimental conditions. Thus, these closely related proteins have strikingly different specificities for recognition of psoralen-cross-linked DNA.

HMGB proteins are thought to be involved in DNA repair, but it is not clear exactly how they impinge on different repair mechanisms. NER repairs a wide variety of DNA lesions induced by UV light and chemical mutagens that lead to distortions in the DNA helix, and there is considerable overlap of these NER targets with the types of DNA lesions

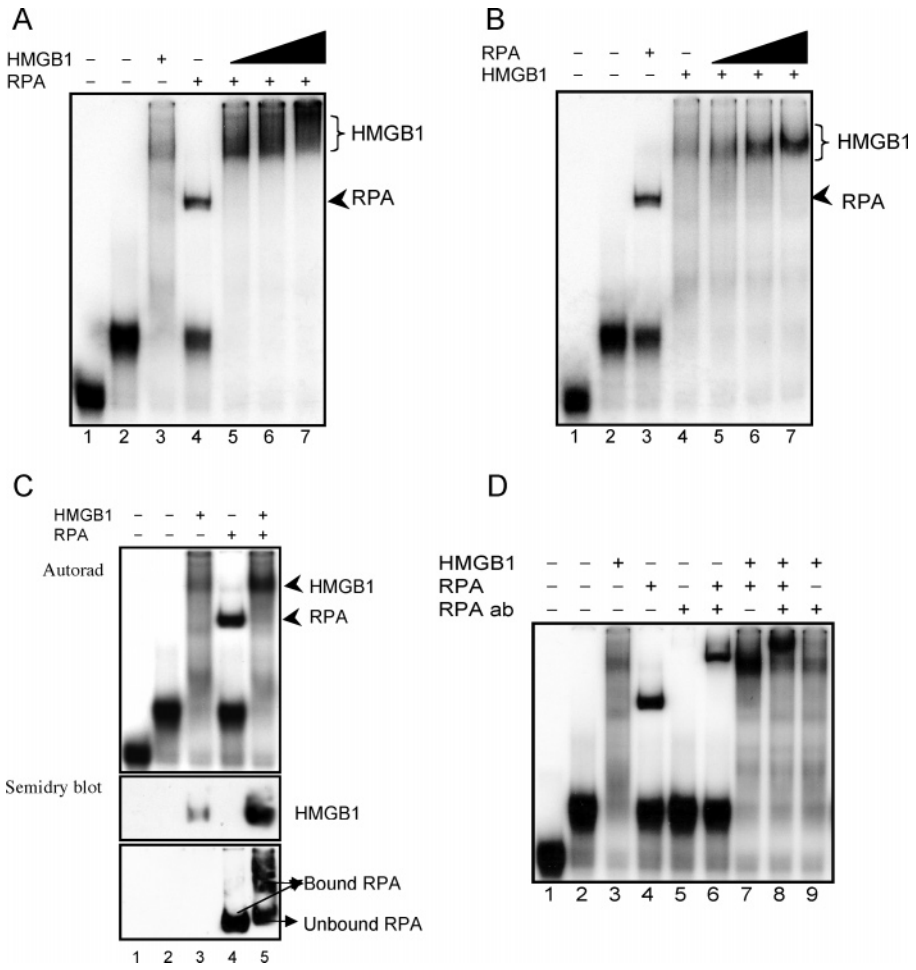

FIGURE 4: Interactions of human HMGB1 and RPA proteins on TFO-directed psoralen ICLs. TFO-directed psoralen cross-links were preincubated with purified recombinant human RPA protein (panel A, lanes 4−7) and purified recombinant human HMGB1 protein (panel B, lanes 4−7) in binding buffer for 20 min at 30 °C. After incubation, increasing concentrations of HMGB1 ($\sim 3 \times 10^{-8}$, 5×10^{-8}, and 7×10^{-8} M; panel A, lanes 5−7) or RPA ($\sim 8 \times 10^{-9}$, 1×10^{-8}, and 2×10^{-8} M; panel B, lanes 5−7) were then added and incubation continued for an additional 20 min at 30 °C. Protein−DNA complexes were separated from free radiolabeled DNA by EMSA on a 6% native polyacrylamide gel in 1× TBE buffer and visualized by autoradiography. (C) Southwestern blot analysis of proteins associated with TFO-directed psoralen ICLs. A competition experiment was performed by incubating purified recombinant human HMGB1 and RPA proteins (at $\sim K_d$ concentrations) with 10^{-8} M psoralen-cross-linked triplex DNA in binding buffer for 20 min at 30 °C. Following the separation of the DNA−protein complexes from free radiolabled substrate by EMSA (top panel C), DNA−protein complexes were transferred to a nitrocellulose membrane by semidry blotting and analyzed by using an anti-HMGB1 (middle panel C) and anti-RPA antibody (bottom panel C). The free RPA protein was also detected on the blot as indicated in the bottom panel lane 5. (D) Identification of RPA in the HMGB1−psoralen-damaged DNA complex by antibody supershift analysis. HMGB1 and RPA were incubated with TFO-directed psoralen ICLs in binding buffer for 20 min at 30 °C. After incubation, anti-RPA p34 antibody (lanes 5, 6, 8, and 9) was added and further incubated at 30 °C for 20 min. Samples were then subjected to gel electrophoresis and autoradiography. Lane 1 (panels A, B, C, and D) contains undamaged duplex DNA only, all remaining lanes contain TFO-directed psoralen ICLs.

and helical distortions recognized by HMGB1. It is unclear whether HMGB1 blocks NER, facilitates it, regulates it in some way, or performs some independent function in binding NER substrates. One of the early events in the multistep processing of DNA lesions by NER is binding by RPA (*44*, *45*, *53*), and we know from recent studies (*41*) that the psoralen-cross-linked triplex structures we observe binding to HMGB1 are recognized by human XPA and RPA proteins.

How or if HMGB1 affects early steps in the NER pathway is unknown, but it has been suggested that the binding of HMGB proteins to DNA lesions may prevent their repair by inhibition of the NER pathway (*1*, *24*−*29*). Inhibition of NER by HMGB proteins would have wide-ranging implications for genome instability and carcinogenesis. For example, DNA adducts formed by the chemical carcinogen BPDE have been strongly implicated in the development of cancers, and it has been shown that the HMGB1 protein binds preferentially to these lesions (*21*). If binding by the HMGB proteins were to block access of the NER machinery required for the repair of BPDE−DNA damage, the result would likely be enhanced tumorigenesis. This notion is supported by evidence that HMGB proteins are overexpressed in a variety of tumor cells (*27*, *54*, *55*). On the other hand, HMGB1 also binds to the DNA adducts of the chemotherapeutic drug cisplatin (*18*, *56*). In this case, HMGB1 binding to cisplatin lesions may provide a therapeutic benefit, because the prevention of their repair in tumor cells correlates with increased efficacy of cisplatin chemotherapy. However, there is currently limited data available on the competitive binding of NER proteins and HMGB proteins on DNA−drug lesions and/or helical distortions, and to our knowledge no reports have been published on the interaction of the HMGB proteins with psoralen ICLs or triple helical distortions.

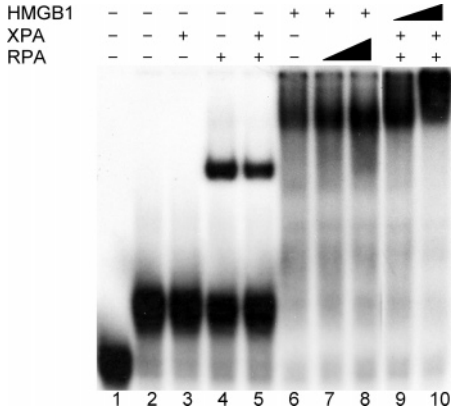

FIGURE 5: Interactions of human HMGB1, XPA, and RPA proteins on TFO-directed psoralen ICLs. Human recombinant HMGB1 protein (lanes 7 and 8) and premixed XPA and RPA proteins (lanes 9 and 10) were incubated with the TFO-directed psoralen ICLs in binding buffer in a 20 μL reaction volume for 20 min at 30 °C. After incubation, increasing concentrations of premixed XPA and RPA proteins ($\sim 1 \times 10^{-8}$ and 2×10^{-8} M; lanes 7 and 8, respectively) and HMGB1 protein ($\sim 5 \times 10^{-8}$ and 7×10^{-8} M; lanes 9 and 10, respectively) were added, and incubation was continued for an additional 20 min at 30 °C. Protein−DNA complexes were subjected to EMSA and visualized by autoradiography. Lane 1 contains undamaged duplex DNA only; all remaining lanes (lanes 2−10) contain TFO-directed psoralen ICLs.

The ability of HMGB1 to bind cisplatin-damaged DNA in the presence of RPA has been reported by Patrick and Turchi (*28*). Neither HMGB1 nor RPA displaced the other in order of addition experiments, but the results obtained when the proteins were preincubated in the absence of damaged DNA were interpreted as decreased RPA binding to the cisplatin-damaged DNA upon addition of HMGB1 (*28*). Their results are not necessarily inconsistent with our observations, i.e., the formation of protein DNA complexes with decreased mobilities when both RPA and HMGB1 are present. We found that the initial RPA−DNA complex is disrupted in both order of addition and competition experiments with HMGB1 (Figure 4). However, the presence of RPA does not prevent the formation of a complex of psoralen-cross-linked triplex DNA with human HMGB1 protein, but rather, it actually stabilized such complexes, resulting in the formation of one or more HMGB1−damaged DNA−RPA ternary complexes with the psoralen ICLs. We have determined that RPA does not enhance the formation of the HMGB1 complex by catalysis but rather participates in the reaction by stabilizing it thermodynamically. It may be that both cisplatin-induced and psoralen-induced lesions can serve as nucleation sites for formation of complexes containing both HMGB1 and RPA, or there may be differences in the interplay of these proteins depending on the nature of the DNA damage (i.e. psoralen vs cisplatin or interstrand cross-link vs intrastrand cross-link).

HMGB proteins can induce local deformation of the DNA helix to facilitate the interactions of DNA binding proteins with their cognate binding sites. For example, p53 binds inefficiently to linear DNA but binds much more efficiently in the presence of HMGB1 (*57*, *58*). HMGB1 plays a similar role for other DNA binding proteins, including TBP and HOX transcription factors, and the RAG1 site-specific recombination protein (*1*, *5*). Remarkably, in our experiments HMGB1 bound to psoralen ICLs and dislocated prebound RPA (Figure 4) and the XPA−RPA complex (Figure 5),

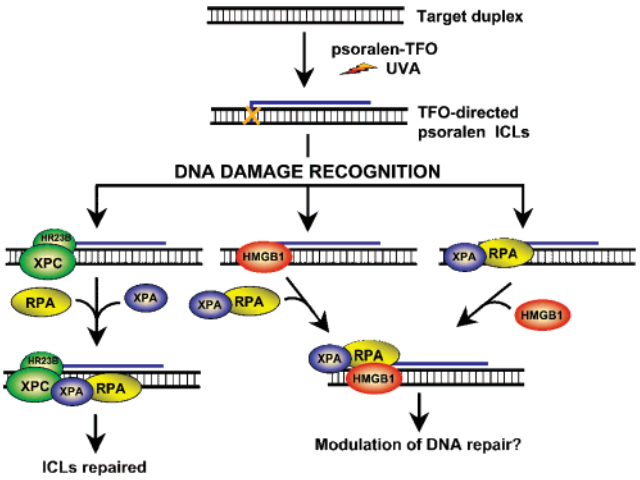

FIGURE 6: Model for the effect of HMGB1 on modulation of DNA repair and/or chromatin structure at sites of TFO-directed psoralen ICLs.

resulting in the formation of a discrete HMGB1−RPA−psoralen damaged DNA (or HMGB1−XPA−RPA−psoralen damaged DNA) ternary complex. Our results are consistent with those of Imamura et al. (*59*), which demonstrated that the p53 protein enhanced the cisplatin-damaged DNA binding activity of HMGB1.

The binding interactions of HMGB1 and RPA on psoralen ICLs may modulate the repair of these lesions by NER. Interestingly, our results suggest that HMGB1, rather than inhibiting NER, may recruit the NER damage recognition factors to the site of the DNA lesion. Because HMGB1 is a known chromatin architectural protein, the binding of HMGB1 to DNA lesions and its interactions with the NER factors as we have shown here suggest that HMGB1 might play a role in chromatin remodeling near the site of DNA damage to allow access of the repair proteins to the damaged site. We have proposed such a model in Figure 6 based on our findings. Here we speculate that if DNA ICLs are first recognized by the DNA damage recognition protein complex XPC−hHR23B, then the ICLs are efficiently repaired by NER (reviewed in ref *60*). However, if the ICL is first bound by HMGB1, then XPA and RPA may be recruited to the lesion (in the presence or absence of XPC-hHR23B), resulting in modulation of DNA repair. If the XPA−RPA complex is the first to bind the ICL and HMGB1 is available to bind the lesion cooperatively with XPA−RPA, this again may lead to modulation of DNA repair (in the presence or absence of XPC−hHR23B). The high concentration of HMGB1 in the nucleus and the strength of interactions as monitored in vitro suggest that the types of complexes we observe are likely to occur in cells with similar types of DNA damage. This previously unrecognized activity of HMGB1 may be one of the important functions that contribute to a need for it at high concentrations in the nucleus, where repair of damaged DNA takes place.

ACKNOWLEDGMENT

We thank Robert Black, Laura Christensen, and Praveen Rao for technical assistance. We thank Dr. Theodore G. Wensel and Dr. Rick A. Finch for advice and critical reading of the manuscript. We thank Sarah Henninger for her assistance in preparing this manuscript.

REFERENCES

1. Agresti, A., and Bianchi, M. E. (2003) HMGB proteins and gene expression, *Curr. Opin. Genet. Dev. 13*, 170−178.
2. Bustin, M. (1999) Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins, *Mol. Cell. Biol. 19*, 5237−5246.
3. Thomas, J. O., and Travers, A. A. (2001) HMG1 and 2, and related 'architectural' DNA-binding proteins, *Trends Biochem. Sci. 26*, 167−174.
4. Yang, H., Wang, H., Czura, C. J., and Tracey, K. J. (2002) HMGB1 as a cytokine and therapeutic target, *J. Endotoxin Res. 8*, 469−472.
5. Degryse, B., and de Virgilio, M. (2003) The nuclear protein HMGB1, a new kind of chemokine?, *FEBS Lett. 553*, 11−17.
6. Andersson, U., and Tracey, K. J. (2003) HMGB1 in sepsis, *Scand. J. Infect. Dis. 35*, 577−584.
7. Czura, C. J., Wang, H., and Tracey, K. J. (2001) Dual roles for HMGB1: DNA binding and cytokine, *J. Endotoxin. Res. 7*, 315−321.
8. Calogero, S., Grassi, F., Aguzzi, A., Voigtlander, T., Ferrier, P., Ferrari, S., and Bianchi, M. E. (1999) The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice, *Nat. Genet. 22*, 276−280.
9. Ronfani, L., Ferraguti, M., Croci, L., Ovitt, C. E., Scholer, H. R., Consalez, G. G., and Bianchi, M. E. (2001) Reduced fertility and spermatogenesis defects in mice lacking chromosomal protein Hmgb2, *Development 128*, 1265−1273.
10. Lilley, D. M. (1992) DNA−protein interactions. HMG has DNA wrapped up, *Nature 357*, 282−283.
11. Cerdan, R., Payet, D., Yang, J. C., Travers, A. A., and Neuhaus, D. (2001) HMG-D complexed to a bulge DNA: An NMR model, *Protein Sci. 10*, 504−518.
12. Gibb, C. L., Cheng, W., Morozov, V. N., and Kallenbach, N. R. (1997) Effect of nuclear protein HMG1 on in vitro slippage synthesis of the tandem repeat dTG x dCA, *Biochemistry 36*, 5418−5424.
13. Payet, D., Hillisch, A., Lowe, N., Diekmann, S., and Travers, A. (1999) The recognition of distorted DNA structures by HMG-D: A footprinting and molecular modelling study, *J. Mol. Biol. 294*, 79−91.
14. Bianchi, M. E., Beltrame, M., and Paonessa, G. (1989) Specific recognition of cruciform DNA by nuclear protein HMG1, *Science 243*, 1056−1059.
15. Bianchi, M. E., Falciola, L., Ferrari, S., and Lilley, D. M. (1992) The DNA binding site of HMG1 protein is composed of two similar segments (HMG boxes), both of which have counterparts in other eukaryotic regulatory proteins, *Embo. J. 11*, 1055−1063.
16. JR, P. o., Norman, D. G., Bramham, J., Bianchi, M. E., and Lilley, D. M. (1998) HMG box proteins bind to four-way DNA junctions in their open conformation, *EMBO J. 17*, 817−826.
17. Hamada, H., and Bustin, M. (1985) Hierarchy of binding sites for chromosomal proteins HMG 1 and 2 in supercoiled deoxyribonucleic acid, *Biochemistry 24*, 1428−1433.
18. Jung, Y., and Lippard, S. J. (2003) Nature of full-length HMGB1 binding to cisplatin-modified DNA, *Biochemistry 42*, 2664−2671.
19. Pil, P. M., and Lippard, S. J. (1992) Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin, *Science 256*, 234−237.
20. Farid, R. S., Bianchi, M. E., Falciola, L., Engelsberg, B. N., and Billings, P. C. (1996) Differential binding of HMG1, HMG2, and a single HMG box to cisplatin-damaged DNA, *Toxicol. Appl. Pharmacol. 141*, 532−539.
21. Lanuszewska, J., and Widlak, P. (2000) High mobility group 1 and 2 proteins bind preferentially to DNA that contains bulky adducts induced by benzo[*a*]pyrene diol epoxide and *N*-acetoxyacetylaminofluorene, *Cancer Lett. 158*, 17−25.
22. Wang, J. F., Bashir, M., Engelsberg, B. N., Witmer, C., Rozmiarek, H., and Billings, P. C. (1997) High mobility group proteins 1 and 2 recognize chromium-damaged DNA, *Carcinogenesis 18*, 371−375.
23. Pasheva, E. A., Pashev, I. G., and Favre, A. (1998) Preferential binding of high mobility group 1 protein to UV-damaged DNA. Role of the COOH-terminal domain, *J. Biol. Chem. 273*, 24730−24736.
24. Nagatani, G., Nomoto, M., Takano, H., Ise, T., Kato, K., Imamura, T., Izumi, H., Makishima, K., and Kohno, K. (2001) Transcriptional activation of the human HMG1 gene in cisplatin-resistant human cancer cells, *Cancer Res. 61*, 1592−1597.
25. Zamble, D. B., Mikata, Y., Eng, C. H., Sandman, K. E., and Lippard, S. J. (2002) Testis-specific HMG-domain protein alters the responses of cells to cisplatin, *J. Inorg. Biochem. 91*, 451−462.
26. Malina, J., Kasparkova, J., Natile, G., and Brabec, V. (2002) Recognition of major DNA adducts of enantiomeric cisplatin analogs by HMG box proteins and nucleotide excision repair of these adducts, *Chem. Biol. 9*, 629−638.
27. He, Q., Liang, C. H., and Lippard, S. J. (2000) Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin, *Proc. Natl. Acad. Sci. U.S.A. 97*, 5768−5772.
28. Patrick, S. M., and Turchi, J. J. (1998) Human replication protein A preferentially binds cisplatin-damaged duplex DNA in vitro, *Biochemistry 37*, 8808−8815.
29. Huang, J. C., Zamble, D. B., Reardon, J. T., Lippard, S. J., and Sancar, A. (1994) HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease, *Proc. Natl. Acad. Sci. U.S.A. 91*, 10394−10398.
30. Joerges, C., Kuntze, I., and Herzinge, T. (2003) Induction of a caffeine-sensitive S-phase cell cycle checkpoint by psoralen plus ultraviolet A radiation, *Oncogene 22*, 6119−6128.
31. Dronkert, M. L., and Kanaar, R. (2001) Repair of DNA interstrand cross-links, *Mutat. Res. 486*, 217−247.
32. Vasquez, K. M., and Wilson, J. H. (1998) Triplex-directed modification of genes and gene activity, *Trends Biochem. Sci. 23*, 4−9.
33. Vasquez, K. M., and Glazer, P. M. (2002) Triplex-forming oligonucleotides: Principles and applications, *Q. Rev. Biophys. 35*, 89−107.
34. Praseuth, D., Guieysse, A. L., and Helene, C. (1999) Triple helix formation and the antigene strategy for sequence-specific control of gene expression, *Biochim. Biophys. Acta 1489*, 181−206.
35. Vasquez, K. M., Narayanan, L., and Glazer, P. M. (2000) Specific mutations induced by triplex-forming oligonucleotides in mice, *Science 290*, 530−533.
36. Vasquez, K. M., Wang, G., Havre, P. A., and Glazer, P. M. (1999) Chromosomal mutations induced by triplex-forming oligonucleotides in mammalian cells, *Nucleic Acids Res. 27*, 1176−1181.
37. Vasquez, K. M., Marburger, K., Intody, Z., and Wilson, J. H. (2001) Manipulating the mammalian genome by homologous recombination, *Proc. Natl. Acad. Sci. U.S.A. 98*, 8403−8410.
38. Datta, H. J., Chan, P. P., Vasquez, K. M., Gupta, R. C., and Glazer, P. M. (2001) Triplex-induced recombination in human cell-free extracts. Dependence on XPA and HsRad51, *J. Biol. Chem. 276*, 18018−18023.
39. Vasquez, K. M., Wensel, T. G., Hogan, M. E., and Wilson, J. H. (1996) High-efficiency triple-helix-mediated photo-cross-linking at a targeted site within a selectable mammalian gene, *Biochemistry 35*, 10712−10719.
40. Oh, D. H., and Hanawalt, P. C. (2000) Binding and photoreactivity of psoralen linked to triple helix-forming oligonucleotides, *Photochem. Photobiol. 72*, 298−307.
41. Vasquez, K. M., Christensen, J., Li, L., Finch, R. A., and Glazer, P. M. (2002) Human XPA and RPA DNA repair proteins participate in specific recognition of triplex-induced helical distortions, *Proc. Natl. Acad. Sci. U.S.A. 99*, 5848−5853.
42. Majumdar, A., Puri, N., Cuenoud, B., Natt, F., Martin, P., Khorlin, A., Dyatkina, N., George, A. J., Miller, P. S., and Seidman, M. M. (2003) Cell cycle modulation of gene targeting by a triple helix-forming oligonucleotide, *J. Biol. Chem. 278*, 11072−11077.
43. Wang, G., Seidman, M. M., and Glazer, P. M. (1996) Mutagenesis in mammalian cells induced by triple helix formation and transcription-coupled repair, *Science 271*, 802−805.
44. Aboussekhra, A., Biggerstaff, M., Shivji, M. K., Vilpo, J. A., Moncollin, V., Podust, V. N., Protic, M., Hubscher, U., Egly, J. M., and Wood, R. D. (1995) Mammalian DNA nucleotide excision repair reconstituted with purified protein components, *Cell 80*, 859−868.
45. Mu, D., Park, C. H., Matsunaga, T., Hsu, D. S., Reardon, J. T., and Sancar, A. (1995) Reconstitution of human DNA repair excision nuclease in a highly defined system, *J. Biol. Chem. 270*, 2415−2418.
46. Schramke, V., Luciano, P., Brevet, V., Guillot, S., Corda, Y., Longhese, M. P., Gilson, E., and Geli, V. (2004) RPA regulates telomerase action by providing Est1p access to chromosome ends, *Nat. Genet. 36*, 46−54.

47. Robbins, J. B., Murphy, M. C., White, B. A., Mackie, R. I., Ha, T., and Cann, I. K. (2004) Functional analysis of multiple single-stranded DNA-binding proteins from Methanosarcina acetivorans and their effects on DNA synthesis by DNA polymerase BI, *J. Biol. Chem. 279*, 6315−6326.
48. Cotmore, S. F., and Tattersall, P. (1998) High-mobility group 1/2 proteins are essential for initiating rolling-circle-type DNA replication at a parvovirus hairpin origin, *J. Virol. 72*, 8477−8484.
49. Cotmore, S. F., Christensen, J., and Tattersall, P. (2000) Two widely spaced initiator binding sites create an HMG1-dependent parvovirus rolling-hairpin replication origin, *J. Virol. 74*, 1332−1341.
50. Christensen, J., Cotmore, S. F., and Tattersall, P. (1995) Minute virus of mice transcriptional activator protein NS1 binds directly to the transactivation region of the viral P38 promoter in a strictly ATP-dependent manner, *J. Virol. 69*, 5422−5430.
51. Christensen, J., and Tattersall, P. (2002) Parvovirus initiator protein NS1 and RPA coordinate replication fork progression in a reconstituted DNA replication system, *J. Virol. 76*, 6518−6531.
52. Vasquez, K. M., Wensel, T. G., Hogan, M. E., and Wilson, J. H. (1995) High-affinity triple helix formation by synthetic oligonucleotides at a site within a selectable mammalian gene, *Biochemistry 34*, 7243−7251.
53. You, J. S., Wang, M., and Lee, S. H. (2003) Biochemical analysis of the damage recognition process in nucleotide excision repair, *J. Biol. Chem. 278*, 7476−7485.
54. Brezniceanu, M. L., Volp, K., Bosser, S., Solbach, C., Lichter, P., Joos, S., and Zornig, M. (2003) HMGB1 inhibits cell death in yeast and mammalian cells and is abundantly expressed in human breast carcinoma, *FASEB J. 17*, 1295−1297.
55. Lotze, M. T., and DeMarco, R. A. (2003) Dealing with death: HMGB1 as a novel target for cancer therapy, *Curr. Opin. Investig. Drugs 4*, 1405−1409.
56. Pasheva, E. A., Ugrinova, I., Spassovska, N. C., and Pashev, I. G. (2002) The binding affinity of HMG1 protein to DNA modified by cis-platin and its analogs correlates with their antitumor activity, *Int. J. Biochem. Cell Biol. 34*, 87−92.
57. Jayaraman, L., Moorthy, N. C., Murthy, K. G., Manley, J. L., Bustin, M., and Prives, C. (1998) High mobility group protein-1 (HMG-1) is a unique activator of p53, *Genes Dev. 12*, 462−472.
58. McKinney, K., and Prives, C. (2002) Efficient specific DNA binding by p53 requires both its central and C-terminal domains as revealed by studies with high-mobility group 1 protein, *Mol. Cell. Biol. 22*, 6797−6808.
59. Imamura, T., Izumi, H., Nagatani, G., Ise, T., Nomoto, M., Iwamoto, Y., and Kohno, K. (2001) Interaction with p53 enhances binding of cisplatin-modified DNA by high mobility group 1 protein, *J. Biol. Chem. 276*, 7534−7540.
60. Thoma, B. S., and Vasquez, K. M. (2003) Critical DNA damage recognition functions of XPC-hHR23B and XPA-RPA in nucleotide excision repair, *Mol. Carcinog. 38*, 1−13.

BI047902N