High-Mobility Group A1 Proteins Inhibit Expression of Nucleotide Excision Repair Factor Xeroderma Pigmentosum Group A

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Abstract

Cells that overexpress high-mobility group A1 (HMGA1) proteins exhibit deficient nucleotide excision repair (NER) after exposure to DNA-damaging agents, a condition ameliorated by artificially lowering intracellular levels of these nonhistone proteins. One possible mechanism for this NER inhibition is down-regulation of proteins involved in NER, such as xeroderma pigmentosum complementation group A (XPA). Microarray and reverse transcription-PCR data indicate a 2.6-fold decrease in intracellular XPA mRNA in transgenic MCF-7 cells overexpressing HMGA1 proteins compared with non–HMGA1-expressing cells. XPA protein levels are also ~3-fold lower in HMGA1-expressing MCF-7 cells. Moreover, whereas a ~2-fold induction of XPA proteins is observed in normal MCF-7 cells 30 min after UV exposure, no apparent induction of XPA protein is observed in MCF-7 cells expressing HMGA1. Mechanistically, we present both chromatin immunoprecipitation and promoter site-specific mutagenesis evidence linking HMGA1 to repression of XPA transcription via binding to a negative regulatory element in the endogenous XPA gene promoter. Phenotypically, HMGA1-expressing cells exhibit compromised removal of cyclobutane pyrimidine dimer lesions, a characteristic of cells that express low levels of XPA. Importantly, we show that restoring expression of wild-type XPA in HMGA1-expressing cells rescues UV resistance comparable with that of normal MCF-7 cells. Together, these data provide strong experimental evidence that HMGA1 proteins are involved in inhibiting XPA expression, resulting in increased UV sensitivity in cells that overexpress these proteins. Because HMGA1 proteins are overexpressed in most naturally occurring cancers, with increasing cellular concentrations correlating with increasing metastatic potential and poor patient prognosis, the current findings provide new insights into previously unsuspected mechanisms contributing to tumor progression. [Cancer Res 2007;67(13):6044–52]

Introduction

The ability to recognize and respond to DNA damage is a ubiquitous property of living organisms, from *Mycoplasma* to multicellular vertebrates. The two pathways of DNA repair thought to be responsible for a majority of day-to-day genomic maintenance are the base excision repair (BER) and nucleotide excision repair (NER) pathways. Whereas BER is known to repair small, single-base modifications, most of which arise from endogenous damage, such as oxidation (1), NER is responsible for detecting and repairing large, helix-distorting lesions, such as cyclobutane pyrimidine dimers (CPD), (6-4) photoproducts, and cisplatin adducts (2).

Deficiencies in proteins involved in NER result in severe sensitivity to DNA-damaging agents, such as UV light and cisplatin, whose lesions are normally repaired via this pathway (3). It was through the identification of patients exhibiting this phenotype that many of the factors involved in NER were, in fact, identified. In particular, patients deficient in functional xeroderma pigmentosum group A (XPA) protein display the most severe symptoms of the human disease xeroderma pigmentosum, for which eight of the NER factors are named (4).

The XPA protein is a highly conserved, 38- to 42-kDa polypeptide containing three separate protein-protein interaction domains, a nuclear localization signal, and a zinc finger domain (5). Although nuclear localization of XPA is observed under many different experimental conditions, nuclear sequestration of this protein is not required for functional participation in NER, during which it is thought to play several different roles (6). Moreover, based on the discovery of a cancer-predisposing human disease characterized by lack of functional XPA, it is apparent that this protein is essential for cellular processes of genome maintenance.

In most normal cells, the XPA gene is constitutively transcribed at very low levels (7, 8) and is not usually induced in response to DNA-damaging agents, such as UV light (9). Nevertheless, it has been convincingly shown that variations in the intracellular concentrations of XPA have dramatic affects on NER efficiency. For example, Cleaver et al. have shown that UV sensitivity in human cells is a linear function of intracellular XPA concentrations (10) with even modest decreases in XPA levels significantly reducing overall NER competence (10, 11).

Previous work from our laboratory has shown that transgenic MCF-7 cells induced to overexpress high-mobility group A1 (HMGA1; also known as HMG-I/Y; ref. 12) proteins, as well as cancerous cell lines that naturally overexpress these proteins, exhibit both increased UV sensitivity as well as compromised NER of CPD lesions (13). Importantly, HMGA1 proteins were directly implicated in mediating both of these cellular phenotypes because artificial reduction of the endogenous concentrations of HMGA1 proteins in overexpressing cells increased both their ability to repair CPD lesions and their ability to survive UV exposure (13). Additionally, recent work by Baldassarre et al. (14) has shown that embryonic stem cells overexpressing HMGA proteins are also more sensitive to dimethylsulfate, an alkylation agent whose base...
modifications are typically repaired via BER. Finally, Boo et al. (15) have also shown that HMGA proteins potentiate genotoxic stress induced by exposure of breast cancer cells to either doxorubicin, a topoisomerase II inhibitor, or cisplatin, a DNA cross-linking agent. Given these highly suggestive data, in combination with results from our previous microarray analyses indicating decreased concentrations of XPA mRNA in cells overexpressing HMGA1 (16), we decided to experimentally address whether HMGA1 overexpression negatively regulates intracellular concentrations of XPA protein and whether XPA deficiency plays a role in the previously shown NER inefficiency displayed by cells that overexpress HMGA1 (13).

Materials and Methods

Cell culture. The human breast adenocarcinoma cell line MCF7-tet (i.e., MCF7/Tet-Off; BD Biosciences) was cultured as described previously (13). Clonal cell line MCF7-7C-Cs is a stably transfected derivative of MCF7/Tet-Off cells containing a tetracycline-regulated pTRE vector encoding hemagglutinin (HA)-tagged HMGA1a cDNA, maintained in the presence of 100 μg/mL hygromycin (13). This cell line expresses high levels of transgenic HA-tagged HMGA1a protein when grown in medium lacking tetracycline (these are referred to as HMGA1 "ON" cells). To prevent expression of HMGA1 transgenes, MCF7-7C-Cs cells were cultured in medium containing 2 μg/mL tetracycline (and are referred to as HMGA1 "OFF" cells).

Cell transfections and luciferase assays. Transfections of the pGL3 Basic luciferase reporter plasmid carrying either the wild-type XPA promoter region (XPA wt-luc) or the negative regulatory mutant XPA promoter construct (XPA mut-luc) were done using Invitrogen’s LipofectAMINE transfection reagent and carried out according to the manufacturer’s recommendations. Briefly, 200 ng of either wild-type or mutant reporter plasmid and 50 ng of pHRB Renilla plasmid (as a transfection efficiency control) were mixed with 2 μL of LipofectAMINE transfection reagent. Complexes were incubated with 70% confluent cells for 5.5 h in the absence of serum. Transfections were rescued by the addition of medium containing 2× serum directly to the transfection medium and allowed to incubate for 48 h. Twelve independent transfection experiments were done with the wild-type XPA wt-luc construct and at least three independent experiments with the mutant XPA mut-luc construct in both the HMGA1 ON and HMGA1 OFF cell lines.

Luciferase reporter assays were done with Promega’s Dual-Luciferase Assay kit according to the manufacturer’s recommendations and read on a Berthold Technologies Lumat LB 9507. Relative light units (RLU) were measured for both the reporter and Renilla plasmids and then calculated for RLUs of reporter per one RLU of Renilla transfection control plasmid.

Multiplex reverse transcription-PCR analysis. HMGA1 ON and HMGA1 OFF cells were cultured as described above until 90% confluent. Total RNA was isolated from cells via Trizol (Invitrogen) extraction according to the manufacturer’s protocol. RNA was resuspended in RNase-free double-distilled water and quantitated via spectrophotometry. Total RNA (1 μg) from each sample was then used as template in a first-strand synthesis reaction using First-Strand cDNA Synthesis kit for reverse transcription-PCR (RT-PCR; Roche) according to the manufacturer’s protocol. Single-stranded cDNA was quantitated by spectrophotometry and 50 ng were used as template in a multiplex PCR to amplify either HMGA1 and hypoxanthine phosphoribosyltransferase (HPRT) sequences or XPA and HPRT sequences. Primers used for HMGA1 amplification were GAD1 (sense, 5′-gtgctttgggtgcttgaagtc-3′) and GAD2 (antisense, 5′-cattggtgcttgaagtc-3′). PCR amplification products were resolved on 1% agarose containing ethidium bromide (0.2 μg/mL) and quantitated using Quantity One software (Bio-Rad). No-template reactions for each primer set were included as negative controls, and reactions using either a construct encoding full-length, wild-type HMGA1 or XPA as template served as positive controls for each primer set.

In vitro site-specific mutagenesis. A 9-bp, suspected HMGA1-binding stretch of A/T-rich DNA (5′-tatttttaaga-3′) located between nucleotides –268 and –276 in the negative regulatory region of the XPA gene promoter (Fig. 2A) was randomly changed to a nonbinding sequence (5′-gctggaactc-3′) by in vitro site-specific mutagenesis using the commercially available QuikChange II XL Site-Directed kit (Stratagene) following instructions provided by the supplier. The MatInspector2 program was used to select perfectly matched DNA sequences of XPA transcription factor–binding sites. The 67-nucleotide-long PCR primers used for in vitro mutagenesis had the following sequences: 5′-gctatatctg-3′ (forward) and 5′-etgatcatatctcaagactgttgctttcgctgcggcggctctgttactcagatacgtgatc-3′ (reverse).

The italicized nucleotides indicate those changed from the wild-type sequence. Both strands of the mutagenized promoter DNA were confirmed by automated sequencing.

Western blot analysis. Levels of intracellular XPA and HMGA1 proteins were determined by Western blot analysis of cell extracts prepared with Trizol according to the manufacturer’s instructions. Total protein (10 μg) from either HMGA1 ON or HMGA1 OFF cells was loaded onto a 12% polyacrylamide gel in the presence of SDS. Proteins were separated electrophoretically at 100 V for 2.5 h before transference to Immobilon-P membrane (Millipore) via tank transfer in buffer containing 25 mmol/LTris (pH 7.5), 0.2 mol/L glycine, and 20% methanol at 100 V for 1 h. Before addition of primary antibodies, membranes were blocked in TBS containing 5% nonfat dry milk. Membranes were probed for XPA, HMGA1, and total actin (as a loading control) using either a monoclonal antibody against XPA (1:1,500 dilution; Santa Cruz Biotechnology), a specific polyclonal antibody against HMGA1 proteins (1:1,000 dilution; ref. 17), or polyclonal anti-actin rabbit antibody (1:5,000; Sigma). Secondary antibodies were either horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:3,000; Santa Cruz Biotechnology) or HRP-conjugated goat anti-mouse (1:10,000; Pierce). Blots were developed using SuperSignal chemiluminescent substrate (Pierce). Films were scanned and quantitated densitometrically using ImageQuant software to determine fold differences observed.

For UV induction, cells were cultured as described above until 90% to 95% confluent. Medium was removed from culture plates and cells were washed once with 1× PBS. Cells were then irradiated using low-pressure Hg lamps (model G30T8; Sylvania) at a dose of 20 J/m² (measured with a Spectroline DM-254N shortwave UV meter; Spectronic Corp.). Immediately after UV exposure, complete medium was replaced, and cells were incubated for 30 min before protein extraction was done as above.

For XPA overexpression, both HMGA1 ON and HMGA1 OFF cells were cultured in serum-free medium for 24 or 48 h before protein extraction was done as above. Two software (Bio-Rad). No-template reactions for each primer set were included as negative controls, and reactions using either a construct encoding full-length, wild-type HMGA1 or XPA as template served as positive controls for each primer set.

Immunocytochemical analyses. HMGA1 ON and HMGA1 OFF cells were cultured on sterile glass coverslips measuring 22 mm × 22 mm under standard culture conditions as described above until 50% to 60% confluent. Medium was removed and cells were washed once with PBS. Coverslips were then irradiated at 20 J/m² (as described above) and either fixed immediately or replaced in culture medium and incubated to allow repair

1. J. E. Adair and R. Reeves, unpublished data.

to occur. At 6 and 24 h after irradiation, coverslips were fixed in ice-cold absolute methanol for 20 min at −20°C followed by washing in PBS for 5 min before being permeabilized using PBS + 0.1% Triton X-100. Coverslips were washed thrice in PBS and subjected to 30 min in 2 mol/L HCl to deproteinize and denature dsDNA. This was followed by a 5-min wash in 1 mol/L borate buffer (pH 8.0) and three washes in PBS for 5 min each. Coverslips were blocked by adding 5% nonfat dry milk in PBS and incubating at room temperature for 30 min. A primary antibody against CPD lesions (18) was added to blocking solution at a 1:500 dilution and incubated for 1 h at room temperature. Coverslips were then washed thrice with PBS + 0.1% Tween 20 before addition of anti-mouse Oregon Green–conjugated secondary antibody (for detection of CPD antibody) was added at a 1:200 dilution in blocking solution and incubated at room temperature in the dark for 1 h. Coverslips were then washed again for 5 min each in PBS + 0.1% Tween 20 before mounting on sterile glass microscope slides in mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) as a nuclear stain. Confocal microscopy was used to visualize cell nuclei via DAPI fluorescence as well as CPD lesions. Individual cells were counted for each of five random fields, and total field fluorescence was divided by the number of cells per field to obtain average per cell fluorescence intensities for each treatment and time after UV irradiation.

For colocalization analyses, micropore irradiation (19) was used as opposed to whole-cell irradiation due to the ubiquitous nature of the chromatin immunoprecipitation analyses. To determine whether HMGA1 was associated with the endogenous XPA promoter, HMGA1 ON cells were cultured as indicated above until complete confluence was obtained. Approximately 4 × 10^6 cells were trypsinized and resuspended in 10 mL serum-free DMEM containing 1% formaldehyde and incubated for 10 min at 37°C. Glycine was added to a final concentration of 125 mmol/L to quench cross-linking, and cells were pelleted by centrifugation. Nuclei were isolated from the cell pellets and extensively sonicated, and the resulting small chromatin fragments were processed following our previously described chromatin immunoprecipitation (ChiP) techniques (20) using rabbit MR19 anti-HMGA1 antibody (17). To evaluate whether HMGA1 was endogenously associated with the XPA promoter, PCR of immunoprecipitated DNA was done using primers specific to a 450-bp region of the 5′-untranslated region (5′-UTR) of the XPA gene, which consists of one identified potential HMGA1-binding site and the only conserved regulatory element described within this promoter, a 100-bp negative regulatory region (21, 22). Primers used were XPA New Neg S1 (sense, 5′-aacgctgaacctactaatcc-3′) and XPA 5′-UTR AS1 (antisense, 5′-ctcctcggtgtctctaa-3′). PCR amplification of a portion of the KIT ligand gene promoter, in which HMGA1 is bound in these cells (20), was done as a positive control to show effective immunoprecipitation. In addition, amplification of a fragment of the HPRT gene promoter, which does not bind HMGA1 in vivo (20), was done as a negative control for the ChiP reactions. Products of the PCRs were resolved by electrophoresis in 1% agarose in the presence of ethidium bromide.

**Results**

XPA mRNA and protein levels are lowered in HMGA1-overexpressing cells. Our previous microarray gene expression analyses indicated that intracellular concentrations of XPA mRNA were decreased 2.6-fold in HMGA1 ON cells compared with HMGA1 OFF cells (16). To confirm this observation, multiplex RT-PCR was used (see Fig. 1A). In these experiments, total RNA was isolated from parental MCF-7 cells as well as from transgenic MCF-7 cells either induced (HMGA1 ON) or not induced (HMGA1 OFF) to overexpress the HMGA1 proteins. Equal amounts of total

![Figure 1](https://www.aacrjournals.org/cancerres/077/13/01/0771301646/Figure1.png)

**Figure 1.** XPA transcript and protein are reduced in HMGA1-expressing MCF-7 cells. A, multiplex RT-PCR analysis of total RNA extracted from MCF-7 cells either noninduced (HMGA1 OFF; lanes 1 and 3) or induced (HMGA1 ON; lanes 2 and 4) to express HMGA1 proteins. Lanes 1 and 2, PCR products from reaction containing primers to both HMGA1 and HPRT gene transcripts; lanes 3 and 4, PCR products from reactions containing primers to XPA and HPRT gene transcripts. B, Western blot analysis of total protein isolated from HMGA1 OFF (lanes 1 and 2) and HMGA1 ON (lanes 3 and 4) cells. Lanes 1 and 3, total protein from untreated cells; lanes 2 and 4, total proteins isolated 30 min after a 20 J/m^2^ dose of UV radiation. C, graphical representation of Western blot densitometric analysis. White columns, HMGA1 OFF cell XPA protein; dark gray columns, HMGA1 ON cell XPA. Columns, mean of three independent experiments for each cell type and treatment; bars, SD.
RNA were used to produce cDNA, which was subsequently used as template in a multiplex PCR to amplify either HMGA1 and HPRT transcript sequences or XPA and HPRT transcript sequences. As shown in Fig. 1A, these analyses indicated that HMGA1 OFF cells expressed only low levels of both HMGA1 (lane 1) and XPA (lane 2) transcripts. In contrast, HMGA1 ON cells (after normalization to levels of "control" HPRT transcripts produced in the same multiplex reaction) showed ~10-fold more HMGA1 transcript (lane 3) and no detectable XPA transcript (lane 4).

As transcript levels are not always indicative of intracellular protein concentrations, Western blot analysis was used to assess intracellular concentrations of XPA in both HMGA1 OFF and HMGA1 ON cells (Fig. 1B). Although both cell lines showed detectable XPA levels, the amount of XPA detected in HMGA1 ON cells was significantly lower than that observed in HMGA1 OFF cells (Fig. 1B, compare lanes 1 and 3). Moreover, when total proteins were isolated from cells 30 min after a 20 J/m² dose of UV radiation, there was a significant increase in intracellular XPA concentrations in HMGA1 OFF cells (Fig. 1B, compare lanes 1 and 2). In contrast, XPA levels seemed unchanged in HMGA1 ON cells under the same conditions (Fig. 1B, compare lanes 3 and 4). Densitometric analyses of three independent assessments of intracellular XPA concentration revealed a basal 3-fold difference between HMGA1 OFF and HMGA1 ON cells before UV irradiation and a 6-fold difference after UV exposure (Fig. 1C). As indicated in Fig. 1B, HMGA1 protein levels were not altered in cells on UV exposure.

HMGA1 is associated with the endogenous XPA gene promoter in living cells. HMGA1 proteins have been shown to participate in both positive and negative regulation of transcription of a large number of mammalian genes by controlling the formation of multicomponent protein-DNA complexes on gene promoter regions (12, 20, 23, 24). Thus, ChIP assays were done to determine if HMGA1 proteins associate with the endogenous XPA promoter (Fig. 2). Briefly, HMGA1 ON cells were exposed to formaldehyde to cross-link proteins to proteins and proteins to DNA wherever close intracellular interactions between the two exist. Isolated chromatin was then sonicated to produce average DNA fragments of ~500 bp in size, and immunoprecipitation of protein and DNA complexes containing HMGA1 was accomplished by incubation in the presence of an antibody specific to HMGA1 proteins. Immunoprecipitated DNA was then purified and subjected to PCR analysis. In these experiments, as shown in Fig. 2, a 450-bp region of the XPA promoter, known to contain a negative regulatory region of ~100 bp that is conserved between mice and humans, was amplified (21, 22). Importantly, this region of the promoter also contains several potential HMGA1-binding domains, as defined by a sequence of six or more adenine or thymine bases (25), one of which is located within the negative regulatory region at nucleotides −268 to −276 (see Fig. 2A). As shown by the ChIP results in Fig. 2B (lane 3), preferential amplification of DNA corresponding to this region of the XPA promoter strongly suggests that HMGA1 proteins either are directly bound to the DNA itself or are in very close proximity to this particular genomic sequence as a result of indirect interactions with other DNA-bound proteins. As positive and negative controls for these ChIP reactions, using the same immunoprecipitation samples, PCR amplifications were also done for the promoters of genes previously shown (20) to either bind (e.g., the KIT ligand) or not (e.g., HPRT) HMGA1 proteins in vivo (Fig. 2B). Additional negative controls (Fig. 2B) included immunoprecipitations with either nonspecific (lane 4) or no (lane 5) antibody in the reactions.

Mutation of an A/T-rich sequence in the XPA promoter relieves transcriptional repression. These ChIP results provide strong support for a close association of HMGA1 with the negative regulatory region of the XPA promoter in living cells but do not directly show that the repressive effects of HMGA1 on XPA gene transcription (Fig. 1A) are mediated through the nine-nucleotide-long stretch of A/T-rich sequence located between nucleotides −268 and −276, the only potential binding site for the HMGA1 protein in this negative control element. To investigate this possibility, in vitro site-directed mutagenesis was used to randomly alter the nine nucleotides in this stretch of DNA so that they would no longer be a potential binding site for either HMGA1 (Fig. 2A) or any other known mammalian transcription factor (see Materials and Methods). Both the wild-type and mutant XPA promoter sequences were ligated into a luciferase reporter plasmid to produce expression vectors XPA wt-luc and XPA mut-luc, respectively. HMGA1 ON and HMGA1 OFF cells were individually transfected with either XPA wt-luc or XPA mut-luc, and 48 h after transfection, the cells were lysed and the amount of luciferase activity in the extracts was determined. The graph in Fig. 3 shows the results obtained when HMGA1 OFF cells were transfected with either XPA wt-luc (Fig. 3, gray column) or XPA mut-luc (Fig. 3, black column) and shows that the transcriptional activity of the XPA promoter significantly increases (by ~50%) when the A/T-rich stretch in the negative regulatory region is mutated to a sequence that will not bind HMGA1. Similar differential transcription trends were also observed when the XPA wt-luc and XPA mut-luc plasmids were transfected into ON cells that are overexpressing HMGA1 protein (data not shown).

HMGA1 proteins do not colocalize with CPD lesions in living cells. In accordance with phenotypes observed in human cells that have been experimentally induced to express low levels of functional XPA protein (10), defective NER of CPD lesions was observed in HMGA1 ON cells, but not in HMGA1 OFF cells or normal MCF-7 cells, both of which express low to undetectable levels of HMGA1 proteins (13). The immunocytochemical results shown in Fig. 4 qualitatively verify this phenomenon. In these experiments, a specific monoclonal antibody was used to detect cis-syn CPDs in nuclei of HMGA1 OFF (Fig. 4A–D) and ON (Fig. 4E–G) cells that had been exposed to UV irradiation (10 J/m²) and then either immediately fixed (time 0; Fig. 4B and F) or allowed to undergo NER for either 6 h (Fig. 4C and G) or 24 h (Fig. 4D and H). Figure 4A and E shows control reactions in which non–UV-treated cells were reacted with the anti-CPD antibody to determine levels of background fluorescence. From these results, two main observations can be made. First, it is evident that at "time 0" the nuclei of both the OFF cells and the ON cells that have been UV irradiated fluoresce with approximately equal intensity, indicating that similar amounts of UV-induced CPDs are present in the global genomic DNA of both cell types. Thus, overexpression of HMGA1 proteins in the ON cells does not seem to enhance the overall amount of UV-induced damage formation. Second, at 6 h after irradiation, fluorescence of HMGA1 ON cell nuclei is much brighter than is the nuclear fluorescence of the OFF cells containing very low levels of HMGA1 proteins (Fig. 4C and G), and even at 24 h after irradiation, a difference in intensity between the ON and OFF cells is still apparent (Fig. 4D and H), although not to the same extent. These highly reproducible results qualitatively confirm, by an independent experimental method, our previously reported quantitative demonstration that overexpression of HMGA1 proteins inhibits global genomic NER of CPD lesions in vivo (13).
We have also previously shown, under in vitro conditions, that HMGA1 proteins exhibit a high binding affinity for non-B-form DNA (12, 24) and that they are capable of binding to, and altering the formation of, CPD lesions in a DNA fragment rich in A/T residues (13). These findings, therefore, raised the question of whether HMGA1 proteins can also selectively bind to CPD lesions in living cells, thus providing a likely explanation for the NER defects observed in HMGA1 ON cells. To investigate this possibility, immunocytochemical analyses were done using specific antibodies to determine whether HMGA1 and CPDs are colocalized in ON cells exposed to 20 J/m² UV irradiation. In these experiments, HMGA1-expressing cells were micropore irradiated (19) rather than whole-cell irradiated as described in Materials and Methods. Micropore analysis was used to avoid potential spurious colocalization artifacts that could arise from several sources (13, 19). In such analyses, colocalization of molecules is examined in time course experiments in only subnuclear "foci" of DNA damage corresponding to small holes in a masking micropore filter placed over the cells before UV irradiation. Figure 5 shows a representative example of such an experiment in which ON cells were replaced in culture medium following UV irradiation and allowed 30 min to equilibrate before being fixed in absolute methanol and examined. Based on kinetic data showing that HMGA1 proteins are remarkably mobile in the nuclei of living cells with rapid on/off rates at most genomic sites (26), this 30-min equilibration period would allow for HMGA1 proteins to diffuse to and/or from DNA lesions induced within micropore-irradiated nuclei. As seen in Fig. 5 (left), numerous sites of HMGA1 accumulation (Fig. 5, x-HMGA1 TR, red) are scattered throughout the nuclei of cells exposed to UV light but these localized concentrations of protein are not (for the most part) induced by the irradiation itself because similar HMGA1 distribution patterns are also observed in the nuclei of normal, nonirradiated cells (27, 28). In the case of CPD lesions, direct support for this conclusion is provided by the results shown in Fig. 5 (middle and right), which show that, although localized areas of CPD lesions were readily visualized within micropore-irradiated nuclei (Fig. 5, a-CPD OG, green), no yellow fluorescence, indicative of HMGA1 and CPD colocalization, was observed in any merged...
fields examined. These results indicate that HMGA1 proteins are not stably associated with sites of CPD damage in living cells soon after UV irradiation.

XPA complimentation restores UV resistance. To assess whether the observed XPA deficiencies associated with HMGA1 overexpression caused the NER defects also seen in these cells, XPA complimentation studies were done. In these experiments, a vector encoding full-length, wild-type XPA, under control of a constitutive CMV promoter, was transfected into both HMGA1 OFF and HMGA1 ON cells and cell viability after UV irradiation at a variety of UV doses was examined (Fig. 6). As shown in the Western blots in Fig. 6A, these transient transfections resulted in increased expression of XPA protein in both ON and OFF cells compared with control cells transfected with an empty pcDNA3.1 vector (Fig. 6A, compare lanes 3–6 with lanes 1 and 2). Importantly, overexpression of XPA did not alter HMGA1 levels in either ON or OFF cells (Fig. 6A, lanes 3–6).

When cell survival after UV irradiation was examined in XPA-overexpressing cells, no significant difference in survival between HMGA1 OFF and HMGA1 ON cells was observed at UV doses between 2 and 100 J/m², indicating that XPA deficiencies are responsible for previously described UV sensitivity in these cells (Fig. 6B; ref. 13). This is exemplified by the significant increase in UV resistance between HMGA1 ON cells overexpressing XPA compared with HMGA1 ON cells transfected with empty pcDNA3.1 vector (Fig. 6B). Moreover, there were no significant increases in cell survival between HMGA1 OFF and nontransgenic parental MCF-7 cells before and after induction of XPA, indicating that the endogenous concentrations of XPA in these cells are sufficient for normal levels of repair to occur. Likewise, additional control experiments showed that there was no difference between survival of parental MCF-7 and transgenic HMGA1 OFF cells that were transfected with an empty pcDNA3.1 vector (data not shown; ref. 13).

Discussion

Previous work from our laboratory showed that MCF-7 cells overexpressing HMGA1 proteins are as much as 50% less efficient in their ability to repair UV-induced CPD lesions compared with non–HMGA1-overexpressing MCF-7 cells (13). These findings, in combination with other work indicating that HMGA1-expressing cells are more sensitive to cisplatin (14), suggest that cells expressing HMGA proteins are compromised in their ability to carry out NER efficiently.

Given the diverse array of protein-protein interactions involving HMGA1, as well as the ability of HMGA proteins to bind DNA in a structure-specific manner, one possible mechanism for the observed NER deficiency in cells overexpressing these proteins would be by interference with repair factor binding. In this model, direct binding of HMGA1 proteins to CPDs could inhibit NER factor access to lesion sites as a consequence of steric hindrance. Indeed, our previously published in vitro analyses indicated that HMGA1 could bind to DNA fragments containing CPD lesions and, moreover, inhibit repair of DNA fragments containing CPD lesions.

Figure 3. Site-specific mutations of the A/T-rich HMGA1-binding site in the negative regulatory region (nucleotides −268 to −276) of the XPA promoter relieve gene transcriptional repression. Results of luminescence assays (depicted in RLUs) obtained with HMGA1 OFF cells that were transfected with either the wild-type XPA wt-luc plasmid construct (gray column) or the mutant XPA mut-luc construct (black column). Similar differential trends were also observed when the XPA wt-luc and XPA mut-luc plasmids were transfected into HMGA1 ON cells (data not shown).

Figure 4. Global genomic NER of CPD lesions is inhibited in MCF-7 cells overexpressing HMGA1 proteins. Immunocytochemistry using anti-CPD primary antibody and Oregon Green secondary antibody in MCF-7 cells induced to either express (bottom) or not express (top) HMGA1a proteins. Cells were exposed to 10 J/m² and then either immediately fixed (F and B; C and G) or allowed to undergo NER for either 6 h (D and H) and then probed for the presence of CPDs. A and E, control reactions in which non–UV-treated cells are reacted with the anti-CPD antibody. Representative of the average total fluorescence observed in five random fields for each treatment at ×600 magnification.
by Xenopus oocyte nuclear extracts (13). The results of immunocytochemical studies reported here, however, do not show colocalization of HMGA1 proteins at CPD lesion sites within MCF-7 cell nuclei after UV irradiation (Fig. 5). These data suggest that in vivo HMGA1 proteins do not directly interfere with binding of repair factors to CPD lesions and, thereby, directly impede repair processes at the sites of damage.

Another possible mechanism for the observed NER deficiency seen in ON cells is that HMGA1 proteins limit the availability of one or more important NER factors, a likely candidate given the well-established role of the HMGA1 proteins as both positive and negative regulators of gene transcription (24). Further evidence supporting this possibility includes microarray analyses that compared basal transcriptome profiles of HMGA1 OFF and ON cells (16). In these experiments, many gene transcripts were found to be either up-regulated or down-regulated in the presence of overexpressed HMGA1 proteins. Of those transcripts that were found to be down-regulated, however, an unexpectedly large number were involved in DNA damage recognition and repair, including the XPA mRNA.

The work described here indicates that not only are XPA transcript levels decreased in MCF-7 cells overexpressing HMGA1 proteins but intracellular XPA protein levels are proportionately decreased as well in these cells (Fig. 1). Moreover, this decrease in intracellular XPA protein concentrations is exaggerated even further following exposure of cells to UV irradiation at 20 J/m², a dose considered to be within the physiologic range of sunlight exposure (Fig. 1B and C). These data provide convincing evidence that, in the presence of HMGA1 proteins, intracellular concentrations of functional XPA protein are decreased, indicating that XPA deficiency should be explored as a cause of the submaximal NER observed in these cells previously (13).

There are several potential mechanisms by which overexpression of HMGA1 proteins could be negatively influencing intracellular XPA protein levels, including possible effects on transcript splicing or stability (29, 30). Nevertheless, given the extensive body of literature defining a role for HMGA1 proteins in controlling gene transcription via interaction with promoter regulatory regions (12, 24), we initially considered this to be the most likely mechanism by which HMGA1 negatively regulates XPA gene activity. Additional arguments for transcriptional control of the XPA gene include microarray and multiplex RT-PCR evidence showing that basal transcript levels are, in fact (as shown in Fig. 1), depleted in HMGA1 ON cells compared with HMGA1 OFF cells, indicating regulation at the level of either transcription or mRNA stability.

Further evidence indicating a direct role for HMGA1 in negatively regulating transcription of the XPA gene in over-expressing cells comes from two important experimental observations. Using ChIP analyses, we showed that in ON cells HMGA1 is associated with an ~450-bp region of the XPA promoter that contains a previously identified negative regulatory region with one potential HMGA1-binding site of A/T-rich nucleotide residues (Fig. 24). Although these ChIP results provide firm evidence physically linking HMGA1 to the endogenous negative regulatory region, they do not, per se, show that the observed repressive effects of HMGA1 on XPA gene transcription are a consequence of binding of this protein to the only A/T residues within this region. Unequivocal in vivo evidence supporting this possibility comes, however, from experiments in which cells were transfected with expression plasmids containing a luciferase reporter gene driven either by the wild-type XPA promoter or by a mutant XPA promoter in which the nine A/T residues have been replaced by a non–HMGA1-binding stretch of random nucleotide sequence (Fig. 24). The graph in Fig. 3 clearly shows that the level of transcription obtained with the mutant XPA promoter is significantly higher (by ~50%) than that from the wild-type promoter. Because great care was taken while designing this random replacement sequence to ensure that no binding site for other known transcription factors was inadvertently introduced into the mutant promoter, the most parsimonious interpretation of these transfection results is that binding of HMGA1 protein to nucleotides –268 to –276 of the negative regulatory region significantly contributes to repression of transcription of the XPA gene in vivo. Together, the results of these ChIP and promoter mutagenesis experiments provide compelling evidence that the
promoter of the *XPAl* gene is a direct cellular target for transcriptional regulation by HMGA1 proteins.

Several groups have previously reported that cells expressing low intracellular concentrations of functional XPA protein show compromised NER which, as a consequence, may only function efficiently in transcriptionally active regions of the genome (10, 11, 31). Consistent with these observations, the immunocytochemical analyses presented here not only verify deficiencies in CPD removal in HMGA1-overexpressing, XPA-depleted, cells (Fig. 4) but also show that HMGA1 proteins do not bind tightly to CPD lesions in HMGA1 OFF cells (Fig. 5). These cellular localization studies indicate that the inhibitory effect of HMGA1 on repair of CPD lesions is indirect and most likely mediated through transcriptional down-regulation of *XPAl*, a critically important gene involved in both global genomic and transcription-coupled NER (1).

Importantly, XPA deficiencies in HMGA1 ON cells seem to be directly responsible for the observed UV sensitivities displayed by these cells. The XPA complementation studies showing that overexpression of XPA completely restores UV resistance of HMGA1 ON cells to a level comparable with that of normal MCF-7 and HMGA1 OFF cells (Fig. 6B) strongly support this biological connection.

The gene coding for HMGA1 is a proto-oncogene whose overexpression induces neoplastic transformation of normal cells and the formation of aggressive lymphoid malignancies when overexpressed in transgenic mice (32). In many different types of human cancers (including thyroid, colorectal, prostate, breast, cervical, and lung carcinomas; neuroblastomas; and leukemias), the constitutive level of HMGA1 gene products is often exceptionally high with increasing concentrations paralleling increasing degrees of metastatic potential and malignancy (33–39). This correlation is so consistent and widespread that elevated HMGA levels are now being investigated as potential diagnostic molecular biomarkers for clinical assessment and management of patients with a variety of cancers (39–43).

Accumulation of genetic mutations (44) and increases in chromosome abnormalities and genomic instabilities (45) are also hallmarks of tumor progression in many malignant cancers, but in most cases, their underlying molecular causes remain obscure. One hypothesis, originally advanced by Loeb (46) and Loeb et al. (47), to partially explain these phenomena is that cancer cells, even in their earliest stages of development, exhibit a "mutator phenotype" that predisposes them to increased mutation rates that are essential to account for the large number of accumulated mutations observed during carcinogenesis. In this connection, HMGA1 overexpression has recently been linked to both chromosomal rearrangements in prostate cancer cells (48) and aneuploidy in colon carcinomas (49). Furthermore, the present data showing that HMGA proteins can repress transcription of the *XPAl* gene in intact cells provide a plausible explanation for why NER is impaired in cells that overexpress these proteins and also suggest that such inhibition likely contributes to the accumulation of mutations and genetic instabilities commonly observed in many human cancers. Work with *XPAl־־* knockout mice, whose phenotype mimics that of humans with *XPAl* mutations, supports this possibility. These NER-deficient mice have a marked predisposition for both spontaneous and genotoxin-induced gene mutations, for large genomic rearrangements, and for developing natural and carcinogen-induced tumors (reviewed in ref. 50).

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High-Mobility Group A1 Proteins Inhibit Expression of Nucleotide Excision Repair Factor Xeroderma Pigmentosum Group A


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