DNA Repair 8 (2009) 822–833

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

Overexpression of transcription factor AP-2 stimulates the P_A promoter of the human uracil-DNA glycosylase (UNG) gene through a mechanism involving derepression

Per Arne Aas[a], Javier Peña-Diaz[a], Nina Beate Liabakk[a], Hans E. Krokan[a], Frank Skorpen[b,*]

[a] Department of Cancer Research and Molecular Medicine, Children's and Women's Health, Faculty of Medicine, Norwegian University of Science and Technology, N-7489 Trondheim, Norway
[b] Department of Laboratory Medicine, Children's and Women's Health, Faculty of Medicine, Norwegian University of Science and Technology, N-7489 Trondheim, Norway

ARTICLE INFO

Article history:
Received 10 December 2008
Received in revised form 28 March 2009
Accepted 30 March 2009
Available online 2 May 2009

Keywords:
Uracil-DNA glycosylase
UNG
AP-2

ABSTRACT

The P_A promoter in the human uracil-DNA glycosylase gene (*UNG*) directs expression of the nuclear form (UNG2) of UNG proteins. Using a combination of promoter deletion and mutation analyses, and transient transfection of HeLa cells, we show that repressor and derepressor activities are contained within the region of DNA marked by P_A. Footprinting analysis and electrophoretic mobility shift assays of P_A and putative AP-2 binding regions with HeLa cell nuclear extract and recombinant AP-2α protein indicate that AP-2 transcription factors are central in the regulated expression of UNG2 mRNA. Chromatin immunoprecipitation with AP-2 antibody demonstrated that endogenous AP-2 binds to the P_A promoter *in vivo*. Overexpression of AP-2α, -β or -γ all stimulated expression from a P_A-luciferase reporter gene construct approximately 3- to 4-fold. Interestingly, an N-terminally truncated AP-2α, lacking the activation domain but retaining the DNA binding and dimerization domains, stimulated P_A to a level approaching that of full-length AP-2, suggesting that AP-2 overexpression stimulates P_A activity by a mechanism involving derepression rather than activation, possibly by neutralizing an inhibitory effect of endogenous AP-2 or AP-2-like factors.

1. Introduction

Human uracil-DNA glycosylase encoded by the *UNG* gene initiates the DNA base excision repair pathway and plays a major role in the removal of uracil resulting from dUMP misincorporation or from U:G mispairs due to cytosine deamination [1,2]. Mice with a disruption in the *UNG* gene have an increased level of uracil in their genome, and older mice display increased incidence of lymphoid organ pathology, abnormal lymphoproliferation, as well as a greatly increased incidence of B-cell lymphomas [3]. Recently it was shown that recessive mutations in the human *UNG* gene are associated with profound impairment in immunoglobulin (Ig) class-switch recombination (CSR), demonstrating an essential role of the nuclear form of UNG in antigen-dependent immunoglobulin gene alterations associated with adaptive B-cell responses [4]. Thus, UNG proteins have important biological functions in diverse cellular processes such as in the maintenance of genome integrity, and in controlled processing of antibody genes.

The human *UNG* gene is localized on the long arm of chromosome 12 (12q24.1) [5], and contains two promoters, P_A and P_B, directing expression of mRNAs for the nuclear (UNG2) and mitochondrial (UNG1) forms of UNG enzymes, respectively [6]. The highest levels of UNG1 mRNA is found in tissues rich in mitochondria, such as skeletal muscle, heart and testis, whereas the expression of UNG2 mRNA is highly correlated with proliferative status, with the highest levels found in testis, placenta, intestine, and thymus [7]. Both *UNG* promoters lack a TATA-box, are GC rich, and are cell cycle regulated with induction at the G_1/S-phase boundary [7,8]. In serum starved cells (G_0 phase) downregulation of UNG2 is mediated by binding of the E2F-4 repressor to the untranslated part of the first exon [9]. Multiple putative sites for the binding of transcription factors have been identified that regulate *UNG* transcription both positively and negatively. Six putative elements for members of the AP-2 family of transcription factors have been identified within the 224 bp P_A genomic fragment by computer searches, suggesting a potential important role of AP-2 in UNG2 mRNA expression. AP-2 transcription factors represent a family of three closely related and evolutionarily conserved sequence-specific DNA-binding proteins, AP-2α, -β and -γ, which regulate genes involved in a spectrum of important biological functions. We demonstrate here a complex array of repressive and dere-

* Corresponding author. Tel.: +47 7273332; fax: +47 72573801.
E-mail address: frank.skorpen@ntnu.no (F. Skorpen).

1568-7864/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.dnarep.2009.03.008

pressive elements located within the *UNG* P_A promoter and show that short-term (24 h) overexpression of AP-2 transcription factors significantly stimulates P_A, but through a mechanism involving derepression rather than activation.

2. Materials and methods

2.1. Construction of plasmids and site-directed mutagenesis

The plasmids pSG5-hAP-2A, directing expression of human AP-2α, and pSG5-hAP-2B were kindly provided by Dr. Michael A. Tainsky. AP-2B is a naturally occurring transdominant-negative mutant which lacks the C-terminal DNA-binding domain but retains its dimerization ability with AP-2 [10]. AP-2B contains the activation domain of AP-2α and part of the DNA-binding domain but lacks the dimerization domain that is necessary for DNA binding. Expression plasmids for human AP-2γ (pSAP-2γ) and murine AP-2β (pSG5mAP-2β) were kindly provided by Dr. Perry Kannan.

The plasmid pSG-hAP-2-DNA, directing expression of the AP-2α DNA-binding domain, was generated by cleavage of pSG5-hAP-2A by restriction endonuclease *Bam*HI, which cleaves in the codon for amino acid 122 in the AP-2α gene. A *Bam*HI compatible linker containing an *Eco*RI site was ligated to the *Bam*HI digested pSG5-hAP-2A. This linker also introduces a new ATG start codon in-frame with the 3′ part of the AP-2α gene.

This construct was then cut by *Eco*RI, and the fragment containing the sequences corresponding to the AP-2 DNA-binding domain was purified and ligated into the *Eco*RI sites of pSG5 to generate pSG-hAP-2-DNA. The correct orientation of the insert was verified by restriction enzyme digestion. This procedure results in the removal the 122 N-terminal amino acids from AP-2α. The amino acids critical for AP-2 transactivation are located between residues 52 and 108 [11].

The construction of pGL2-P_A and pGL2-P_B has been described previously [7]. Site-directed mutagenesis to generate pGL2-$P_{A\text{-}AP\text{-}2}$ mutants was performed using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Mutagenic oligonucleotide primers, each complementary to opposite strands of the pGL2-P_A construct, were designed and synthesized for introducing specific mutation in putative AP-2 sites as shown in Fig. 6. For the introduction of mutation in several AP-2 sites of one construct, appropriate pGL2-$P_{A\text{-}AP\text{-}2\ mut}$ constructs were used as template in the mutagenesis reaction. Following an initial denaturation at 95 °C for 1 min, the cycling parameters for the mutagenesis reaction were 95 °C for 30 s, 55 °C for 60 s, and 68 °C for 14 min. The 18 thermal cycles were performed on a PerkinElmer GeneAmp PCR System 2400. Following temperature cycling, the product was treated with 1 μl of the restriction endonuclease *Dpn*I (10 U/μl) to cleave the parental DNA template, and was then used to transform heat-shock competent *Esherichia coli* DH5α cells. Plasmid DNAs were purified using Qiagen EndoFree™ Plasmid Kit. The orientation and the sequence of wild-type and mutated plasmids were confirmed by DNA sequencing.

Constructs containing deletions of P_A were generated by cleavage of pGL2-P_A with the restriction endonucleases *Sma*I (position 490), *Fsp*I (position 553), or *Bsr*BI (position 597), to generate the constructs pGL2-P_A-Δ-149, pGL2-P_A-Δ-86, and pGL2-P_A-Δ-42, respectively. Nucleotide positions are give according to GenBank accession no. X89398. pGL2-P_A-Δ-149 was generated by cleaving pGL2-P_A with *Sma*I, followed by religation of the vector. This removes the 5′ portion of the P_A promoter. pGL2-P_A-Δ-86 and pGL2-P_A-Δ-42 were generated by ligating the *Fsp*I/*Sal*I or the *Bsr*BI/*Sal*I promoter fragments of pGL2-P_A into the *Sma*I/*Sal*I sites of pGL-2-Basic. Deletion constructs pGL2-P_A-Δ-130, pGL2-P_A-Δ-115, and pGL2-P_A-Δ-94 were generated by PCR using *Pfu* polymerase and forward primers with 5′ positions at 509, 524 or 546, along with a pGL-2-Basic vector reverse primer. Amplified PCR products were treated with the restriction endonuclease *Xho*I, and ligated into the *Sma*I/*Xho*I sites of the pGL2-Basic vector. The correct sequences of all deletion constructs were verified by DNA sequencing.

2.2. Cell culture and transient transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; 4500 mg glucose/l) supplemented with 10% fetal calf serum, fungizone (1 μg/ml) (Gibco) and gentamicin (50 μg/ml) (Sigma), and maintained at 37 °C in 5% CO_2 and 95% air. Retinoic acid (RA, all-*trans*, Sigma) was prepared as 1 mM stock solution in 96% ethanol and stored at −20 °C in the dark until use. For experiments involving RA, culture medium was replaced with new medium containing RA immediately before transfection. The final concentration of ethanol in cell cultures did not exceed 0.1%.

Transient transfections were performed using the nonliposomal formulation FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 7.5×10^4 HeLa cells were seeded in 24-well culture dishes in 0.5 ml medium and grown overnight before transfection. Each FuGENE 6 Reagent/plasmid DNA suspension (170 μl) was prepared by mixing 0.75 μg of the luciferase reporter plasmid, 50 ng of the internal control plasmid pRL-TK (encoding *Renilla* luciferase; Promega), and varying amounts of the indicated expression plasmids or empty vector (in a total of 20 μl), with 150 μl serum-free DMEM containing 3 μl FuGENE 6 for each μg of DNA. Each mixture was sufficient for triplicate transfections (55 μl were added to each 0.5 ml cell culture). The cell extracts were prepared 24 h post-transfection, using 1× passive lysis buffer (100 μl, Promega). The dual luciferase reporter assays were carried out on each lysate (20 μl) using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA).

2.3. Analysis of uracil-DNA glycosylase activity in retinoic acid treated cells

HeLa cells were cultured until near confluence in DMEM 4500 mg glucose/l with 10% charcoal/dextran treated fetal calf serum (Hyclone) for 24 h prior to addition of the retinoic acid. Trans-retinoic acid (Calbiochem) was prepared in DMSO as 100 mM stock solution and stored at −80 °C in the dark until use. Serial dilutions of retinoic acid were made and added to the cell culture, with DMSO used as a negative control. The final concentration of DMSO did not exceed 0.1%. Cells treated and untreated were grown for additional 24 h and collected. Whole cell extracts were prepared essentially as described in Ref. [12]. Briefly, cells were washed twice with PBS and pelleted at 450 × *g*. Cell pellet was resuspended at 1× packed volume in buffer I [10 mM Tris–HCl (PH 8.0), 200 mM KCl] and 1× packed volume of buffer II [10 mM Tris–HCl (pH 8.0), 200 mM KCl, 2 mM EDTA, 40% (v/v) glycerol, 0.5% NP-40, 2 mM DTT, Complete® protease inhibitor]. The mixture was rocked at 4 °C for 2 h and the cell debris was pelleted at 22,000 × *g* at 4 °C for 10 min. The supernatant was recovered and protein concentration measured using the Bio-Rad protein assay. Whole cell extracts were diluted to 2 μg/μl by adding equal amounts of Buffer I and Buffer II and stored at −80 °C until used. UDG activity was measured as described [13]. Briefly, 200 ng of whole cell extract were used in a 20 μl assay mixture containing (final) 62.5 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA and 1.8 μM [^{3}H]dUMP:A-containing calf-thymus DNA substrate (specific act. 0.5 mCi/μmol). The mixture was incubated for 10 min at 30 °C and the amount of uracil released was measured. One unit of UDG activity was defined as the amount of enzyme releasing 1 nmol of uracil per min at 30 °C.