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Regulation of expression of nuclear and mitochondrial forms of human uracil-DNA glycosylase

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ABSTRACT

Promoters P_A and P_B in the *UNG* gene and alternative splicing are utilized to generate nuclear (UNG2) and mitochondrial (UNG1) forms of human uracil-DNA glycosylase. We have found the highest levels of UNG1 mRNA in skeletal muscle, heart and testis and the highest UNG2 mRNA levels in testis, placenta, colon, small intestine and thymus, all of which contain proliferating cells. In synchronized HaCaT cells mRNAs for both forms increased in late G_1/early S phase, accompanied by a 4- to 5-fold increase in enzyme activity. A combination of mutational analysis and transient transfection demonstrated that an E2F-1/DP-1–Rb complex is a strong negative regulator of both promoters, whereas 'free' E2F-1/DP-1 is a weak positive regulator, although a consensus element for E2F binding is only present in P_B. These results indicate a central role for an E2F-DP-1–Rb complex in cell cycle regulation of UNG proteins. Sp1 and c-Myc binding elements close to transcription start areas were positive regulators of both promoters, however, whereas overexpression in HeLa cells of Sp1 stimulated both promoters, c-Myc and c-Myc/Max overexpression had a suppressive effect. CCAAT elements were negative regulators of P_B, but positive regulators of P_A. These results demonstrate differential expression of mRNAs for UNG1 and UNG2 in human tissues.

INTRODUCTION

Uracil in DNA may result from misincorporation of dUMP or deamination of cytosine (1). Removal of uracil by uracil-DNA glycosylase (UDG) or other damaged bases by other DNA glycosylases represents the first step in base excision repair (BER). Presumably the subsequent steps in BER are common for different DNA glycosylases (reviewed in 2). Recently the BER pathway for repair of uracil residues in DNA has been reconstituted using purified proteins (3,4). UDG activity from the *UNG* gene was found to be critical for reconstitution of the BER pathway for repair of uracil in DNA and could not be substituted by other proteins reported to have UDG activity (4). The gene for human uracil-DNA glycosylase (*UNG*) consists of seven exons and encodes a nuclear (UNG2) and a mitochondrial (UNG1) form of UDG (5,6). mRNAs for UNG2 and UNG1 result from transcription from alternative promoters and alternative splicing and the enzymes differ in their N-terminal amino acid sequences, which are required for nuclear and mitochondrial import respectively. The 269 amino acids downstream of the variant N-terminal sequences are common for UNG1 and UNG2 and include a short region that binds replication protein A (RPA) (7) and a larger and very compact catalytic domain (8) of known structure (9,10). The possible functional significance of RPA binding by UNG proteins has not yet been determined (7). The UDG activity in mammalian cells appears to be cell cycle regulated, increasing 2- to 3-fold early in S phase (11,12). It is also in general significantly higher in proliferative as compared with non-proliferative tissues (13). *UNG* expression has been proposed to be mainly regulated at the transcription level (12). However, the probe used in these studies is now known to detect both UNG1 and UNG2 transcripts, which are of very similar size (6), such that they cannot be separated by agarose gel electrophoresis. Furthermore, enzymatic assays for UDG activity do not discriminate between mitochondrial and nuclear activities and consequently the pattern of expression of UNG1 and UNG2 during the cell cycle, as well as their contribution to UDG activity in different tissues (14,15), are unknown. The promoter upstream of exon 1B (P_B) exhibits typical features of promoters in housekeeping genes. It is very GC-rich, lacks a TATA box and contains both activating and inhibitory regions. In addition to four CCAAT boxes, several regulatory elements binding E2F, Sp1, Ap2 and c-Myc have been identified (5,16). The putative promoter A (P_A) upstream of exon 1A contains putative binding elements for Sp1, c-Myc, c-Myb and Ap2 and one CCAAT box (6). P_A and P_B are both located within a partially methylated CpG island (5).

In the present study we have examined cell cycle expression of mRNAs for UNG1 and UNG2, as well as their expression in various tissues. Furthermore, we have demonstrated that promoters P_A and P_B are both functional and regulated by both positive and negative *cis*- and *trans*-acting factors.

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MATERIALS AND METHODS

Probes

The probes used to detect UNG2 transcripts were a 271 bp genomic *Sma*I fragment (positions 675–946; 5) containing 167 bp of the first exon (exon 1A) and 104 bp of the first intron of the human *UNG* gene (6,16), for cell cycle studies, and exon 1A prepared by PCR, for probing tissue blots. The probe used to detect UNG1 transcripts was the 181 bp *Eco*RI–*Pst*I fragment of pUNG15 (17), corresponding to the first 176 bp of exon 1B of the human *UNG* gene. *UNG2* cDNA (6) of 2058 bp was used to detect UNG1 and UNG2 transcripts in a single band. The probe used to detect c-Myc transcripts was a 1.5 kb *Cla*I–*Eco*RI genomic fragment comprising most of the third exon of the human c-*MYC* gene and some of the 3′ sequences. All probes were labeled in random primer extension reactions (Rediprime™ labeling kit; Amersham, UK) with [α-^{32}P]dCTP (Amersham).

Expression plasmids

pCMV-E2F-1 and pCMV-HADP-1, directing expression of E2F-1 and DP-1 respectively, were kindly provided by Dr Kristian Helin (18,19). pCMV-E2F-1(Y411H), which expresses a point mutated form of E2F-1 unable to bind Rb, was kindly provided by Dr Wen-Hwa Lee (20). p290-Myc and pHeBoCMV-β-globin-Max, directing expression of c-Myc and Max respectively, were kindly donated by Dr Jack Streitman. pRBWT3HA/SVE, expressing Rb, and expression constructs for the corresponding deletion mutants Δ389–580 and Δ662–775 (21,22) were kindly donated by Dr Dennis Templeton. pRSVSp1 has been described previously (23). pCI-Myb, expressing c-Myb, was kindly provided by Dr Odd Gabrielsen.

Construction of plasmids

All promoter constructs were made in the pGL2-Basic vector (Promega) carrying the coding region for firefly luciferase. pGL2-P_A, carrying P_A, was constructed by insertion of a *Pvu*II–*Nhe*I fragment (positions 418 and 660 respectively; 5) from the promoter region of the *UNG* gene into a blunted *Mlu*I site and a *Nhe*I site of pGL2-Basic. Construction of pGL2-P_B carrying P_B has been described elsewhere (16). A construct containing both promoters, pGL2-P_{AB}, was prepared by insertion of a *Pvu*II–*Mlu*I fragment (positions 418–1035; 5) from the promoter region of the *UNG* gene into the *Sma*I and *Mlu*I sites of pGL2- P_B. In this construct only transcripts from P_B will result in an in-frame luciferase protein.

Cell cycle experiments

Aliquots of 5×10^5 HaCaT cells were plated in 60 mm dishes and grown overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 0.3 mg/ml glutamine. After removal of the medium cells were washed once with phosphate-buffered saline (PBS) and once with DMEM. The cells were then grown in DMEM supplemented with 0.3 mg/ml glutamine without FCS for 3 days. Then the medium was replaced by medium containing 10% FCS. Effects of TGFβ-1 were studied after addition of TGFβ-1 (2 ng/ml) in medium containing 10% FCS. For the mimosine experiments the drug was added to the medium to a final concentration of 0.4 mM. To monitor DNA synthesis cultures in 60 mm dishes were pulse labeled with 20 μCi [^{3}H]thymidine (Amersham) for 15 min at 37°C at various time points after addition of serum to starved cells. The cultures were then washed three times with PBS, the cells harvested, resuspended in 100 μl PBS and precipitated by addition of 1 ml 5% trichloroacetic acid. Precipitates were then collected on GFC filters (Whatman) and incorporated activity quantified by scintillation counting. Uracil-DNA glycosylase assays were performed as described (24). Total RNA was isolated as described for northern blot analysis.

Site-directed mutagenesis

The promoter fragments from pGL2-P_A and pGL2-P_B were inserted into the pALTER plasmid and site-directed mutagenesis was performed with the Altered Sites system as recommended by the manufacturer (Promega). All promoter constructs were sequenced using an Applied Biosystems Model 373A Sequencing System to verify the correct structure.

Northern blot analysis

For analysis of UNG1 and UNG2 mRNAs in cell cycle studies total RNA was isolated according to the RNeasy™ protocol (Qiagen Inc., Chartsworth, CA). Total RNA (20 μg) was electrophoresed on 1% agarose gels containing formaldehyde, stained with ethidium bromide, blotted onto Hybond N^+ membrane (Amersham, UK) by vacuum and crosslinked to the membrane by baking (20 min at 120°C). Hybridization was carried out overnight in ExpressHyb™ hybridization solution (Clontech Laboratories Inc., CA) to radio-labeled UNG1 or UNG2 probes. Membranes were washed five times for 20 min in 2× SSC, 1% SDS at 65°C and twice for 20 min in 0.1× SSC, 0.5% SDS at 65°C, enclosed in plastic wrap and exposed on a Molecular Dynamics PhosphorImager SF. Prior to rehybridization the membranes were stripped of probe by boiling in 0.5% SDS for 10 min followed by an additional 10 min in the hot solution after removing it from heat. For analysis of UNG1 and UNG2 transcripts in different human tissues we used pre-made Multiple Tissue Northern Blots (MTN Blots) from Clontech. These were hybridized to radiolabeled probes specific for UNG2 or UNG1 mRNAs, as well as probes for c-MYC and β-actin mRNAs. Conditions for hybridization and washing were as described above, except that 60°C was used in the final wash. Membrane stripping was as described above.

Transfection assays

Plasmid DNA was prepared using the Midi Kit (Qiagen) and phenol/chloroform extractions. Transfection of HeLa cells was performed as previously described (16) except that 4 μg DNA were used per 60 mm culture dish. CCD1070 cells (normal human fibroblasts; ATCC) were transfected under the same conditions as for HeLa cells except that only 2.5×10^5 cells were plated in each 60 mm dish and medium containing FCS was added after 6 h for maximal survival. Aliquots of 0.2 μg pRL-TK vector encoding *Renilla* luciferase were used as an internal control in all transfections. Activities expressed from the firefly (*Photinus pyralis*) luciferase reporter gene and the *Renilla* luciferase control were detected with a luminometer (Turner) using the Dual Luciferase Assay as recommended by the manufacturer (Promega). For the co-transfection experiments 2 μg reporter gene construct were mixed with the specified amount of expression construct (0.05–2 μg). Expression vectors without insert were added when required to

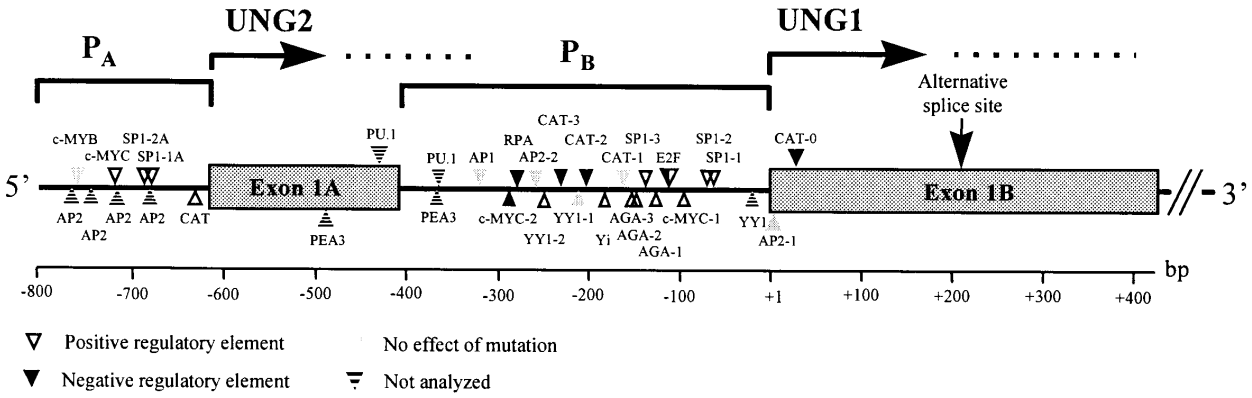

Figure 1. Structure of promoters P_A and P_B and the mechanism for generation of mRNAs for UNG1 and UNG2. P_A is located upstream of exon 1A, which encodes 44 N-terminal amino acids required for sorting of UNG2 to nuclei (26). Transcripts from exon 1A are spliced into exon 1B after codon 35. P_B is located in the intron between exons 1A and 1B and directs expression of mRNA for UNG1, which contains 35 N-terminal amino acids not found in UNG2, derived from the first part of exon 1A. Identified and putative transcription factor binding elements are indicated by triangles.

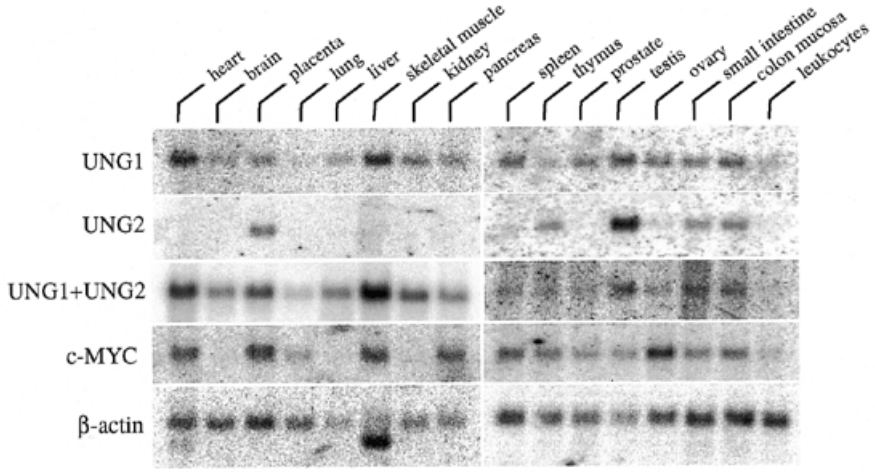

Figure 2. Expression of mRNA for UNG1 and UNG2, as well as mRNAs for c-Myc and β-actin in different tissues. Each lane contains ~2 µg poly(A)$^+$ RNA. The blots were successively hybridized with probes specific for UNG1, UNG2, UNG1+UNG2, c-Myc, and β-actin, with complete stripping of the membranes between each hybridization. Quantification of band densities was performed on a Molecular Dynamics PhosphorImager SF.

ascertain that all transfections were performed with the same amount of DNA. Constructs expressing c-Myc and Max (in identical vectors) in a 10:1 ratio have been demonstrated to be optimal, probably because of the higher stability of Max protein (25), but transfections were also carried out with other ratios and c-Myc alone. For the E2F experiments equal amounts of E2F-1 and DP-1 expression constructs were used.

RESULTS

The human gene *UNG* encodes both nuclear (UNG2) and mitochondrial (UNG1) forms of human uracil-DNA glycosylase. These forms are generated by alternative splicing and the use of two promoters (6). The structures of the promoter regions are outlined in Figure 1 and have been described in detail (6).

Expression of mRNAs for UNG1 and UNG2 in human tissues

cDNAs for UNG1 and UNG2 are of very similar size (2061 and 2058 bp respectively) and consequently the corresponding mRNAs are not resolved as two species in gel electrophoresis. Therefore, to investigate the pattern of expression of the two mRNAs in different tissues probes specific for each form were hybridized to northern blots of poly(A)$^+$ RNAs from various tissues. In addition, a cDNA probe detecting both forms (in one band) was also applied (Fig. 2). The results demonstrate differential expression of mRNAs for UNG1 and UNG2. Mitochondrial UNG1 mRNA is apparently expressed in all tissues examined, with the highest levels in skeletal muscle, heart and testis. Expression of UNG1 mRNA was also examined using a Human RNA Master Blot™ (dot blot of 50 adult and fetal tissues) from Clontech. These results indicated that UNG1 mRNA is expressed in all tissues represented in this blot and confirmed results from northern blots. In addition, very high

expression was found in the adrenal gland and the thyroid (data not shown). In contrast, expression of appreciable levels of nuclear UNG2 mRNA in northern blots was limited to proliferating tissues. Among tissues tested the level was highest in testis, followed by placenta, colon, small intestine and thymus. Using the short probe specific for UNG2 we were not able to demonstrate a clear expression of UNG2 mRNA in other tissues, neither in northern blots (Fig. 2) nor in dot blots (data not shown). When using the longer UNG2 cDNA probe that detects both forms the expression pattern for most tissues resembled the UNG1 pattern, again indicating that UNG1 mRNA is the predominant form in tissues with low or no proliferation. However, even in tissues with low or no proliferation the transcript pattern, as evaluated by phosphorimaging, was not identical to the pattern obtained with the UNG1-specific probe. This indicates that there may be a low level of UNG2 transcripts not detected by the short UNG2-specific probe even in non-proliferative tissues. In tissues where UNG2 mRNA expression was detected by northern blotting the relative contribution of this form did not exceed 50% except in testis, probably because even in normal proliferating tissues only a fraction of the cells actually proliferate. We also examined whether there was a correlation between UNG1 or UNG2 mRNA expression and expression of mRNAs for transcription factors E2F-1, Sp1 and Rb, but no clear correlation was found (data not shown). c-Myc mRNA expression was relatively high in most, but not all, tissues expressing either both forms or high levels of UNG1 (Fig. 2).

Table 1. Relative activities of P_B-constructs mutated in putative transcription factor binding motifs in HeLa cells and normal fibroblasts (CCD1070)[a]

Promoter construct	Normal element (position of first nucleotide relative to start of exon 1B)	Mutation (bold)	HeLa luciferase activity (%)	CCD1070 luciferase activity (%)
pGL2-P_B			100 ± 12	100 ± 6
pGL2-P_B-muCAT-0	CCAAT (+41)	CC**GC**T	126 ± 15	133 ± 6
pGL2-P_B-muAP2-1	CCCAGCCC (+1)	CTT**AG**CCC	98 ± 3	95 ± 43
pGL2-P_B-muSP1-2	GGGCGG (–78)	GG**AT**GG	127 ± 10	93 ± 13
pGL2-P_B-muMYC-1	CACGTG (–96)	CA**TA**TG	25 ± 3	24 ± 4
pGL2-P_B-muE2F	GCCGCGAAAA (–109)	GC**AT**CGAAAA	118 ± 6	126 ± 17
pGL2-P_B-muAGA-1	AGAGGG (–124)	AG**CT**GG	78 ± 6	104 ± 30
pGL2-P_B-muSP1-3	GGGCGG (–134)	GG**AT**GG	98 ± 6	73 ± 7
pGL2-P_B-muAGA-2	AGAGGG (–140)	AG**CT**GG	79 ± 12	102 ± 22
pGL2-P_B-muAGA-3	AGAGGG (–147)	AG**CT**GG	73 ± 4	78 ± 24
pGL2-P_B-muCAT-1	CCAAT (–153)	C**TG**AT	100 ± 25	131 ± 2
pGL2-P_B-muYi	CCCTCCTGGCT (–175)	CCCTC**GA**GGCT	64 ± 6	53 ± 11
pGL2-P_B-muCAT-2	CCAAT (–194)	C**TG**AT	127 ± 19	129 ± 13
pGL2-P_B-muYY1-1	CCAT (–202)	**AT**AT	93 ± 22	77 ± 10
pGL2-P_B-muCAT-3	CCAAT (–225)	C**AT**AT	127 ± 33	94 ± 22
pGL2-P_B-muYY1-2	CCAT (–242)	**TT**AT	62 ± 10	78 ± 6
pGL2-P_B-muAP2-2	CCCATGGG (–253)	CCC**GC**GGG	106 ± 12	99 ± 5
pGL2-P_B-muRPA	AGCCGCCGCT (–267)	AGCC**AT**CGCT	116 ± 8	108 ± 11
pGL2-P_B-muMYC-2	CACGTG (–281)	CA**TA**TG	107 ± 2	128 ± 11
pGL2-P_B-muAP1	CTGACTCG (–321)	CTGA**GG**CG	106 ± 6	101 ± 2
pGL2-P_B-muSP1-1+2	GGCGGG/GGGCGG	G**AT**GGG/GG**AT**GG	66 ± 7	74 ± 25
pGL2-P_B-muSP1-2+3	GGGCGG/GGGCGG	GG**AT**GG/GG**AT**GG	74 ± 19	54 ± 5
pGL2-P_B-muSP1-1+2+3	Position for SP1-1 (–63)		89 ± 13	72 ± 10
pGL2-P_B-muE2F/muMYC-1	GCCGCGAAAA/CACGTG	GC**AT**CGAAAA/CA**TA**TG	57 ± 8	92 ± 10
pGL2-P_B-muE2F/muYi	GCCGCGAAAA/CCCTCCTGGCT	GC**AT**CGAAAA/CCCTC**GA**GGCT	107 ± 6	134 ± 6
pGL2-BASIC	No promoter		0.8 ± 0.4	13 ± 2

[a]HeLa cells and normal human fibroblasts (CCD1070) were transiently transfected with promoter–luciferase constructs (4 µg/dish) and luciferase activity measured after 21 h. Results are given as percent of luciferase activity expressed from pGL2-P_B alone. Designations of constructs indicate the element which was mutated. Nucleotide changes in mutated elements are indicated in bold. Mutations in pGL2-P_B-SP1-1-2-3 (all mutations in a single vector) were identical to those shown in the two lines above (G**AT**GGG/GG**AT**GG/GG**AT**GG). Data for each element are presented as the mean ± SD of eight (HeLa cells) or three (fibroblasts) separate experiments, each carried out in triplicate.

Table 2. Relative activities of P_A-constructs mutated in putative transcription factor binding motifs in HeLa cells[a]

Promoter construct	Normal element (position of first nucleotide relative to start of exon 1B)	Mutation (bold)	HeLa luciferase activity (%)
pGL2-P_A			100 ± 2
pGL2-P_A-muCAT	CCAATT (–626/–15)	CCAA**GG**	69 ± 9
pGL2-P_A-muSP1-1	CCGCCC (–675/–64)	CCG**TCT**	52 ± 8
pGL2-P_A-muSP1-2	CCGCCC (–682/–71)	CCG**TCT**	80 ± 11
pGL2-P_A-muMYC	CACATG (–704/–93)	C**G**CATG	34 ± 12
pGL2-P_A-muMYB	CCGTTG (–754/–143)	CCG**CG**G	91 ± 7

[a]HeLa cells were transiently transfected with promoter–luciferase constructs (4 µg/dish) and luciferase activity measured after 21 h. Results are given as percent of luciferase activity expressed from pGL2-P_A alone. Designations of constructs indicate the element which was mutated. Nucleotide changes in mutated elements are indicated in bold. Data are means ± SD of three experiments, each carried out in triplicate.

Promoters P_A and P_B are functional independently of each other

Promoters P_A and P_B are both located within a partially methylated CpG island (5). We have previously shown that P_B is a competent promoter by transfection of P_B–luciferase constructs (16), but the putative promoter P_A has not been directly shown to be active alone. To study the function of this putative promoter a fragment extending from the first upstream *Alu* element (5) to the start of exon 1A was inserted into the reporter vector pGL2-Basic. The resulting construct (pGL2-P_A) directed expression of luciferase activity, as measured in cell extracts prepared from transiently transfected HeLa cells, to a level of 182 ± 11% when compared with the corresponding construct containing P_B (pGL2-P_B). A construct containing both promoters as well as the intervening exon 1A (pGL2-P_{AB}) expressed luciferase activity to a level of 154 ± 6% of that of pGL2-P_B alone. Since possible translation products of transcripts from P_A in the pGL2-P_{AB} construct are not in-frame with the luciferase coding frame (as verified by sequencing of the construct), the measured luciferase activity most likely results from transcription from promoter P_B. These results indicate that both P_A and P_B constitute functional promoters able to direct transcription separately and that P_A may stimulate transcription from P_B to some 54%, indicating a possible functional interaction between the two promoters. The significance of different putative binding elements for transcription factors in P_B and P_A was examined by mutational analysis (Tables 1 and 2). These studies demonstrated that putative c-Myc binding elements, as well as SP1 elements, in both promoters are required for full expression, whereas mutation of the E2F binding element in P_B resulted in a weak but significant increase in expression. Mutations in other putative elements also affected expression, as described in more detail later.

Cell cycle-regulated expression of total UDG activity and mRNAs for UNG1 and UNG2

To examine cell cycle regulation of mRNAs for UNG1 and UNG2 we measured DNA synthesis, total UDG activity and transcript levels for UNG1 and UNG2 during the cell cycle in synchronized HaCaT cells. Figure 3A demonstrates that total UDG activity was low in early G_1 phase, started to increase late in G_1 phase and was some 4- to 5-fold higher in S phase, as compared with early G_1 phase. mRNAs for UNG1 and UNG2 increased 2.5- and 5-fold respectively in late G_1/early S phase and UNG2 transcripts declined more rapidly than UNG1 transcripts after the middle of S phase. This indicates that both induction of transcription from P_A and P_B and degradation of UNG transcripts are differentially regulated. Mimosine completely blocked S phase progression, but only delayed UDG induction and accumulation of UNG1/UNG2 transcripts, demonstrating that UDG induction is not dependent upon ongoing DNA synthesis. However, transcript levels decreased more slowly, indicating a possible requirement for S phase progress for transcript degradation (Fig. 3B). Early in G_1 phase, Rb is known to be present in a hypophosphorylated form that binds E2F. In late G_1/early S phase Rb is stepwise phosphorylated and E2F is released (27). Furthermore, TGFβ-1 treatment of responsive cells results in accumulation of hypophosphorylated Rb late in G_1 phase (28) and a complex of this form and E2F is believed to repress genes regulated by E2F (29). As a first attempt to examine the possible role of Rb in regulation of *UNG* expression synchronized HaCaT cells were treated with TGFβ-1 (Fig. 3C). TGFβ-1 delayed S phase as well as induction of UDG activity and UNG1/UNG2 transcript accumulation by some 4–5 h. Although this effect is modest, it was observed in two independent experiments, each in triplicate, one of which is shown in Figure 3C. This result may be consistent with a mechanism in which inhibition of Rb phosphorylation inhibits *UNG* expression, but is clearly no proof of such a mechanism. We also probed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is often used as a normalization marker. However, GAPDH also tended to increase late in G_1/S phase, as reported previously by others (26). Ethidium bromide staining of gels did not reveal evidence for uneven loading except for lane 11 in Figure 3A (42 h) (data not shown).

An E2F-1/DP-1–Rb complex inhibits both promoters, while 'free' E2F-1/DP-1 stimulates P_B

E2F is a heterodimeric protein composed of an E2F and a DP family member and E2F-1/DP-1 has been shown to lead to cooperative transactivation (20). In order to analyze the significance of Rb and E2F-1/DP-1 on *UNG* expression in more detail, we co-transfected promoter–luciferase constructs with plasmids that either overexpressed DP-1 and E2F-1 or Rb. pGL2-P_A, pGL2-P_B and a P_B construct mutated in the E2F element (pGL2-P_B-muE2F) which

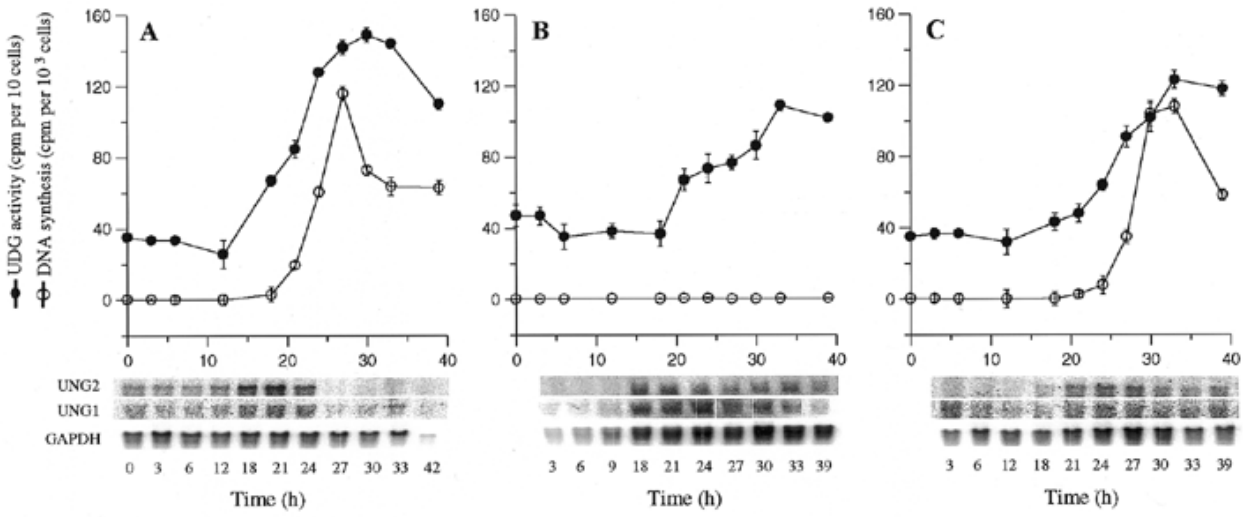

Figure 3. Cell cycle regulation of mRNAs for UNG1 and UNG2 and total UDG activity during the cell cycle. HaCaT cells arrested by serum starvation were forced to enter the cell cycle by addition of 10% FCS to the medium and total UDG activity, DNA synthesis and UNG1 and UNG2 mRNA expression determined at different time points after release as described in Materials and Methods. As a rough estimate of RNA loading the northern blots were hybridized with a cDNA probe specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts. The relationship between total UDG activity, UNG1 and UNG2 mRNA expression and DNA synthesis was assessed by including mimosine (0.4 mM) (**B**) or TGFβ-1 (2 ng/ml) (**C**) in the medium at the time of release. The results presented in (**A**) are without drug supplement. All results are from one experiment with measurements of UDG activity and DNA synthesis performed in triplicate. Similar results were obtained in a separate experiment.

does not bind E2F (16) were co-transfected with constructs expressing E2F-1 and DP-1. The most striking effect observed was a dose-dependent inhibition of expression from pGL2-P_B and pGL2-P_B-muE2F, while the constitutive promoter in pRL-TK was not affected by overexpression of E2F-1/DP-1 (Fig. 4A). The inhibitory effect of E2F-1/DP-1 on P_A as well as on the P_B mutant pGL2-P_B-muE2F demonstrates that the negative effect of E2F-1/DP-1, at least when overexpressed, is not dependent upon a functional E2F binding element in P_B, although we cannot exclude a possible residual binding capacity of the mutated promoter. However, the negative regulatory effect of E2F-1/DP-1 on P_B is apparently caused by an E2F-1/DP-1–Rb complex, since overexpression of DP-1 and a mutated E2F-1 protein (Y411H) unable to bind to Rb, but with an intact DNA binding domain, in fact resulted in a small but significant increase in P_B activity. No significant effect was observed when the construct expressing this mutant E2F-1 was co-transfected with pGL2-P_B-muE2F (Fig. 4A). These results indicate that 'free' E2F-1/DP-1 stimulates UNG1 expression through the E2F binding element. As a complement to these experiments we co-transfected pGL2-P_A or pGL2-P_B and an Rb expression plasmid (pRBWT3HA/SVE) into HeLa cells (Fig. 4B). This resulted in significantly reduced expression from P_B, while deletion mutants of Rb (Δ389–580 and Δ662–775) unable to bind E2F had a marginal stimulatory effect. Co-transfection of pRBWT3HA/SVE and pGL2-P_B-muE2F or pGL2-P_B-muE2F/muMYC1 also had a marginal stimulatory effect. Somewhat surprisingly, overexpression of E2F-1/DP-1 also inhibited expression from pGL2-P_A (which does not contain a consensus E2F binding element), although not as strongly as inhibition of expression from pGL2-P_B and pGL2-P_B-muE2F. A small inhibitory effect was also observed on Rb overexpression (Fig. 4C). While our results (Fig. 4A) strongly indicate that the inhibitory effect of E2F-1/DP-1 is mediated via an E2F 1/DP-1–Rb complex, we cannot exclude the possibility that unmutated E2F-1 may also play a role in negative regulation in an Rb-independent manner. In conclusion, these data indicate that 'free' E2F-1/DP-1 positively regulates P_B through the E2F element, while P_A and P_B are both negatively regulated by an E2F-1/DP-1–Rb complex and this inhibition is in part independent of an E2F binding element in the *UNG* promoter, at least when E2F-1/DP-1 is overexpressed.

Other regulatory elements in P_B and P_A

To examine the role of other putative *cis*-acting elements in promoters P_A and P_B we mutated a number of the elements (Tables 1 and 2) and also carried out transfection experiments with promoter–luciferase constructs carrying intact or mutated promoters. HeLa cells have ~10-fold higher total UDG activity than the human fibroblast cell line used and luciferase activities after transfection were also ~10-fold higher in HeLa cells. Mutations of various elements gave qualitatively essentially similar results for human fibroblasts and HeLa cells. Mutations in c-MYC-1, Yi, YY1-2 and the SP1 elements in P_B reduced transcription to 24–25, 53–64, 62–78 and 54–74% of the controls respectively, indicating that these elements are required for maximal expression of UNG1 both in normal fibroblasts and HeLa cells. Each of three repeated AGAGGG motifs located close to each other was also required for full expression in HeLa cells, but only one was required in fibroblasts. To our knowledge a transcription factor binding to this putative motif has not been identified. In contrast, mutations in the E2F and c-MYC-2 elements, as well as the CCAAT-2 and -3 boxes, tended to enhance expression, as shown in Table 1, indicating negative regulatory functions. P_A contains putative binding elements for c-Myc, c-Myb, Ap2 and Sp1 and a CCAAT box (Fig. 1). Mutations in the c-MYC, SP1-1A and SP1-2A elements in P_A reduced promoter activities to 34, 52 and 80% of the controls, whereas a double mutation of the CCAAT element reduced the activity to 69% of the control, indicating that all these elements

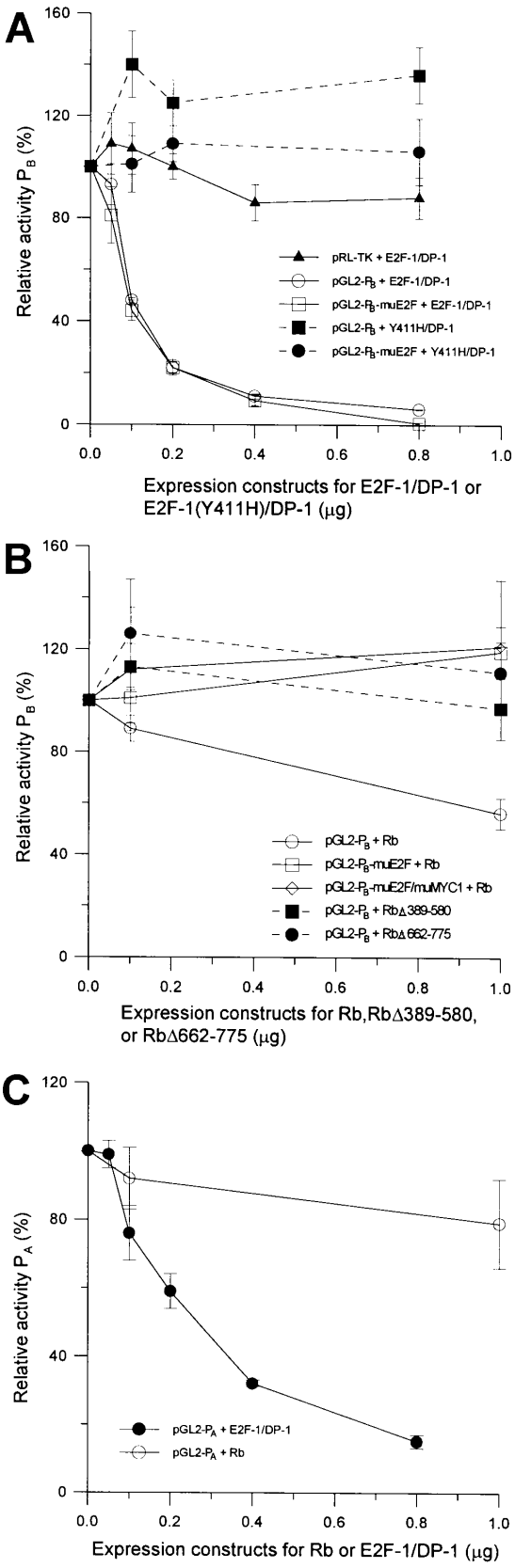

Figure 4. Effects of overexpression of transcription factors E2F-1/DP-1 and Rb and mutants of E2F-1 (Y411H) and Rb (RbΔ389–580 and RbΔ662–775) on activities of P_A and P_B in transiently transfected HeLa cells. HeLa cells were co-transfected with expression vectors and promoter–luciferase constructs as indicated in (A)–(C). An identical amount of total DNA was transfected in each experiment, as described in Materials and Methods. At 48 h after transfection luciferase activities were quantified in cell extracts. Experiments in which cells were transfected for 21 h were also carried out and gave essentially similar results (data not shown). Experiments involving transfection with E2F-1/DP-1 and E2F-1 (Y411H)/DP-1 expression vectors (**A**) were carried out eight and three times respectively, each time in triplicate. Experiments involving transfection with vectors expressing Rb or RbΔ389–580 and RbΔ662–775 (**B**) were carried out four times, each in triplicate. Results displayed in (**C**) are from three experiments, each carried out in triplicate. To make comparisons easier the activity of each promoter without co-transfection of the transcription factor expression plasmid (0 μg value) is set to 100%. However, the activity of promoter construct pGL2-P_B-muE2F alone was 118% of that of pGL2-P_B alone, whereas the activity of pGL2-P_B-muE2F/muMYC1 was 57% of that of pGL2-P_B alone.

are required for maximal expression of UNG2 in HeLa cells. In agreement with these results, co-expression of Sp1 together with pGL2-P_A or pGL2-P_B increased the activity of both promoters (Fig. 5). Since E2F is involved in regulation of c-Myc expression (30), the c-Myc binding elements were considered as possible candidates for mediating repression. However, the double mutant altered in both the E2F and the MYC-1 elements is also inhibited by overexpression of E2F-1/DP-1 (data not shown). Furthermore, co-transfection of constructs expressing c-Myc/Max and pGL2-P_B, pGL2-P_A or the corresponding promoter mutants not binding c-Myc (pGL2-P_B-muMYC-1, pGL2-P_B-muMYC-2, pGL2-P_B-muE2F/muMYC-1 and pGL2-P_A-muMYC) all resulted in decreased promoter activity (Fig. 5). Similarly, co-transfection of c-Myb and pGL2-P_A or pGL2-P_A-muMYB also resulted in decreased promoter activity (data not shown).

DISCUSSION

We have found that expression of mRNAs for nuclear UNG2 and mitochondrial UNG1, controlled by promoters P_A and P_B respectively, are cell cycle regulated and differentially expressed in human tissues. Due to its more widespread expression, mRNA for UNG1, and probably also UNG1 protein, are apparently the quantitatively dominant forms of *UNG* gene products in human tissues, most cells of which are non-proliferating even in proliferating tissues. In the relatively few replicating cells in normal tissues UNG2 is likely to be the predominant form, as in HeLa cells, in which at least 70% of the total UDG activity is located in the nuclei (8,31).

One inherent weakness in transient transfection studies is that cells at different stages in the cell cycle are transfected and the results therefore represent an 'average effect' on cells in different stages. Expression and modification of different transcription factors are often cell cycle regulated and consequently cells in different phases of the cell cycle may respond differently upon transfection. Our results demonstrate that P_A is a stronger promoter than P_B and also indicate that it may enhance expression from P_B. Functional interactions between promoters located close to each other is not without precedence. Thus, for the c-*MYC* gene, which also contains two promoters, transcription from promoter 2 has been demonstrated to impede transcription from the upstream promoter 1 (32). Whether transcription from P_B may inhibit transcription from P_A in the *UNG* gene is currently not known, but at least in proliferating cells this can hardly be the

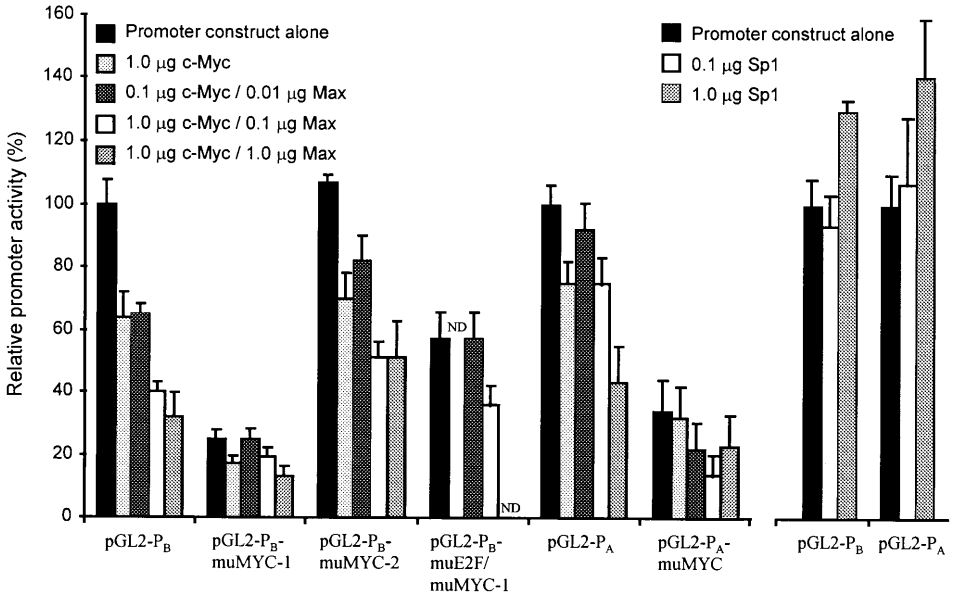

Figure 5. Effects of overexpression of transcription factors c-Myc, Max and Sp1 on activities of P_A and P_B in HeLa cells. Promoter constructs were co-transfected with expression vectors for c-Myc and Max (pHebo-CMV-Myc alone or with pHebo-CMV-Myc/pHebo-CMV-Max as specified) for 48 h before measurement of luciferase activities in cell extracts. Results are given as percent of luciferase activity expressed from pGL2-P_B alone or pGL2-P_A alone and are derived from three experiments (c-Myc/Max) or two experiments (c-Myc), each in triplicate. ND, not determined. The relative activity of P_A was 1.8-fold higher than that of P_B, but for comparison the activity of both are set to 100% in the absence of Myc expression vector.

case, since transcription from both promoters is enhanced late in G_1 phase. E2F-1/DP-1 is a critical determinant for the G_1/S phase transition (29), in part due to its involvement in regulation of cyclins and also by directly interacting with Rb and related proteins (33). Our experiments strongly suggest that Rb complexed to E2F-1/DP-1 is central in negative regulation of expression from both promoters, whereas 'free' E2F-1/DP-1 is apparently a positive regulator of P_B and this effect is dependent on the E2F element in P_B. Recently the sequence of the mouse *UNG* gene promoter was reported (34). The structures of P_B in mouse and man are very similar and the elements found to be important in regulation of the human gene are also present in the mouse gene. Interestingly, P_B in mouse and man contain an identical, but structurally uncommon, E2F element of 14 bp which is duplicated in mouse. The inhibitory effect of overexpression of E2F-1/DP-1 could possibly be mediated by altered expression of *trans*-acting factors from genes involved in regulation of the *UNG* gene, such as c-*MYC*. It is also well established that overexpression of E2F proteins under certain conditions may induce apoptosis and suppress proliferation (35,36). Previously it was reported that UDG activity is increased some 10-fold in SV40-transformed human fibroblasts, as compared with normal human fibroblasts (37). This may be caused by the known ability of SV40 large T antigen to inactivate Rb proteins by complex formation, resulting in an increase in 'free' E2F-1/DP-1 that may stimulate expression of UNG proteins.

Overall, our results are consistent with a model in which the E2F element alternates between an activating and an inhibitory function depending on the phosphorylation status of Rb proteins in the cell cycle (30,38,39). Thus E2F elements may recruit Rb to the promoter region and this in turn may inhibit the activating function of other transcription factors such as Sp1 (39,40). It is also well established that Rb may activate Sp1- and Sp3-dependent transcription (41,42). This 'superactivation' is in part dependent on the same regions in Rb required for E2F-1/DP-1 binding (42). It is therefore possible that overexpression of E2F-1/DP-1 not only results in formation of inhibitory E2F–Rb complexes, but in addition it may inhibit a possible superactivation by binding most of the available Rb. Since both P_A and P_B have Sp1 binding elements, this could contribute to down-regulation of both promoters. It should also be noted that the Sp, E2F and Rb families as well as CCAAT binding proteins comprise several members that may regulate the *UNG* gene differently; the effects of these on UNG mRNA expression have not been examined so far.

P_B contains two canonical c-MYC elements, whereas the putative c-MYC element in P_A is non-canonical, but an identical sequence has been shown to bind c-Myc (43). Mutational analysis indicates that c-Myc and Sp1 binding elements in both promoters are positive regulatory elements, but whereas overexpression of Sp1 enhanced mRNA expression, overexpression of c-Myc/Max inhibited expression from both promoters. This indicates that c-Myc is a positive regulator of expression of UNG proteins when present at physiological concentrations, but inhibitory when overexpressed. In conclusion, our tissue studies, as well as the combination of mutational analyses and transient transfections, demonstrate differential regulation of nuclear and mitochondrial human uracil-DNA glycosylase.

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