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Structure of the gene for human uracil–DNA glycosylase and analysis of the promoter function

Terje Haug, Frank Skorpen, Henning Lund, Hans E. Krokan*

UNIGEN Center for Molecular Biology, University of Trondheim, N-7005 Trondheim, Norway

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Abstract The gene for human uracil–DNA glycosylase (UNG) contains 4 exons and has an approximate size of 13 kb. The promoter is very GC rich and lacks a TATA box. Nested deletions of the promoter demonstrated that two SP1 elements and a putative c-*MYC* element proximal to the transcription initiation region were sufficient to support some 27% of the promoter activity, while a clone that in addition contained the elements E2F/SP1/CCAAT increased expression to almost 90% of the full-length construct. A region upstream of these elements appears to exert a negative control function.

Key words: Uracil–DNA glycosylase; Gene expression; Promoter function

1. Introduction

Uracil–DNA glycosylase (UNG) initiates the base excision repair pathway for removal of uracil from DNA. The successive steps in the base excision repair pathway are thought to involve action of an AP endonuclease, DNA deoxyribophosphodiesterase, a DNA polymerase and a DNA ligase [1,2]. Uracil in DNA results from deamination of cytosine [3] or incorporation of dUMP instead of dTMP [4,5]. Deamination will result in a GC to AT transition mutation unless uracil is removed before the next round of replication. It has been hypothesized that incorporated dUMP, or strand breaks resulting from repair of newly replicated DNA, may have a role in strand selection in mismatch repair (reviewed in [6]), but to our knowledge there is no direct experimental evidence supporting this view. *E. coli* mutants in the *ung*-gene encoding uracil–DNA glycosylase show some 5-fold increased spontaneous mutation rate [7] rising to 30-fold at certain bases [8], whereas a 20-fold increase in spontaneous mutations has been found in similar yeast mutants [9]. Mammalian mutants in the *UNG*-gene are not available. UNG has been purified from human placenta [10] and the corresponding cDNA cloned [11]. This cDNA encodes the major nuclear form, as well as the mitochondrial form of the enzyme [12,13]. The UNG-protein shows a striking similarity to analogous enzymes from *E. coli* [14], *Streptococcus pneumoniae* [15], animal viruses [16–19] and *Saccharomyces cerevisiae* [20]. Although all human cells and tissues investigated have measurable UNG activities, significant interindividual as well as interorgan variation in activities has been observed in extracts from human adult tissues [21,22]. Furthermore, stimulation of peripheral lymphocytes with phytohemagglutinin, and studies of cells with different proliferation rates have demonstrated a correlation between UNG activity and DNA synthesis [23–25]. Transcription of the *UNG* gene is induced late in the G_1-phase resulting in an 8–12 fold increase in the transcript level, and the pattern of UNG accumulation indicated that the gene expression is regulated mainly at the transcriptional level [12]. These results together with the experiments demonstrating an association between UNG and replicating SV40 minichromosomes [26], indicate a coordinated expression of UNG and proteins involved in DNA synthesis. However, specific inhibition of DNA replication by aphidicolin indicated that although the accumulation of UNG is temporally correlated with induction of the S-phase, it is not dependent on active DNA synthesis [12].

In the present work, we present the structure of the *UNG* gene and the organization of a TATA-less and very GC-rich upstream sequence containing a highly active promoter with recognition sites for several transcription factors.

2. Materials and methods

2.1. Southern analysis of the UNG gene structure

High molecular weight DNA was prepared from human leukocytes by phenol/chloroform extraction and isopropyl alcohol precipitation using a model 340A Nucleic Acid Extractor (Applied Biosystems). 10 μg DNA were digested with 30–60 U of restriction enzyme and separated on 0.8% agarose gels. The DNA was transferred to GeneScreenPlus membranes and hybridized with ^{32}P-labelled cDNA probes as described [28], except that 65°C and 1% SDS were used. According to the numbering of the cDNA [11], the following probes were used: 1–301, 301–546, 546–1140, 1140–2074, 1468–2074. The membranes were subjected to a final wash in 0.1 × SSC/0.5% (w/v) SDS, 65°C for 1 h and autoradiographed on Kodak X-Omat film for 6–10 days with intensifying screens.

2.2. Inverse PCR

High molecular weight DNA (20 μg) was digested with 32 U *Rma*I overnight and separated on a 0.8% low melting point agarose gel. The region of the gel corresponding to 1.3 kb was cut out and DNA isolated from the gel using Gelase (Epicentre Technologies). The DNA was dissolved in 20 μl dH_2O. 200 ng of the isolated *Rma*I fragments were circularized in a 50 μl ligation mixture overnight at 16°C. After addition of carrier tRNA to a final concentration of 50 ng per μl, the DNA was finally precipitated with NH_4Ac and ethanol. The PCR reaction was carried out on a Perkin Elmer thermo cycler in 100 μl with the supplied 1 × Vent buffer, 200 μM of each dNTP, 30 pmol of primer 1+ (5′-CCAAGGCAGAAGACGCCCATTTGT-3′) and primer 1− (5′-CGCCTCTGCGGGGACCACTTGC-3′) using the circularized *Rma*I fragments resuspended in dH_2O as template. The template was denatured for 5 min at 95°C and the temperature lowered to 90°C before addition of 2 U Vent polymerase (New England Biolabs) and two droplets of light mineral oil. Conditions for the 35 cycles were 1 min at 95°C, 1.5 min at 65°C and 2 min at 74°C. To ensure the specificity of the product, a nested PCR reaction was carried out under the same conditions using about 1 ng of the first PCR product as template together with primer 2+ (5′-CCCAGCAAAAGCGGACAGTAAGAGC-3′) and primer 2− (5′-CGCTGAGTGCCGAGCAGTTGGA-3′).

*Corresponding author. Fax: (47) (73) 598705.

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This PCR product was cloned into the plasmid pCR1000 (Invitrogen), transformed into an *E. coli* strain (Epicurian Coli SURE from Stratagene) and sequenced by Sanger's ddNTP method using 7-deaza-guanosine triphosphate and primer walking with 14 primers. The sequence was determined on both strands.

2.3. Preparation of a promoter-reporter gene construct and transient transfections

By adding an *Xho*I and a *Hin*dIII linker, the sequence from 42 to 440 was cloned in the correct orientation into the polylinker of pGL2-Basic (Promega) and named pGL2-ProB. The pGL2-Basic vector carries the coding region for firefly (*Photinus pyralis*) luciferase which was used to monitor transcriptional activity in transfected cells. Using the Erase-A-Base system (Promega), several deletion clones were generated and transiently transfected into HeLa cells using Transfectam (Promega). 5 μg DNA were mixed with 25 μg Transfectam in 1 ml medium without serum and added to 10^6 adherent HeLa cells. After 7 h the DNA–Transfectam mixture was removed and medium with serum added. After 14 h the cells were lyzed in 250 μl of 25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid, 10% glycerol and 1% Triton X-100. Cell debris was removed from the supernatant by centrifugation. 20 μl of the supernatant were added to 100 μl Luciferase Assay substrate (Promega). Photons were counted by scintillation counting (Packard, Model 1900CA) using a ^{3}H-window and single photon counting mode. The samples were counted in 10 consecutive periodes of 20 s immediately after addition of the substrate. The activity detected in the fourth period was used for comparison with the other samples.

2.4. Analysis of protein binding

Binding of transcription factors to the promoter region was investigated using gel mobility shift assays. SP1 analysis was performed by incubating 200 pg of a 150 bp radioactively labelled *Bgl*I/*Ava*I fragment (position 218 to 368), containing the putative SP1 binding sites, with 4 ng of purified Sp1 (Promega). The incubation took place in a buffer consisting of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 10% glycerol, 0.05% NP-40 and 3 μg poly(dI-dC)·poly(dI-dC) at room temperature for 20–30 min in a total volume of 20 μl. The AP2 assay was similarly performed with a 149 bp *Eco*NI/*Nar*I fragment (position 42 to 191) incubated with 560 ng purified Ap2 in 25 mM Tris-HCl (pH 8), 6.25 mM MgCl, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT and 1 pmol AP1 oligonucleotide (Promega) as unspecific competitor. The reactions were separated on a 5% non-denaturing polyacrylamide gel. For analysis of E2F binding, 0.05 pmol of a radioactively labelled 24-mer oligonucleotide (corresponding to position 270 to 294) containing the putative binding site for E2F was incubated in a total volume of 20 μl with HeLa nuclear extract containing approximately 25 ng protein in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 10% glycerol, 0.05% NP-40 and 2 pmol Oct1 oligonucleotide (Promega) as unspecific competitor. For supershift assays 2 μg E2F-1 Tranz Cruz Gel Supershift Antibody (Santa Cruz Biotechnology) were added to the binding reaction after 30 min for additional 60 min incubation. Separation of various products was carried out on a 7% non-denaturing polyacrylamide gel.

3. Results

The organization of the *UNG* gene was determined by extensive Southern analysis with 12 different restriction endonucleases as described in section 2. Previous attempts to isolate genomic sequences from two phage libraries and a cosmid library were unsuccessful, apparently due to an extreme under-representation of *UNG* genomic sequences in the library. At this level of resolution, the gene was found to be 13 kb long and to contain 4 exons (Fig. 1). The complete gene was contained on a 17 kb *Xba*I fragment. The approximate size of the last exon was verified by PCR amplification of a 725 bp region corresponding to position 1,230 to 1,955 in the cDNA.

Digestion with *Rma*I and hybridization with the 5′-terminal cDNA probe identified a fragment of 1.3 kb containing the first exon, and parts of the promoter and first intron. Using this information, we cloned the promoter region by inverse PCR. The PCR product was sequenced and found to contain 387 bp of the sequence upstream of the cDNA start, 280 bp of the first exon, the first exon/intron boundary and 480 bp of the first intron. The upstream sequence (Fig. 2) is very GC rich, lacks a TATA box, and was identified by the 'CpG plot' program [29] as a part of a CpG island (Fig. 3) [30]. The upstream sequence contains putative binding sites for several proteins regulating transcription (Table 1). Except for the putative AP1 site, all elements listed are identical to the reported consensus sequences. The proposed AP1 consensus site is 5′-CTGACT-CA-3′ and the putative site in the *UNG* promoter is

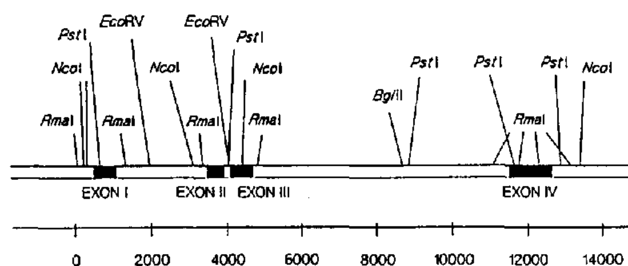

Fig. 1. Structure of the *UNG* gene. Model of the gene structure as determined by extensive Southern analysis of human genomic DNA using *Taq*I, *Rma*I, *Nco*I, *Eco*RV, *Eco*RI, *Bgl*II, *Sac*I, *Pvu*II, *Hin*dIII, *Bgl*I, *Pst*I and *Bam*HI. All known sites for *Rma*I, *Nco*I, *Eco*RV, *Pst*I and *Bgl*II are shown.

Table 1
Putative recognition sites for transcription factors in the regulatory region of the *UNG* gene

ELEMENT	POSITION	SEQUENCE	REF.
PEA3	18 - 23	AGGAAG	[47]
AP1	67 - 74	CTGACTCG	[32]
AP2	135 - 142	CCCATGGG	[48]
YY1	136 - 139	CCAT	[43]
YY1	146 - 149	CCAT	[43]
CCAAT	163 - 167	CCAAT	[49]
YY1	186 - 189	CCAT	[43]
CCAAT	194 - 198	CCAAT	[50]
Yi	213 - 223	CCCTCCTGGCT	[44]
CCAAT	235 - 239	CCAAT	[45]
SP1	253 - 258	GGGCGG	[51]
E2F	279 - 288	GCCGCGAAAA	[46]
c-MYC	292 - 297	CACGTG	[52]
SP1	308 - 313	GGGCGG	[51]
SP1	318 - 323	GGCGGG	[51]
YY1	377 - 380	CCAT	[43]
CCAAT	428 - 432	CCAAT	[45]

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   1 ACTAGGGGGT GAAGGGGGAG GAAGGCGGTG GGCCCCGCCT GACGGAGGGC GTGCAGGATC GCGCCTCTGA   70
  71 CTCGGTAAAC CCGGGCTCCG CTTTCCAAAT AGCCTCCACG TGTTCAAAAT AGCCGCCGCT GTCCCCCATG  140
 141 GGCCGCCATG CTAAAGGGCC AGCCAATGGG AACGCGTCTC GGGGCCCATG GCGCCAATCC GCGCGCCACA  210
 211 GGCCCTCCTG GCTCGGTGCG CTGTCCAATC AGAGGGGAGA GGGGGCGGGA CCCAGAGGGA GGTTTTTTGC  280
 281 CGCGAAAAGA CCACGTGGGG ACGCGGTGGG GCGGGTCTGG CGGGGGTGGG GCACCTCTGT GCAGGGTTCC  350
 351 CGGTCACCGC GACGCTCCTC GGGAAGCCAT AGGGCGCCTC CCAGCCCGTC TCCCCGCTCC AGTTTAGAAC  420
 421 CTAATTCCCA ATTCCCGGAC CGGGCCCAGC CCTGGGCTCT TACTGTCCGC TTTTGCTGGG ACCTGTTCCA  490
                                         <-------------------------]          <
                                                   primer 2+
 491 CAAATGGGCG TCTTCTGCCT TGGGCCGTGG GGGTTGGGCC GGAAGCTGCG GACGCCTGGG AAGGGGCCGC  560
     ------------------------]
         primer 1+
 561 TGCAGCTCTT GAGCCGCCTC TGCGGGGACC ACTTGCAGGC CATCCCAGCC AAGAAGGCCC CGGCTGGGCA  630
                [-------------------->
                     primer 1-
 631 GGAGGAGCCT GGGACGCCGC CCTCCTCGCC GCTGAGTGCC GAGCAGTTGG ACCGGATCCA GAGGAACAAG  700
                                 [-------------------->
                                        primer 2-
 701 GCCGCGGCCC TGCTCAGACT CGCGGCCCGC AACGTGCCCG TGGGCTTTGG AGAGAGCTGG AAGAAGCACC  770
 771 TCAGCGGGGA GTTCGGGAAA CCGTATTTTA TCAAGgtaaa tatggaaatg caccttccat aagggtaaat  840
 841 gtggaggctg ccggcccttt tgacttgtta gtgtagccgg ccaagttcat gtttccgtag gcttaggttg  910
 911 tacccccttc cacctccttt actcacaaag ggggtaaaag aaagtcatta tgtttcactc tgcggcttta  980
 981 tattggttaa agttgttaac gacccgcgag atgatatcat ggattcattt aagtcacata gtctattgtc 1050
1051 caggaaaggc tggcgtagta aaatcaccaa ctatcctgaa tgaaacctgg cttgagcttt aaaaagccgt 1120
1121 gaggagtggc actgtcagga cccagcccag agaaagaggc aaggaattga ccttattgaa ccacttaggt 1190
1191 gggggggcag gcactgtttt tgtttgtagt tttttaaaag aatttggaca taacatgaca aagaactaat 1260
1261 gatgttccaa ataacttgca cta
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Fig. 2. The nucleotide sequences of the *UNG* promoter, first exon and a part of the first intron. The first exon is shown in bold letters and the first intron in small letters. The start of translation is indicated by double underlining at position 495. Primers used for inverse PCR are also shown. The nucleotide sequence will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence databases under Accession Number X79093.

5′-CTGACTCG-3′. However, yeast and Chinese hamster analogs of human Ap1 are known to bind to this sequence [31,32]. Interestingly, the promoter contains an E2F-binding sequence upstream of two SP1 elements close to the transcription initiation region.

A number of clones obtained as nested deletions were sequenced to accurately define their structure and the most informative clones were used for transient transfection of HeLa cells. The observed activities relative to the activity detected with pGL2-ProB are given in Fig. 4. The two SP1 sites located close to the site of transcription initiation and a putative c-MYC element were sufficient to support a transcription at some 27% using pGL2- ProB as reference (100%). Other important elements are localized between position 229 and 290. When this sequence, which contains putative sites for Sp1, E2F and a CCAAT-box, is present in addition to the proximal SP1/c-MYC elements, 90% of the activity directed by the full-length construct is restored. Sequences between 136 and 229 (containing two CCAAT boxes and an AP2 element) appear to exert negative control functions, since deletion of this region increases transcription. Finally, sequences between position 42 to 136, which contain a putative site for binding of Ap1, enhances transcription approximately 2-fold.

Purified Sp1 was used in band shift assay with a promoter fragment containing the putative SP1 sites proximal to the transcription initiation site. A strong, specific binding to the promoter fragment was observed (Fig. 5) indicating a role for Sp1 in regulation of transcription from the *UNG* gene. A specific binding of Ap2 was detected using the same strategy with purified Ap2 and another fragment containing the AP2 site in the distal part of the upstream sequence. To identify binding of proteins of the E2F-family, nuclear extracts from HeLa cells and a 24-mer oligonucleotide containing the putative E2F site were used in a mobility shift assay. The shifts were quantitatively demonstrated using a phosphor-imager (Fig. 6). This analysis revealed three bands of which at least two (peak 2 and 3) contain proteins binding to the E2F element as they are diminished upon addition of unlabelled E2F oligo and unaffected by addition of an unspecific competitor (panel A). When an E2F-1 antibody was included in the band shift mixture (panel B), peak 3 was shifted to peak 4. Thus, it appears that at least two different protein complexes may bind specifically to the E2F element. Furthermore, changing different combinations of each of two GC's in the putative E2F site with AT's (position 279/281, 281/282, 278/ 279, 280/282), the oligonucleotides lost their ability to compete out the specific binding to the labelled E2F oligo (data not shown).

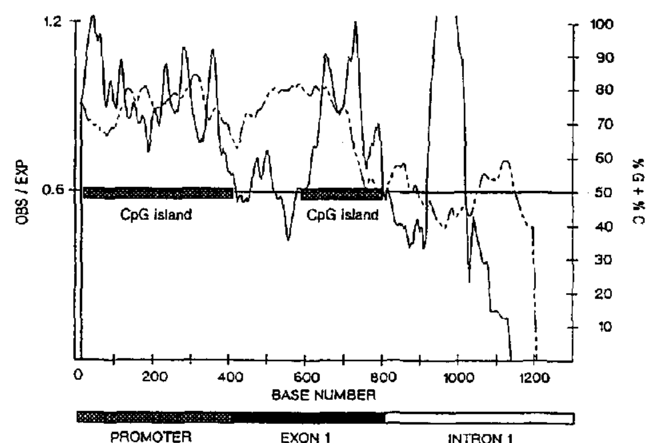

Fig. 3. CpG plot of the promoter, first exon and a part of the first intron. Observed/expected CpG dinucleotides (unbroken line) and% G+C (broken line) are plotted against position in the sequence of the promoter, first exon and a part of the first intron. One CpG island covers the whole upstream sequence and another one is present in the first exon.

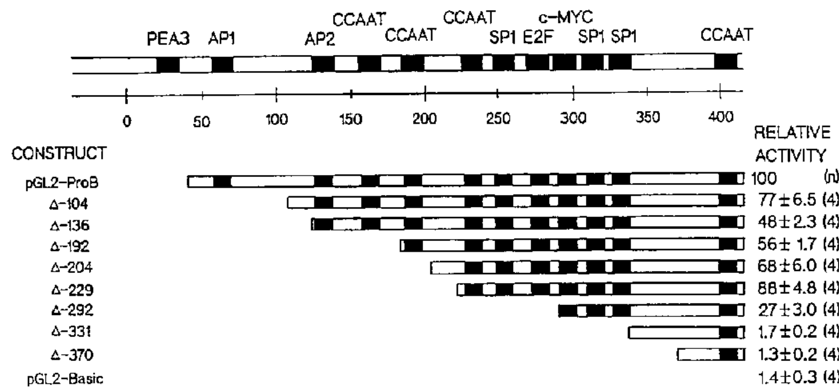

Fig. 4. Promoter structure and relative activities of deletion clones. The model shows putative recognition sites in agreement with proposed consensus sequences and sites present in the different constructs used in transient transfection of HeLa cells. The detected luciferase activity from each construct relative to the activity directed by the full length construct (pGL2-ProB) is given together with the standard deviation calculated from four experiments. The numbering indicates the start position of each construct. pGL2-Basic, not containing the *UNG* promoter, is used as negative control. The luciferase activity in extracts from HeLa cells transfected with pGL2-ProB (100%) was about 15 ng luciferase/mg protein (7.5×10^{11} cpm/mg protein) as calculated from experiments in which purified luciferase protein was used under similar buffer conditions.

4. Discussion

The *UNG* promoter lacks a TATA box and is located within a CpG island. This finding, together with the ubiquitous expression of UNG classifies the *UNG* gene as a house keeping gene [29]. Promoters containing CpG islands are thought to have an open chromatin structure facilitating the binding of transcription factors to the promoter [33].

The promoter proximal SP1 elements were clearly necessary for transcription, in agreement with the role of Sp1 as an ubiquitous transcription factor that seems to be essential in many TATA-less promoters [34–36]. The E2F element in position 279 to 288 may explain the previously observed cell cycle regulation of the *UNG* gene, which is induced late in the G_1-phase [12]. E2F sites have been found in several genes essential for the S-phase, and which exhibit a similar pattern of cell cycle regulation (reviewed in [37]).

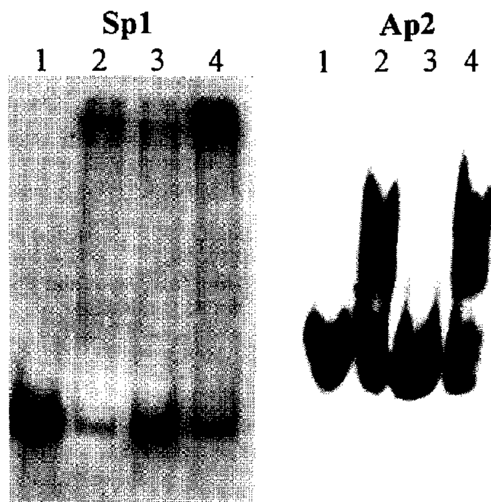

Fig. 5. Sp1 and Ap2 binding analysis. In both autoradiograms lane 1 contains the labelled promoter fragment; lane 2 contains the fragment incubated with the purified factor; lane 3 contains the labelled fragment incubated with purified factor and a 10-fold excess of a specific competitor (Sp1 or Ap2 binding oligonucleotide (Promega)), lane 4 contains the labelled fragment incubated with purified factor and 10-fold excess of an unspecific competitor (GRE binding oligo (Promega)).

In addition to E2F, elements like AP1 and PEA3 are common to some of the genes that accumulate their protein products in the S-phase. The DNA polymerase α promoter contains binding sites for Ap1, Ap2, Sp1, E2F, Pea3 as well as CCAAT-boxes [38, 39], all of which are present in the *UNG* promoter. Transient transfections revealed that the region from 42 to 136, which contains a putative AP1 element, enhances transcription from the *UNG* promoter some 2-fold. Pea3 seems to recognize enhancer elements and often cooperates with Ap1 [40,41]. PEA3 is shown to be activated by different factors, including the oncogene products of c-Ha-*ras* and v-*src* [42], indicating a role for Pea3 in the control of proliferation.

Furthermore, the upstream sequence in the *UNG* gene contains a AP2 site (position 135 to 142) which was verified in band shift assays with purified Ap2, and three CCAAT-boxes separated by approximately 30 base pairs. When parts of this region are deleted from the constructs (constructs called Δ-192, Δ-204 and Δ-229), the transcription activity increases, indicating the presence of a negatively regulating region in the promoter. Furthermore, the regulatory factor YY1, shown to activate and repress promoter activity by bending DNA, is binding to the weak consensus CCAT [43] present at three places from position 136 to 189. We have also noticed that a putative site for the Yi protein is present. A G_1/S specific binding to the Yi site in the murine thymidine kinase promoter has been reported [44].

In conclusion, the regulation of *UNG* transcription seems to involve several activating and repressing regulators. Noticably, an E2F element known to be involved in cell cycle regulation of other genes induced late in the G_1-phase is also present in the *UNG* gene, which otherwise resembles typical cell cycle regulated genes and house keeping genes that often lack a TATA box and frequently contain one or more CpG islands in the 5′-part of the gene.

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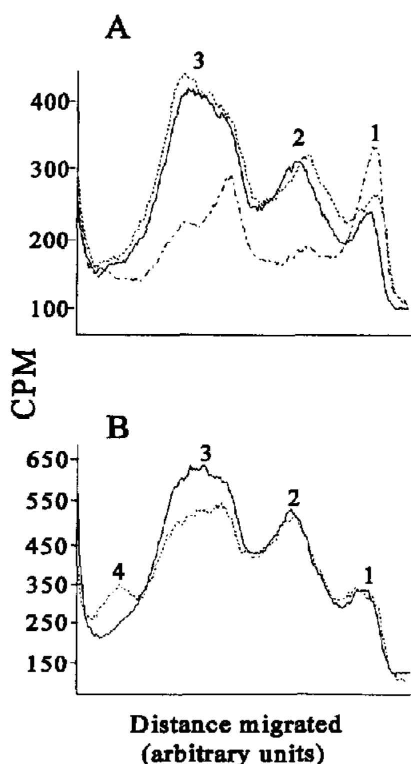

Fig. 6. E2F binding analysis. The figures show phosphor-imager scans of E2F mobility shift gels. Scans are performed along the lanes, so that direction of migration is from left to right in the figure. The graphs show the intensity of the radioactivity along the lanes, and the shifted bands are represented as peaks. Panel A: control of specificity. The E2F oligo was incubated with HeLa nuclear extract and no competitor (——), nuclear extract and unlabelled E2F oligo as specific competitor (-.-.-) or nuclear extract and an Oct1 oligo as unspecific competitor (.....). Radioactivity of the unshifted oligo is not shown. Panel B: gel mobility shift with the putative E2F oligo and HeLa nuclear extract with (.....) and without E2F-1 specific antibody (——).

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