Endogenous DNA Lesions Can Inhibit the Binding of the AP-1 (c-Jun) Transcription Factor†

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ABSTRACT: The repair of DNA damage, caused by both endogenous and exogenous sources, is necessary to remove lesions that either miscode or block DNA or RNA polymerases. We propose that damage also must be repaired to maintain sequence-specific DNA−protein interactions. In this paper, we have systematically studied two lesions that interfere with one important DNA landmark, the thymine methyl group. Oxidation of the thymine methyl group in DNA generates 5-hydroxymethyluracil (HmU) whereas the misincorporation of dUMP into DNA generates uracil (U), replacing the methyl group with a hydrogen. Both substitutions are shown to inhibit binding of the AP-1 (c-Jun) transcription factor. The energy cost of the perturbation, approximately 0.4 kcal/mol, is similar in magnitude for both U and HmU substitutions and is additive when multiple substitutions are present. A third lesion, substitution of the central C:G base pair of the AP-1 DNA binding domain with the pro-mutagenic U:G mispair, unexpectedly increases AP-1 binding, allowing the transcription factor to interfere with uracil DNA glycosylase activity. Our results support the hypothesis that an additional role for DNA repair systems is to maintain the integrity of sequence-specific DNA−protein interactions, a role of particular importance in long-lived organisms.

Sequence-specific DNA binding proteins mediate the complex process of transcriptional control in eukaryotes, particularly during development and differentiation. These transcription factors recognize and bind to their promoter regions based upon specific contacts formed with the DNA duplex. Among the possible interacting functional groups, the methyl group of thymine is known to be important in mediating the binding specificity of transcription factors, including steroid hormone receptors (*1*−*15*). The methyl group of 5-methylcytosine also has been demonstrated to either enhance or inhibit sequence-specific DNA−protein interactions (*16*−*21*).

Endogenous processes, including misincorporation of dUMP and oxidative thymine damage, could potentially interfere with thymine-specific interactions. DNA polymerases can misincorporate dUMP by utilizing dUTP as an alternative substrate to dTTP. This misincorporation could happen when the cell is deficient in folate cofactors (*22*) or particularly when dTTP metabolism is inhibited by chemotherapy agents such as methotrexate or 5-fluorouracil (*23*, *24*). In addition, polymerase-mediated DNA repair can cause dUMP misincorporation, a problem of greater significance in neurons (*25*, *26*). The oxidation of the methyl group of thymine can generate 5-hydroxymethyluracil (HmU),[1] one of the more frequent oxidation damage products found in DNA (*27*−*34*). This oxidation can happen endogenously through the action of free radicals, which are generated by normal cellular metabolic processes (*30*). Because of the importance of the thymine methyl group in varied DNA−protein interactions, either oxidation of the methyl group (generating HmU) or elimination of the methyl group (resulting in U) would be expected to interfere with transcription factor binding.

Cells contain complex mechanisms for the repair of both endogenous and exogenous DNA lesions (*35*). Most of the DNA-repair literature focuses on the need to remove potentially miscoding lesions as well as lesions that could block the progression of either DNA or RNA polymerases. However, the replacement of thymine by either U or HmU is not inherently mutagenic (*36*, *37*), nor does it impede DNA or RNA polymerases (*36*, *38*). Substitution of thymine with uracil results in only a slight decrease in DNA melting temperature (*39*), and neither U nor HmU paired with adenine substantially perturb DNA structure (*40*, *41*). There are even certain bacteriophages that completely replace T by either U (*42*) or by HmU (*43*) in their genomes. Yet, repair activities exist in most cells to remove U paired with either adenine or guanine (*36*, *44*−*50*), and higher organisms have an additional capacity to remove HmU (*51*−*56*). In fact, the removal of U or HmU in cells treated with antimetabolites or with HmdU (5-hydroxymethyl-2′-deoxyuridine), respec-

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[1] Abbreviations: HmU, 5-hydroxymethyluracil; HmdU, 5-hydroxymethyl-2′-deoxyuridine; U, uracil; I, inosine; RP-HPLC, reversed-phase high-performance liquid chromotography; GC/MS, gas chromatography/mass spectrometry; UDG, uracil DNA glycosylase; TDG, thymine DNA glycosylase.

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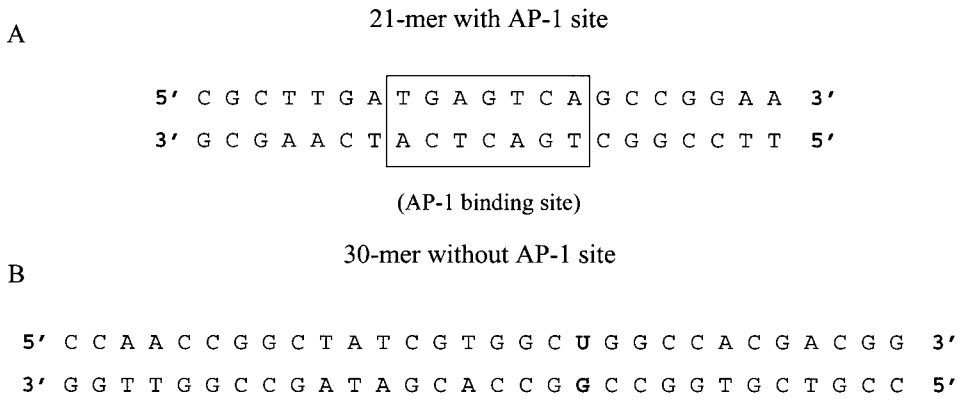

FIGURE 1: (A) Unsubstituted control 21-mer oligonucleotide sequence containing the palindromic AP-1 binding site 5′-TGA$^{G}/_{C}$TCA-3′. (B) A 30-mer oligonucleotide sequence not containing the AP-1 site but containing one U:G mispair.

tively, can trigger apoptosis as a result of repair-induced chromosome breaks (*23*, *24*, *57*). Repair gaps resulting from HmU repair can also result in deletion mutations (*58*).

These observations suggest that an additional role for DNA repair, particularly in the cells of complex, long-lived organisms, is the maintenance of sequence-specific DNA−protein interactions. In this paper, we systematically examine the influence of both U and HmU substitutions on the binding of the transcription factor AP-1 (c-Jun). We assayed binding using c-Jun homodimers, which are part of the family of AP-1 transcription factors. AP-1 contains the basic leucine zipper (bZIP) motif and is ubiquitous in cells. Components of this family are activated in response to many signals, ranging from cellular and oxidative stress to mitogenic growth factors. In vivo, AP-1 functions primarily as a heterodimer of c-Jun and c-Fos monomers, but c-Jun can also form homodimers that bind to DNA (*59*). It has been shown that the DNA binding domains of both c-Jun and c-Fos contain conserved residues that make identical contacts to the DNA bases (*60*). We have used the c-Jun homodimer binding as a model system to investigate the effects of thymine methyl group perturbation on transcription factor binding.

We observe that both U and HmU substitutions do interfere with AP-1 binding and that the magnitude of the perturbation is sequence-dependent and similar for both lesions. Although previous studies have shown inhibitory effects on DNA−protein interactions resulting from U in place of thymine, our study is the first to show inhibition of transcription factor binding by replacing thymine with HmU. In addition, we observe that substitution of the central C:G base pair for a U:G mispair in the AP-1 binding sequence enhances AP-1 binding and that this enhanced binding can inhibit repair initiated by uracil DNA glycosylase.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis and Characterization. Oligonucleotides were prepared by automated phosphoramidite synthesis and purified by RP-HPLC. HmU phosphoramidite synthesis was done according to a method developed by this laboratory (*61*). See Figure 1 for the sequence of the 21-mer unsubstituted oligonucleotide sequence containing an AP-1 binding site and the 30-mer sequence without an AP-1 site. One strand of each oligonucleotide duplex pair was 5′-^{32}P end-labeled by T4 polynucleotide kinase (New England Biolabs) with [γ-^{32}P]ATP (ICN). Labeled oligonucleotides were purified after labeling reactions using P6 Micro Bio-Spin chromatography columns (Bio-Rad) or Sephadex G-25 Quick Spin (TE) columns (Boehringer Mannheim). Unlabeled oligonucleotides were hydrolyzed at 140 °C in formic acid, heated at 140 °C in acetonitrile and bis(trimethyl silyl)-trifluoroacetamide for silylation, and analyzed by GC/MS in order to confirm the presence of the correct bases. For HmU bases, an additional step was required following formic acid hydrolysis, as reported by LaFrancois et al. (*33*).

To obtain duplexes, labeled oligonucleotides were heated with a 2-fold molar excess of their unlabeled complementary oligonucleotides in 10 mM Tris−HCl (pH 7.5) and 0.67 to 1.0 mM EDTA at 95 °C for 5−10 min and allowed to cool slowly to room temperature to promote annealing. To confirm that duplexes were being formed correctly, a sampling of uracil-substituted oligonucleotides were assayed by DNA glycosylase cleavage experiments. Uracil DNA glycosylase (UDG) from *Escherichia coli* (USB) and thymine DNA glycosylase (TDG) from *Methanobacterium thermoautotrophicum* (Trevigen) were used to discriminate between duplexes with U:A pairs and U:G mispairs, because UDG cleaves both pairs but TDG cleaves only U:G mispairs (*56*). Reactions were incubated at 37 °C for 2 h in a total volume of 10 μL, adding 3.0 pmol duplex to appropriate enzyme buffers and 1 μL of UDG (1 U/μL) or 5 μL TDG (1 U/μL) for respective experiments. UDG reactions (including buffers) contained 3 mM Tris−HCl (pH 7.5), 5 mM HEPES−KOH (pH 7.4), 16 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol (v/v); TDG reactions (including buffers) contained 10 mM HEPES−KOH (pH 7.4), 100 mM KCl, 10 mM EDTA, 0.1 mg/mL BSA, and 5% glycerol (v/v). After incubation, the following was added: 15 μL of 10 mM Tris−HCl (pH 7.5), 1 mM EDTA, 5 μL of 0.1 M NaOH, and 25 μL of 98% formamide loading buffer (includes 0.025% xylene cyanole, 0.025% bromophenol blue, and 0.01 M EDTA). This mixture was incubated at 90 °C for 30 min, then at −70 °C for at least 30 min. After thawing the reactions, unlabeled single-stranded competitor oligonucleotides were added in about 400*x* molar excess and reactions were placed at 95 °C for 15 min, cooled quickly on ice, and loaded onto a 20% denaturing polyacrylamide gel run with 1*x* TBE buffer (89 mM Tris base, 89 mM boric acid, and 2.8 mM EDTA). Gels consisted of 20% 19:1 acrylamide/bisacrylamide (National Diagnostics), 1*x* TBE

buffer, 4.6 mM ammonium persulfate, and 30 μL TEMED in a 50 mL solution and were allowed to polymerize for at least 20 min prior to use. Gels were prewarmed at 400−450 V for about 30 min before 15 μL of each sample were loaded and run at 519 V for at least 90 min. Wet gels were wrapped in plastic and exposed on a phosphor screen for 30 min, and the radioactivity was visualized using a PhosphorImager (Molecular Dynamics).

Gel Mobility Shift Assay. Binding of AP-1 transcription factor to end-labeled annealed oligonucleotides was assayed by incubating Human Recombinant AP-1 c-Jun (Promega) with the appropriate oligonucleotides and resolving the complexes on 13% nondenaturing polyacrylamide gels in 1*x* TBE buffer. Gels consisted of 13% 19:1 acrylamide/bisacrylamide, 1*x* TBE buffer, 4.0 mM $MgCl_2$, 2.6 mM ammonium persulfate, and 30 μL TEMED in a 50 mL solution and were allowed to polymerize for at least 1 h prior to use. Gels were prewarmed at 200 V for 25−40 min before samples were loaded and run at 275 V for 75−90 min. In all cases, the total volume of each sample was loaded onto the gel. Wet gels were wrapped in plastic and exposed to a phosphor screen for 10−60 min, and the radioactivity was visualized.

In experiments using increasing concentrations of c-Jun, between 1 and 4.5 μL of c-Jun (0.32 mg/mL) was added to 0.55 pmol duplex oligonucleotides, and mixtures were incubated on ice for about 30 min in a final volume of 15 μL, containing 10 mM Tris−HCl (pH 7.5), 0.50 mM EDTA, 50 mM NaCl, 17 mM KCl, 3.3 mM $MgCl_2$, 5.0 mM dithiothreitol, 170 mM guanidine hydrochloride, 10% glycerol (v/v), and 0.002 mg/mL competitor Poly d(I-C). Before c-Jun was added, mixtures were preincubated on ice for at least 45 min to equilibrate. Controls without c-Jun included 2 μL nonformamide loading buffer (0.1% xylene cyanole, 0.1% bromophenol blue, and 0.01 M EDTA), but no loading buffer was added to reactions containing c-Jun, as it has been known to compete with some proteins for binding (*62*).

In experiments done to quantitatively measure binding differences between unmodified and modified oligonucleotides, mixtures were preincubated on ice as previously mentioned, after which 4.5 μL c-Jun was added and incubated on ice for about 30 min in a final volume of 14 μL, containing 11 mM Tris−HCl (pH 7.5), 0.54 mM EDTA, 54 mM NaCl, 18 mM KCl, 3.6 mM $MgCl_2$, 5.4 mM dithiothreitol, 180 mM guanidine hydrochloride, 11% glycerol (v/v), and 0.002 mg/mL competitor Poly d(I-C). Again, loading buffer was added only to controls without c-Jun. Because of the variation in binding ability between aliquots of c-Jun, and in order to normalize each experiment, at least 4 or 5 separate reaction mixtures were simultaneously incubated as mentioned for both the unmodified and modified oligonucleotide duplexes. Then, all reactions were run on the same gel under the same conditions, and binding of the unmodified sequence was compared to binding of the modified sequence. Because the binding ability of c-Jun is highly sensitive to freezing and thawing, all reactions contained c-Jun that had gone through no more than 2 freeze−thaw cycles.

Assay for UDG Activity Inhibition by AP-1. In addition to oligonucleotides containing the AP-1 binding site, a 30-mer oligonucleotide lacking the binding site was used as a control for nonspecific binding of AP-1. (See Figure 1B for sequence.) Binding reactions were preincubated and incubated with c-Jun as previously mentioned, using 0.50 pmol (for reactions with one duplex) or 1.0 pmol duplex oligonucleotides (for reactions with two duplexes) and 4.5 μL of c-Jun (0.32 mg/mL) in a final volume of 15 μL, containing 10 mM Tris−HCl (pH 7.5), 0.50 mM EDTA, 50 mM NaCl, 17 mM KCl, 3.3 mM $MgCl_2$, 5.0 mM dithiothreitol, 170 mM guanidine hydrochloride, 10% glycerol (v/v), and 0.002 mg/mL competitor Poly d(I-C). One set of binding reactions was run as a control to visualize binding on a 13% nondenaturing polyacrylamide gel according to protocol previously mentioned. A second set of binding reactions was mixed with 2 μL of UDG dilution buffer (50 mM HEPES−KOH (pH 7.4), 10 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol) and 1 μL UDG (1 U/μL) to a final volume of 20 μL and incubated at 37 °C for 15 min. Then 20 μL of 98% formamide loading buffer and 5 μL of 0.1 M NaOH was added and mixtures were incubated at 90 °C for 30 min, after which they were incubated on ice for a short time. Unlabeled competitor oligonucleotides were added in at least 400*x* molar excess and mixtures were heated to 95 °C for 5 min, then put on ice for 1 min, after which 30 μL of the mixture was loaded onto a 20% denaturing polyacrylamide gel and run as previously mentioned for denaturing gels.

Binding Model and Data Analysis. The quantitation of the fraction of labeled oligonucleotide bound by c-Jun homodimer was accomplished with the use of ImageQuant 5.0 (Molecular Dynamics) and Microsoft Excel by dividing the amount of bound labeled oligonucleotide by the sum of the bound and unbound labeled oligonucleotide

$$\text{fraction bound oligonucleotide} = \text{(bound oligonucleotide)}/[\text{(unbound oligonucleotide)} + \text{(bound oligonucleotide)}] \quad (1)$$

This fraction was plotted as a function of c-Jun monomer added to the reaction. The scheme for the following binding model is one in which the monomers bind to DNA sequentially and then dimerize once bound to DNA (*63*, *64*)

$$U + O \rightleftharpoons AO$$
$$U + AO \rightleftharpoons A_2O$$

where U = unbound AP-1 (c-Jun) monomer, O = unbound oligonucleotide, AO = oligonucleotide bound by AP-1 (c-Jun) monomer, and A_2O = oligonucleotide bound by AP-1 (c-Jun) homodimer. This scheme leads to the following equilibrium and mass action equations:

$$K_1 = [O][U]/[AO] \quad (2)$$

$$K_2 = [U][AO]/[A_2O] \quad (3)$$

$$[\text{c-Jun}]_{\text{total}} = [U] + [AO] + 2[A_2O] \quad (4)$$

where K_1 and K_2 are two dissociation constants for the above binding model. A version of the Langmuir equation can be used as a theoretical curve in describing the cooperative association of two ligands in the binding of a macromolecule (*63*−*67*)

$$y = ([U]/K_d)^2/(1 + ([U]/K_d)^2) \quad (5)$$

where y = fraction bound labeled DNA, [U] = concentration of unbound AP-1 (c-Jun) monomer, and $K_d = (K_1K_2)^{1/2}$, which is the effective dissociation constant, the value of c-Jun concentration at which $y = 0.5$, or where 50% of the labeled oligonucleotide is bound by c-Jun. Because the concentration of c-Jun is at least 15 times that of the oligonucleotide (~36 nM) in the binding reactions with varying amounts of c-Jun (0.55−2.5 μM) and about 70 times that of the oligonucleotide in those reactions which contained 4.5 μL c-Jun (2.6 μM), the approximation $[U] \cong [\text{c-Jun}]_{total}$ can be made; thus, eq 5 can be rewritten as

$$y = ([\text{c-Jun}]_{total}/K_d)^2/(1 + ([\text{c-Jun}]_{total}/K_d)^2) \quad (6)$$

To obtain K_d values for the purpose of quantitative analysis, at least 4 or 5 measurements were taken per oligonucleotide at [c-Jun] = 2.6 μM (4.5 μL c-Jun in a 14 μL reaction total, where molecular weight of c-Jun = 39 kD) and averaged together to obtain the best K_d using the following equation (obtained by solving eq 6 for K_d):

$$K_d = ([\text{c-Jun}]_{total}^2(1 - y)/y)^{1/2} \quad (7)$$

The K_d for a particular substituted oligonucleotide can be compared with the K_d value for the unsubstituted control oligonucleotide to obtain a $\Delta\Delta G$ between the binding of the two different oligonucleotides using the following equation:

$$\Delta\Delta G = -RT \ln(K_{d,control}/K_{d,substitution}) \quad (8)$$

where the gas constant $R = 1.987 \times 10^{-3}$ kcal mol^{-1} K^{-1} and temperature $T = 277$ K (4 °C, the temperature of the ice−water bath). This $\Delta\Delta G$ value is the energy cost per mole in binding by c-Jun due to each substitution in the oligonucleotide. A positive $\Delta\Delta G$ indicates decreased binding compared to the unsubstituted sequence, and a negative value indicates increased binding. Errors on the $\Delta\Delta G$ values were calculated according to the general method of propagation of errors given by Bevington (*66*, *68*).

RESULTS

Measurement of AP-1 Binding to Oligonucleotides. In Figure 2A, we show the effect of increasing AP-1 concentration on the amount of labeled oligonucleotides bound in a DNA−protein complex. In Figure 2B, the fraction of bound oligonucleotide is plotted versus the concentration of AP-1 (c-Jun monomer). The experimental points are plotted over a theoretical curve generated according to eq 6 (see Experimental Procedures). The dissociation constant, K_d, was obtained by fitting the experimental data to this equation. Free energy changes ($\Delta\Delta G$) between unmodified and modified oligonucleotides were obtained from the K_d's by methods described in the experimental procedures section. Data generated from the gel assays can therefore be used to obtain binding constants and free energy changes associated with binding of AP-1 to oligonucleotides.

Substitutions of Thymine with Uracil and Hydroxymethyluracil. *(1) Single Substitutions*. A series of oligonucleotides were constructed containing single or multiple substitutions. Free energy changes were determined from the gel mobility shift assays and are presented in Table 1 as differences in the free energy ($\Delta\Delta G$) of binding of the unsubstituted oligonucleotide (duplex 21) with each of the substituted oligonucleotides. In oligonucleotide duplex 1, substitutions of T to HmU and T to U outside the AP-1 consensus binding domain had no significant effect on binding. All single substitutions made within the AP-1 binding site (duplexes 2−5) significantly reduced binding relative to the unsubstituted AP-1 sequence, with $\Delta\Delta G$ values ranging from 0.19 to 0.37 kcal/mol.

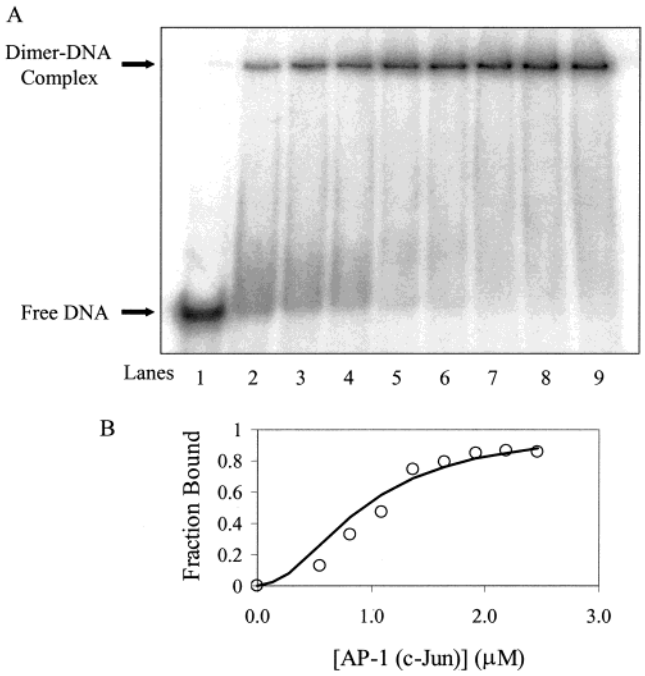

FIGURE 2: Dependence of oligonucleotide binding on increasing amounts of AP-1. (A) Gel mobility shift assay with 13% nondenaturing polyacrylamide gel, using unsubstituted oligonucleotide duplex with increasing amounts of AP-1 (c-Jun). Each lane contained ~0.55 pmol duplex DNA. AP-1 (c-Jun at 0.32 mg/mL) was added in increments of 0.5 μL, starting with 1 μL. Lane 1 is a minus AP-1 control. (B) Binding curve for AP-1 (c-Jun) binding, taken from data in gel of (A). Circles are the data points. Solid line represents the equation y = fraction bound oligonucleotide = (bound oligonucleotide)/[(unbound oligonucleotide) + (bound oligonucleotide)] = $([\text{c-Jun}]_{total}/K_d)^2/(1 + ([\text{c-Jun}]_{total}/K_d)^2)$ for K_d = 0.92 μM.

(2) Multiple Substitutions. Pairwise multiple substitutions were made, each resulting in a greater reduction in binding than for single substitutions. Duplexes 6 and 7 in Table 1 correspond to substituting for the two half-sites to which AP-1 binds (*60*), while duplexes 8 and 9 correspond to substitutions on the 5′-TGAGTCA and 5′-TGACTCA strands, respectively. Duplex 10 corresponds to substitutions for all four thymines within the AP-1 binding site. All double substitutions significantly reduced binding, with $\Delta\Delta G$ values ranging from 0.46 to 0.81 kcal/mol. Reduction in binding was the most dramatic when all four thymines were substituted, resulting in as much as 1.5 kcal/mol. Comparisons of single and multiple substitutions for T by either U or HmU indicate that the effects of multiple substitutions are approximately additive. Figure 3 qualitatively shows the effects of single and multiple T to HmU substitutions on the binding of c-Jun.

As an additional control experiment, gel mobility shift assays were repeated under denaturing conditions following the binding reactions. Oligonucleotides were treated with 0.1 M NaOH at 90 °C for 30 min and subsequently run on

Table 1: Single and Multiple Oligonucleotide Substitutions Made at Thymine Residues Both Inside and Outside the c-Jun Binding Site[a]

duplex number	duplex sequence	ΔΔ*G* (kcal/mol)
21	5′-CGCTTGA|TGAGTCA|GCCGGAA-3′ 3′-GCGAACT|ACTCAGT|CGGCCTT-5′	
1	5′-CGCT**X**GA|TGAGTCA|GCCGGAA-3′ 3′-GCGAACT|ACTCAGT|CGGCCTT-5′	X = H: 0.05 ± 0.07 X = U: −0.03 ± 0.04
2	5′-CGCTTGA|**X**GAGTCA|GCCGGAA-3′ 3′-GCGAACT|ACTCAGT|CGGCCTT-5′	X = H: 0.30 ± 0.05 X = U: 0.36 ± 0.08
3	5′-CGCTTGA|TGAG**X**CA|GCCGGAA-3′ 3′-GCGAACT|ACTCAGT|CGGCCTT-5′	X = H: 0.34 ± 0.08 X = U: 0.19 ± 0.07
4	5′-CGCTTGA|TGAGTCA|GCCGGAA-3′ 3′-GCGAACT|AC**X**CAGT|CGGCCTT-5′	X = H: 0.23 ± 0.10 X = U: 0.27 ± 0.07
5	5′-CGCTTGA|TGAGTCA|GCCGGAA-3′ 3′-GCGAACT|ACTCAG**X**|CGGCCTT-5′	X = H: 0.37 ± 0.09 X = U: 0.25 ± 0.11
6	5′-CGCTTGA|**X**GAGTCA|GCCGGAA-3′ 3′-GCGAACT|AC**X**CAGT|CGGCCTT-5′	X = H: 0.72 ± 0.14 X = U: 0.81 ± 0.15
7	5′-CGCTTGA|TGAG**X**CA|GCCGGAA-3′ 3′-GCGAACT|ACTCAG**X**|CGGCCTT-5′	X = H: 0.66 ± 0.09 X = U: 0.48 ± 0.10
8	5′-CGCTTGA|**X**GAG**X**CA|GCCGGAA-3′ 3′-GCGAACT|ACTCAGT|CGGCCTT-5′	X = H: 0.57 ± 0.08 X = U: 0.70 ± 0.12
9	5′-CGCTTGA|TGAGTCA|GCCGGAA-3′ 3′-GCGAACT|AC**X**CAG**X**|CGGCCTT-5′	X = H: 0.46 ± 0.14 X = U: 0.79 ± 0.11
10	5′-CGCTTGA|**X**GAG**X**CA|GCCGGAA-3′ 3′-GCGAACT|AC**X**CAG**X**|CGGCCTT-5′	X = H: 1.30 ± 0.15 X = U: 1.51 ± 0.11

[a] ΔΔ*G* is calculated by comparing binding of the substituted oligonucleotide duplex of interest with binding of the unsubstituted control oligonucleotide duplex using the formula described in the experimental procedures section. (H = hydroxymethyluracil, U = uracil.)

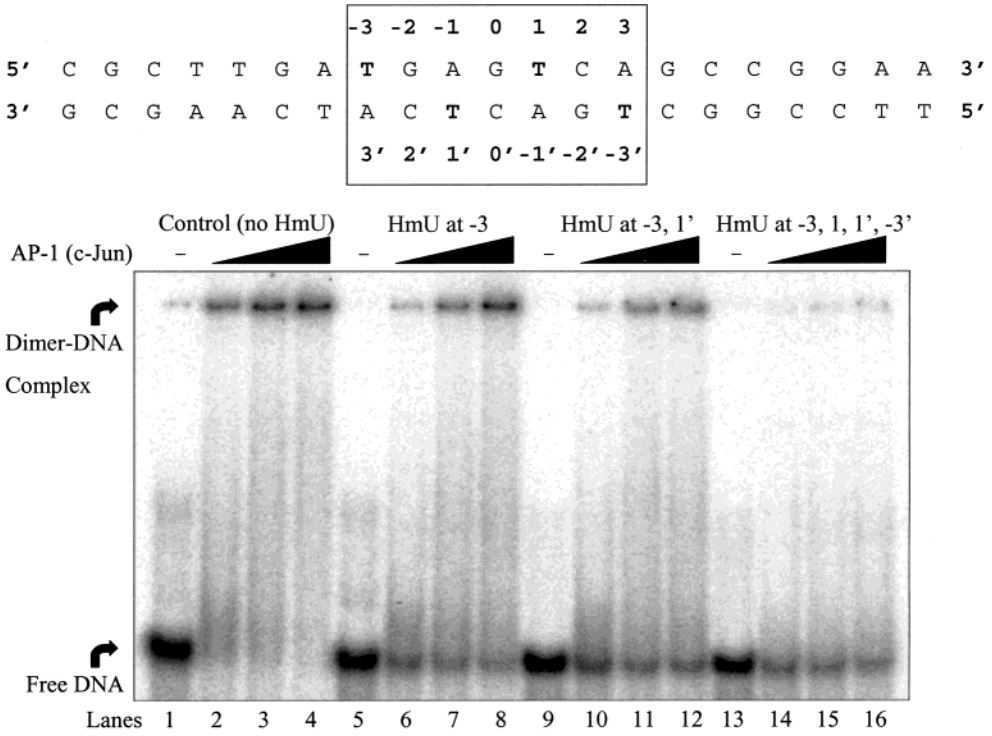

FIGURE 3: Inhibition of AP-1 binding by single and multiple T to HmU substitutions in the AP-1 binding site. Gel mobility shift assay with 13% nondenaturing polyacrylamide gel compares the unsubstituted oligonucleotide duplex with duplexes containing 1, 2, and 4 HmU bases in place of T using increasing amounts of c-Jun. Each lane contains ~0.55 pmol duplex DNA. Lanes 1, 5, 9, and 13 are minus AP-1 controls. Lanes with AP-1 (c-Jun at 0.32 mg/mL) contain 2, 3, and 4 μL protein in that order (corresponding to 1.1, 1.6, and 2.2 μM c-Jun, respectively, during binding reactions).

denaturing polyacrylamide gels. No cleavage was observed with T-, HmU-, or U-containing oligonucleotides, ruling out the possibility that the AP-1 fraction contained glycosylase activity, which could account for the inhibition of transcription factor binding.

Substitutions at Nonthymine Positions. In addition to substitutions at thymine, the effects of cytosine deamination to uracil and adenine deamination to inosine were also tested with the c-Jun binding and gel mobility shift assays. Table 2 contains free energy changes for these substitutions. Both C to U and A to I substitutions result in base pairing with wobble geometry instead of Watson−Crick geometry (*69*). Three single C to U substitutions were made, all within the AP-1 binding site, leading to a range of effects on binding. Surprisingly, substitution at the central C:G base pair with U:G (duplex 25) resulted in a modest but significant increase

Table 2: Oligonucleotide Substitutions Made at Residues Other than Thymine Inside the c-Jun Binding Site[a]

duplex number	duplex sequence	$\Delta\Delta G$ (kcal/mol)
21	5′-CGCTTGA|TGAGTCA|GCCGGAA-3′ 3′-GCGAACT|ACTCAGT|CGGCCTT-5′	
22	5′-CGCTTGA|**C**GAGTCA|GCCGGAA-3′ 3′-GCGAACT|**G**CTCAGT|CGGCCTT-5′	0.59 ±0.15
23	5′-CGCTTGA|TGAGTCA|GCCGGAA-3′ 3′-GCGAACT|**I** CTCAGT|CGGCCTT-5′	0.41 ± 0.11
24	5′-CGCTTGA|TGAGT**U**A|GCCGGAA-3′ 3′-GCGAACT|ACTCAGT|CGGCCTT-5′	0.41 ± 0.09
25	5′-CGCTTGA|TGAGTCA|GCCGGAA-3′ 3′-GCGAACT|ACT**U**AGT|CGGCCTT-5′	−0.19 ± 0.08
26	5′-CGCTTGA|TGAGTCA|GCCGGAA-3′ 3′-GCGAACT|A**U**TCAGT|CGGCCTT-5′	0.02 ± 0.06

[a] As in Table 1, $\Delta\Delta G$ is calculated by comparing binding of the substituted oligonucleotide duplex of interest with binding of the unsubstituted control oligonucleotide duplex using the formula described in the experimental procedures section. (I = inosine, U = uracil.)

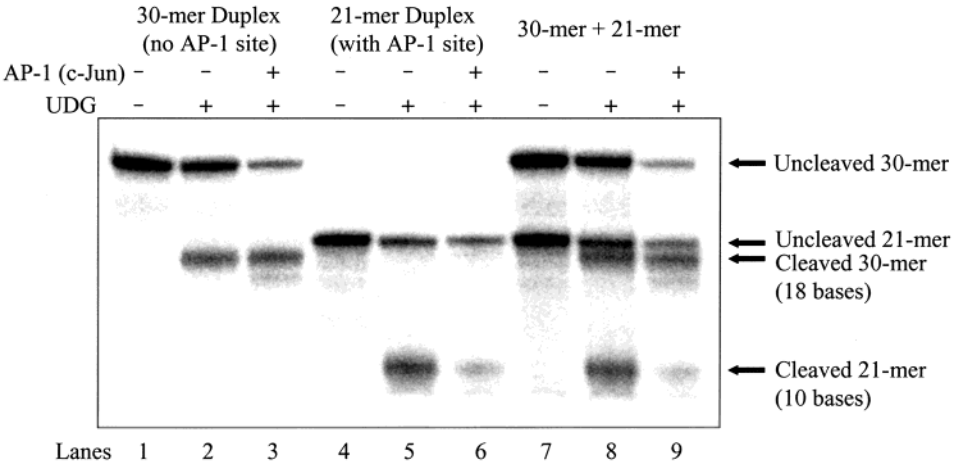

FIGURE 4: Assay for inhibition of uracil DNA glycosylase (UDG) activity by prebound AP-1. Lanes 1−6 contain ~0.50 pmol duplex DNA and 7−9 contain ~1.0 pmol duplex DNA. Lanes with AP-1 were incubated with 4.5 μL c-Jun (2.5 μM) before assaying activity of UDG. Lanes 1−3 contain duplex DNA with no AP-1 site but with one U:G mispair (see Figure 1B), 4−6 contain duplex DNA with a U:G mispair in the center of the AP-1 site (duplex 25, Table 2), and 7−9 contain both duplexes, having been assayed together in all the same reactions.

in binding ($\Delta\Delta G = -0.19$ kcal/mol). Of the other two, one (duplex 24) reduced binding ($\Delta\Delta G = 0.41$ kcal/mol) and the other (duplex 26) had no significant effect on binding. The substitution of adenine (A) with inosine (I) in duplex 23 resulted in a binding reduction equal to that of duplex 24 ($\Delta\Delta G = 0.41$ kcal/mol). Duplex 22, which corresponds to an A:T → G:C transition at an outer thymine, significantly reduced AP-1 binding, as $\Delta\Delta G = 0.59$ kcal/mol.

Inhibition of UDG Cleavage by AP-1. When a uracil residue is found within an AP-1 consensus site, both AP-1 and uracil DNA glycosylase (UDG) could compete for binding. We therefore constructed an experiment to examine potential protein−protein competition. Because the 21-base oligonucleotide (duplex 25, Table 2) contains a U:G mispair and showed an increase in the binding of c-Jun over the unsubstituted sequence (see Table 2), UDG was used to cleave the sequence with and without a previous incubation with c-Jun. A longer, 30-base sequence lacking the AP-1 site but also containing a U:G mispair was used as a control for nonspecific binding of c-Jun. As seen in Figure 4, we assayed each oligonucleotide separately and also in the same reaction in order to observe any differences due to possible changes in protein−protein and DNA−protein interactions. Binding reactions were visualized on a nondenaturing gel, showing c-Jun to selectively bind the 21-mer duplex over the 30-mer that did not contain the AP-1 site (data not shown). Figure 4 displays the UDG activity assays performed on AP-1 binding reactions that contained each oligonucleotide separately and one in which both were present. Prior incubation of c-Jun with the 21-mer duplex inhibited the activity of UDG, while prior incubation of c-Jun with the 30-mer duplex actually enhanced the activity of UDG. Assays on the separate oligonucleotides yielded the same results as the assay performed on both simultaneously.

We considered the possibility that the lane to lane variability in total ^{32}P signal might result from residual exonuclease activity in the AP-1 fraction. A control experiment was performed in which the oligonucleotide was incubated with AP-1 as described in Figure 4. When incubated at 37 °C for 1 h, slight exonuclease activity was observed. However, similar activity was observed for both T- and U-containing oligonucleotides. The observed AP-1 inhibition of UDG activity on the U:G-containing oligonucleotide is based upon the ratio of cleaved and uncleaved bands within each lane and is independent of the total signal intensity.

DISCUSSION

Implications of U:A Base Pairs in DNA. Uracil is one of the most common lesions found in DNA. Although uracil paired with guanine is mutagenic, uracil paired with adenine

is neither mutagenic nor polymerase-blocking (*36*). To date, at least five different activities have been identified in human cells that remove uracil from DNA: UDG, SMUG1, MBD4, TDG, and a partially purified HmU glycosylase that has activity against U:G pairs (*56*, *70*). The role of uracil removal is usually ascribed to the need for repair of deaminated cytosine, which results in pro-mutagenic U:G mispairs. However, although some uracil glycosylases selectively remove uracil mispaired with guanine resulting from cytosine deamination (*45*), the predominant uracil glycosylase in mammalian cells (UDG) removes uracil paired opposite either adenine or guanine with similar efficiency (*47*). Recently, it has been demonstrated that elimination of UDG does not increase the mutation frequency in some mammalian cells (*48*, *50*), suggesting that the main role of this activity is not to remove pro-mutagenic U:G lesions. However, in some human cells (*49*) as well as *E. coli ung* mutants (*36*), reduced UDG activity is associated with increased C:G to T:A mutations.

On the basis of previous studies, and in concert with data presented here, we propose that UDG's role in removing uracil from U:A base pairs is to protect the role of the thymine methyl group in DNA−protein interactions, because replacement of T by U inhibits transcription factor binding (*1*−*15*). The magnitude of the effect for a single T to U substitution can be modest or profound. Quantitative studies done on binding of lac repressor to the lac operator containing T to U substitutions yielded $\Delta\Delta G$ values of up to 1.5 kcal/mol for a single substitution (*1*, *2*). Later studies done with Cro and λ repressors binding to their DNA consensus sequences yielded values ranging from 0.3 to 2 kcal/mol for a single T to U substitution (*4*, *5*, *13*). Using the DtxR-*tox* operator system, Chen et al. showed that four thymine methyl groups within the binding region of the operator, when substituted with uracil, account for a total of 3.4 kcal/mol. These effects were greater than additive, however, because modification of one thymine alone resulted in a $\Delta\Delta G$ of only 0.33 kcal/mol (*14*). Qualitative binding studies have also been done, showing a range of inhibition resulting from T to U substitutions (*6*−*10*, *12*).

In our studies, we observe that replacement of a single T residue decreases binding affinity anywhere from approximately 0.2−0.4 kcal/mol and that inhibition is of the same magnitude for all sites within the binding region of the AP-1 (c-Jun) transcription factor. Furthermore, the decrease in binding affinity for multiple substitutions is approximately additive. Calculations done by Plaxco and Goddard on the energy cost of solvating the thymine methyl group in the context of helical DNA resulted in a predicted $\Delta\Delta G$ of about 0.9 kcal/mol, whereas van der Waals effects were thought to contribute more modestly to changes in free energy (*11*). Information from the crystal structures of c-Jun/c-Fos bound to DNA (*60*) in combination with studies done on T to U substitutions in the AP-1 sequence (*6*, *15*) show that two conserved alanines contact the thymine methyls in the AP-1 site, with one alanine contacting each methyl group. Because of protein dimerization, the two alanines in each arm of c-Jun and c-Fos together make contact with all four thymines in the DNA binding site, with one alanine per thymine methyl. The crystal structure also suggests that a more distant serine residue makes contact with one of the two methyl groups in a half-site of the DNA binding domain. Though these conserved residues contact the thymine methyl group, the contacts would not be able to completely desolvate each methyl group from its surrounding water cage in an aqueous environment. These data, along with our observed binding reduction of 0.2−0.4 kcal/mol, are evidence that primarily van der Waals interactions, and perhaps only to a small degree changes in solvation, are responsible for the energy cost when a thymine methyl is removed in the AP-1 binding site.

With a binding reduction of ~0.4 kcal/mol upon uracil substitution, the cell would therefore require the presence of 2−3 times as much AP-1 to achieve a normal level of binding. The AP-1 transcription factor is inducible and protein levels increase when cells are placed under stress, increasing 2−5-fold over baseline levels, depending on the stimulus (*59*, *71*, *72*). Thus, a single T substitution could abrogate the impact of upregulated AP-1 levels. Failure to repair such endogenous lesions would result in their accumulation and multiplication of the negative impact on transcription factor binding.

Inhibition of transcription factor binding resulting from the replacement of T by U would be a problem primarily in replicating cells. High levels of dUMP can be incorporated into DNA during replication in cells that are folate deficient (*22*). Furthermore, the activity of nuclear UDG, the main glycosylase that removes U from U:A lesions, is cell cycle dependent (*73*, *74*), suggesting that most U:A lesions are formed during replication. However, other studies suggest that replacement of T by U could also effect nonreplicating cells (*8*, *25*, *26*). Focher et al. have shown that mammalian DNA polymerase β, involved in repair synthesis, is present in adult neurons and does not make a distinction between dTTP or dUTP as a substrate, incorporating the nucleotides as a function of their local intracellular concentration. Their observations indicate that UDG levels fall considerably during development of neurons and that misincorporation of dUMP by polymerase β during DNA repair could result in uracil accumulation and inhibition of DNA-binding proteins. This group further reported that levels of dUTPase, which catalyzes the conversion of dUTP to dUMP, increase as neurons develop, thus minimizing dUMP incorporation. However, failure of the activity of dUTPase would result in increased accumulation of uracil in DNA. This mechanism has been proposed to be a possible contributor in some neurodegenerative diseases (*25*, *26*).

Implications of HmU in DNA. The thymine methyl group can also be modified by oxidation to the more hydrophilic hydroxymethyl group, an event that occurs several hundred times per day in every living cell (*30*). As is the case with U paired with A, HmU paired with A is not a miscoding (*37*) or blocking (*38*) lesion. In fact, studies done with SPO1 bacteriophage, which contains HmU in place of T in its genome, and its transcription factor TF1 have shown that the naturally occurring HmU is actually required for transcription factor binding (*75*, *76*). This interesting preference for HmU-containing sequences is probably not a result of direct DNA−protein contacts involving HmU. Instead, HmU was found to confer more flexibility to the DNA and thus increase binding of TF1 (which is known to bend DNA) to the HmU-containing site relative to thymine-containing DNA (*76*, *77*). NMR studies done by Vu et al. suggest that the extra flexibility conferred by HmU in the TF1 DNA-binding

sequence is due to *intra*-strand hydrogen bonds formed by HmU and its neighboring adenine, which weakens the *inter*-strand Watson−Crick hydrogen bonds of HmU and its base pairing partner, adenine (*77*). Despite these nondeleterious and even beneficial (in the case of SPO1 phage) effects of HmU in DNA, higher organisms have repair activities that remove HmU paired with A (*51*−*56*). In fact, studies show that mammalian cells will excise HmU from their DNA to the extent that significant levels of exogenous HmdU incorporation followed by excision will trigger apoptosis (*57*) and deletion mutations (*58*). Why are cells of higher organisms willing to commit suicide or to risk accumulating deletion mutations by excising HmU out of their DNA?

Previously, it has been demonstrated that HmU substitution can alter restriction endonuclease cleavage (*78*). To our knowledge, the effects of selective T to HmU substitution on binding of transcription factors to the DNA have not previously been examined. As is the case with T to U substitution, we observed that T to HmU substitution does indeed inhibit transcription factor binding. Interestingly, even though replacement of the thymine methyl with a hydroxymethyl group (T to HmU) results in a bulkier, more hydrophilic moeity than does replacement with hydrogen (T to U), the two substitutions result in an approximately equivalent change in the free energy of binding by AP-1. As previously discussed, replacement of the thymine methyl with hydrogen results in loss of van der Waals contacts between the protein and the DNA and perhaps a slight loss of favorable desolvation differences between free DNA and protein-bound DNA. However, the bulkier, hydrophilic hydroxymethyl group would sterically hinder ordinary protein binding to DNA, replacing favorable DNA−protein van der Waals contacts with unfavorable interactions between a hydrophilic and hydrophobic group.

The replacement of T by HmU in cellular DNA occurs by oxidation of T in A:T base pairs. Oxidative DNA damage products other than HmU, such as 8-oxoguanine, have also been known to inhibit transcription factor binding (*79*). If unrepaired, HmU residues from thymine oxidation would accumulate, presenting a challenge for long-lived organisms and particularly in cells that do not dilute the amount of HmU through DNA replication. In agreement with this hypothesis, the highest levels of HmU glycosylase in mammals were found in their neurons (*52*, *54*). Furthermore, unlike UDG, HmU glycosylase activity is not regulated by the cell cycle (*80*), suggesting that its predominant role is not to repair lesions in the DNA of replicative cells. Failure of this repair pathway could result in the development of neurodegenerative diseases. Accumulation of HmU in DNA is observed in Werner's syndrome (adult progeria), an autosomal recessive disease that is characterized by early onset of accelerated aging (*81*). Defects in DNA repair pathways are likely associated with several neurodegenerative diseases (*82*).

U:G Mispairs Can Have a Range of Effects on Transcription Factor Binding. While uracil opposite adenine in DNA results in a Watson−Crick base pair, uracil paired with guanine, resulting from deamination of cytosine, assumes wobble geometry (*69*). Three C to U substitutions that were made in the AP-1 binding site (see Table 2) resulted in a range of effects on binding of c-Jun. The DNA damage product arising from deamination of adenine is inosine (I), which also results in wobble geometry (*69*). One substitution of this kind was also shown to inhibit c-Jun binding. Comparison of these changes in free energy to that of a single T to U or T to HmU substitution demonstrates that removal or oxidation of the 5-methyl group on thymine can cause a reduction in transcription factor binding approximately equal to the binding reduction caused by a lesion that changes the base-pairing geometry, such as U:G or T:I.

Interestingly, we show here that one deamination event in which a C:G base pair is transformed to a U:G wobble pair *increases* AP-1 binding (duplex 25). Because AP-1 uses arginine side chains to make specific contacts with this central G base and with the phosphate backbone of the central C (not the base itself) (*60*, *83*), deamination of C does not result in removal of a specific DNA−protein contact. Instead, it is possible that this central base pair is a hinge region in the AP-1 site that becomes more flexible following deamination, leading to the increase in binding. Our study shows that when both UDG and AP-1 are present, the increased binding of AP-1 protects the uracil from repair. Other studies have shown similar phenomena. Verri et al. showed that a C to U substitution in the C:G central base pairs of the CRE sequence (very similar to the AP-1 sequence) increased binding by HeLa nuclear extract (*8*). Protection of a U:A base pair from UDG cleavage by DNA binding proteins such as λ-repressor has also been previously shown (*10*). In cells, multiple DNA-binding proteins compete, increasing the complexity of DNA repair pathways in vivo.

CONCLUSION

The three endogenous lesions described here (incorporation of uracil in place of thymine, oxidation of thymine to HmU, and deamination of cytosine to uracil) have all been shown to have important effects on AP-1 transcription factor binding. These effects have been previously shown for the two lesions involving uracil (U:A and U:G base pairs) and are now demonstrated for the HmU:A base pair. The results presented here suggest that an additional role for DNA repair is the maintenance of sequence-specific DNA-binding contacts and that reduction of this capacity could result in human disease, including neurodegeneration. Because neurons do not replicate, damage to their DNA is most important in the context of the loss of their ability to control gene expression as a result of an accumulation of lesions that inhibit transcription factor binding. As organisms age and repair mechanisms fail, the impact of damage-induced perturbations in DNA−protein interactions increases substantially.

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