DNA repair takes place in the context of chromatin. Previous studies showed that histones impair base excision repair (BER) of modified bases at both the excision and synthesis steps. We examined BER of uracil in a glucocorticoid response element (GRE) complexed with the glucocorticoid receptor DNA binding domain (GR-DBD). Five substrates were designed, each containing a unique C→U substitution within the mouse mammary tumor virus promoter, one located within each GRE half-site and the others located outside the GRE. To examine distinct steps of BER, DNA cleavage by uracil-DNA glycosylase and Ape1 endonuclease was used to assess initiation, dCTP incorporation by DNA polymerase (pol) β was used to measure repair synthesis, and DNA ligase I was used to seal the nick. For uracil sites within the GRE, there was a reduced rate of uracil-DNA glycosylase/Ape1 activity following GR-DBD binding. Cleavage in the right half-site, with higher GR-DBD binding affinity, was reduced ~5-fold, whereas cleavage in the left half-site was reduced ~3.8-fold. Conversely, uracil-directed cleavage outside the GRE was unaffected by GR-DBD binding. Surprisingly, there was no reduction in the rate of pol β synthesis or DNA ligase activity on any of the fragments bound to GR-DBD. Indeed, we observed a small increase (~1.5–2.2-fold) in the rate of pol β synthesis at uracil residues in both the GRE and one site six nucleotides downstream. These results highlight the potential for both positive and negative impacts of DNA-transcription factor binding on the rate of BER.

Large bulky adducts between adjoining nucleotides have been shown to physically block access of proteins necessary for cellular maintenance such as replication and transcription (2). Indeed, we have previously shown that binding of transcription factor IIIA to the 5 S rRNA gene decreases the rate of repair of UV radiation-induced cyclobutane pyrimidine dimers located in the internal control region (3). Furthermore, introduction of single cyclobutane pyrimidine dimer lesions at six different sites in the internal control region of 5 S rDNA allowed us to map the binding strength of transcription factor IIIA to its cognate sequence (4, 5). Although overall binding was unaltered, cyclobutane pyrimidine dimer damage at certain sites increased the dissociation rate of transcription factor IIIA by up to 4-fold. Moreover, the higher off rate correlated with an increased repair rate (4, 5), suggesting that the rate of DNA damage recognition is determined, in part, by existing protein-DNA complexes.

Base excision repair is responsible for the repair of DNA lesions that result from modification of the nitrogenous bases. These lesions are caused by a variety of sources, including exogenous agents like alkylating chemicals and endogenous events like replication errors and oxidative damage. In eukaryotic cells, damaged bases are recognized and removed by specialized enzymes, collectively known as DNA glycosylases. Each glycosylase recognizes one or more specific lesions and removes the base as a template to insert the correct nucleotide. Finally, DNA polymerase β (pol β)-dependent short patch repair (one-nucleotide replacement). During short patch repair, pol β displaces Ape1, removes the deoxyribose sugar, and uses the complementary base as a template to insert the correct nucleotide. Finally, DNA ligase I seals the nick (10).

Similar to examples involving bulky adducts, the binding and affinity of regulatory proteins such as transcription factors are sometimes altered when the regulatory element contains oxidative damage (11, 12). In addition, these protein-DNA interactions can block access of repair proteins to DNA lesions. Indeed, we demonstrated that the activity of pol β is completely suppressed when damaged DNA is associated with a nucleosome (13). This is most likely due to the prevention of signifi-
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cant DNA bending by the DNA-histone complex, which is a requirement for pol β function (14). Thus, protein interactions with damaged DNA can affect the recognition and subsequent repair of DNA.

In this study, we examined BER in a DNA-protein complex where the protein binds just one side of the DNA helix. We used the well characterized glucocorticoid receptor and its cognate sequence, the glucocorticoid response element (GRE), to test the activity of BER enzymes UDG, Ape1, pol β, and DNA ligase I on uracil residues located within (or just outside) a GRE. We showed that the impact of the glucocorticoid receptor DNA binding domain (GR-DDB) binding on opposite sides of the DNA helix from uracil yields both negative and positive effects on the UDG/Ape1 and pol β steps, respectively.

**EXPERIMENTAL PROCEDURES**

**Oligos**—Oligomers were purchased from Integrated DNA Technologies. Radiolabeling of the 5′-end was achieved by T4 polynucleotide kinase (Fermentas) and [γ-32P]ATP (PerkinElmer Life Sciences). Oligos were annealed to their complement and gel-purified. Briefly, bands were excised and eluted from the gel in 0.3 M NaOAc overnight at 37 °C. The supernatant was extracted with phenol/chloroform, ethanol-precipitated, and subjected to further purification on a spin column via the Nucleotide Removal kit (Qiagen). Samples were spotted onto Whatman paper and assayed by scintillation counting, and the specific radioactivity was quantified.

**GR-DDB Proteins**—Segment Cys440–Gly525 of the rat glucocorticoid receptor was overexpressed from plasmid pGR440 (a gift from Dr. Keith Yamamoto, University of California, San Francisco) in *Escherichia coli* and purified using ammonium sulfate saturation prior to separation on CM-Sepharose. Peak fractions containing GR-DDB were further purified on a Superdex 75 column (supplemental Fig. S1). Pure GR-DDB was dialedyzed against 10 mM Hepes (pH 7.6), 1 mM DTT, 10% glycerol, 10 mM ZnCl2, and 0.1 mM EDTA and then stored at −80 °C. Only fractions containing homogeneous GR-DDB (e.g. fractions 19–24; supplemental Fig. S1) were utilized in these studies.

**EMSA**—Radiolabeled damaged and undamaged DNA substrates at varying concentrations were incubated with 80 pmol of GR-DDB in binding buffer (10 mM Hepes, pH 7.9), 2.5 mM MgCl2, 0.05 mM EDTA, 10% glycerol, 50 mM NaCl, 0.1% Triton X-100, 1 mM DTT, 2.5 ng/ml poly(dI-dC), and 10 mM ZnCl2 for 30 min at 37 °C. Complexes were resolved on 5% polyacrylamide gels. Gels were soaked in 5% acetic acid, 10% methanol, and 0.5% glycerol after electrophoresis and dried before exposing to a PhosphorImager screen.

**DNA Ligase I Assay**—DNA ligase I was a gift from Dr. Samuel Wilson, Niehs, National Institutes of Health. Unlabeled substrate was generated by annealing the two oligonucleotides and subsequent treatment with a Qiagen Nucleotide Removal kit. The reaction mixtures contained the same buffer as described above and 16 pmol substrate. After cleavage for 1 h with 10 nM UDG and Ape1, 1 μM GR-DDB was added for 15 min at 37 °C. The time course began with addition of 0.05 nM pol β and 1 μl of 2000 Ci/ml [α-32P]dCTP (PerkinElmer Life Sciences). At various times, aliquots were removed and PCI-extracted to stop the reaction. Gels were run and treated as described above. The concentration of pol β was optimized so that we could examine linear enzyme activity, which was achieved at 0.05 nM (supplemental Fig. S2B).

**DNA Ligase I Assay**—DNA ligase I was a gift from Dr. Samuel Wilson, Niehs, National Institutes of Health. Unlabeled annealed substrate was treated with 10 nM UDG, 10 nM Ape1, 0.05 nM pol β, and 1 μl of 2000 Ci/ml [α-32P]dCTP. The resulting labeled substrate was purified by PCI extraction and subsequent ethanol precipitation. Using the same buffer as described above, 4 pmol of substrate were exposed to 100 nM DNA ligase I in the presence or absence of GR-DDB. At various times, aliquots were removed and PCI-treated to stop the reaction. Gels were run and treated as described above. Percent
The MMTV long terminal repeat promoter is a rich source of regulatory elements and has been used extensively in the characterization of the interaction between the GR and its cognate DNA sequence, the GRE (17, 18). Indeed, there are four GREs within ~200 bases upstream from the transcriptional start site, each consisting of a 15-bp sequence with two conserved hexamer sites were mutated to CACCTC (left) and CAUCTC (right) to abrogate specific GR-DBD binding while maintaining the uracil in a position similar to that in the RH. We note that concentrations over 1 μM GR-DBD resulted in a smear of nonspecific binding of the (−) GRE construct (data not shown). We concluded that each GRE half-site was occupied at 1 μM GR-DBD, and this concentration was used for all subsequent experiments.

Finally, methylation protection assays were performed to evaluate, in detail, the GR-DBD binding characteristics of the uracil-containing constructs. As shown in Fig. 2C, the guanine bases within the GRE were protected from methylation by dimethyl sulfate when GR-DBD was present. Specifically, guanines 185 and 175 were protected in both the RH and WT constructs in the top strand. The same was observed for the complementary guanines in the bottom strand opposite cytosines 172 and 181 (data not shown). Together with the EMSA data, these results indicate that the presence of uracil in the GRE of our constructs had little effect on the binding of GR-DBD.

**UDG/Ape1 Cleavage of Damaged Substrates in Presence or Absence of GR-DBD**—The early steps of BER can be reconstituted in vitro using commercially available recombinant enzymes. Our initial analysis utilized *E. coli* UDG and human Ape1 (New England Biolabs). Using 5′-end-labeled GRE fragments, we examined the reduction of full-length product following incubation with UDG/Ape1 in the presence or absence of GR-DBD. Interestingly, only uracil residues located within the RH and LH portions of the GRE showed a significantly reduced rate of cleavage. Fig. 3A shows representative denaturing gels of the reduction of full-length product over time (insets) as well as plots of the fraction of full-length (FL) fragment remaining. From the initial slopes of these data, a “rate of cleavage” (Δpercent FL/Δunit time) was obtained. We note that the LH and RH substrates are located on opposite sides of the DNA helix from the GR-DBD binding surface (Fig. 1B). These locations have not been identified as regions of interaction with the GR-DBD and are not expected to interfere with GR-DBD binding to the GRE (see below).

**Binding of GR-DBD to dU-damaged Substrate**—We initially determined the DNA binding characteristics of GR-DBD to the high affinity RH substrate. For these studies, the binding constant of GR-DBD to damaged and undamaged constructs was determined by EMSA using increasing amounts of DNA and a constant amount of protein (21). In each experiment, binding of the uracil-containing construct was compared with that of the undamaged GRE construct (WT). As seen in Fig. 2A, the binding constants for undamaged GRE (WT) and RH are 0.82 and 0.85 nm, respectively. Thus, the binding affinity of GR-DBD to the uracil-damaged GRE constructs is equivalent to that of the undamaged constructs.

To ensure that both half-sites of the GRE were bound in subsequent repair experiments, saturating concentrations of GR-DBD were used. A concentration of 1 μM GR-DBD shifted all free DNA into a complex as demonstrated for the US21 construct in Fig. 2B (left panel). On the other hand, the non-GRE-containing constructs yielded no specific gel shift at this concentration (Fig. 2B, right panel). In the latter case, the conserved hexamer sites were mutated to CACCTC (left) and CAUCTC (right) to abrogate specific GR-DBD binding while maintaining the uracil in a position similar to that in the RH. The three variable nucleotides serve as a spacer that places both hexamers on the same side of the DNA helix (15), a configuration that facilitates receptor dimerization (20–22). The affinity of the GR is higher for the right half-site, a consensus hexamer with the sequence TGTTC (right) to abrogate specific GR-DBD binding while maintaining the uracil in a position similar to that in the RH.
there was an initial drop in cleavage by ~10% at time 0. In addition, cleavage was reduced by ~5-fold for RH and ~3.8-fold for LH in the presence of GR-DBD protein. In contrast, the rate of cleavage of uracil residues in locations just outside of the GRE was only slightly affected by the presence of GR-DBD (Fig. 3B).

We repeated these experiments using human UDG in combination with the human Ape1. Not only did we obtain inhibition of human UDG/Ape1 cleavage with GR-DBD binding but the human enzyme showed a more pronounced inhibition (Fig. 3C). Indeed, the human UDG/Ape1 reaction was almost completely inhibited in the presence of GR-DBD binding.

In an attempt to determine whether the reduced rate of cleavage was UDG- or Ape1-dependent, we assayed the independent activities of UDG and Ape1 plus or minus GR-DBD. In all experiments, reaction conditions and enzyme concentrations were as described for the UDG/Ape1 assay. To test UDG activity, substrates LH and RH were treated with UDG ± GR-DBD, and aliquots were removed at various time points. After PCI extraction, they were treated with Ape1. To test Ape1 activity, substrates LH and RH were treated with UDG and then PCI-extracted prior to the addition of Ape1 ± GR-DBD (supplemental Fig. S3).

Clearly, separation of these activities resulted in reduced efficiency for both UDG and Ape1. Furthermore, the presence of GR-DBD appeared to affect both the UDG and Ape1 steps (although because of the greatly reduced efficiency of these reactions, the data were not analyzed for rate determination).

*Pol β Activity in Presence or Absence of GR-DBD—* Short patch repair synthesis can be promoted *in vitro* by the addition of purified pol β to substrates containing nicked abasic sites. By adding pol β and a radiolabeled cytosine to unlabeled substrates, we monitored the incorporation of cytosine at nicked abasic sites over time in DNA with and without GR-DBD bound. Initially, EMSA was used to show that, as expected, GR-DBD was able to bind nicked abasic DNA similarly to unnicked DNA in the presence of the high concentrations of UDG and Ape1 used in the following assay (supplemental Fig. S4). We then examined pol β-dependent incorporation of radiolabeled cytosine at the nicked abasic sites in the GR-DBD complex. As shown in Fig. 4A, pol β incorporation at the abasic site located in the RH portion of the GRE (see Fig. 1A) was not inhibited by GR-DBD binding. Indeed, when band intensities from several experiments, normalized to the band intensity at 20 min for naked DNA, were plotted as percentage of incorporation versus time, there appeared to be an enhancement of pol β incorporation in the presence of GR-DBD (Fig. 4A). The initial slopes of these lines, reflecting Δpercent incorporation/Δunit time of pol β incorporation for the various substrates shown in Fig. 1A, are given in Fig. 4B. Surprisingly, the rates for pol β incorporation show a small, yet statistically significant increase for both the RH and DS6 samples (1.5- and 2.2-fold, respectively, with *p* < 0.05). Thus, in contrast to our results with cleavage by UDG and Ape1, we found no inhibition of pol β activity by the binding of GR-DBD but instead saw a small enhancement.

**DNA Ligase I Activity in Presence or Absence of GR-DBD—** DNA ligase I has been found to be associated with pol β in complexes isolated from bovine testis (24) as well as to perform the ligation function *in vitro* (25–27). In the present study, we examined the activity of DNA ligase I on the dU-containing
substrates LH and RH. After treatment by BER enzymes through the pol β step, all enzymes were removed from the reaction by PCI extraction, and the nicked fragments were treated with DNA ligase I. Complete repair was measured by the accumulation of full-length product, which did not exceed 65% under the limiting conditions used. As shown in Fig. 5, the activity of DNA ligase I was unaffected by the presence of GR-DBD in either the LH or RH of the GRE.

DISCUSSION

Upon binding of ligand, the GR translocates into the nucleus where it binds to its cognate sequence, the GRE. Although there is evidence that the receptor can dimerize before it interacts with DNA (28), recognition of the GRE by the GR-DBD has been shown to be cooperative (20), and binding to the low affinity half-site is dependent on the high affinity site being bound (21). The GR-DBD contains five residues that are required for receptor dimerization (22) and behaves similarly to the intact protein, making it ideal for studying GR interactions with DNA. Based on the crystal structure of GR-DBD (15), the damaged nucleotides used in this study do not have specific contacts with the GR-DBD protein. In support of this, we saw no effect of U₃C substitution on the ability of GR-DBD to bind to the GRE. Using EMSA and methylation protection assays, we found that GR-DBD bound to dU-damaged DNA with the same affinity as to undamaged DNA.

Based on their crystal structures, both UDG and Ape1 bind one surface (or side) of the DNA molecule (7, 29). However, we found that they were strongly affected by the binding of the GR-DBD to the opposite side of the helix, suggesting that these proteins cannot bind the GRE simultaneously. In the case of UDG, the higher affinity of GR-DBD (5.7 versus 48 nM for UDG) (7, 30) must create a steric block to the access of the GRE. In contrast, the binding affinity of Ape1 is significantly lower than that of GR-DBD, allowing it to bind the GRE in the presence of GR-DBD.
higher (0.8 nM $K_d$ (31)), but GR-DBD binding appears to dominate. Thus, competition between GR-DBD and UDG/Ape1 may be the source of slower cleavage.

If competition for GRE binding yields the lag in cleavage activity by UDG/Ape1, there may be a correlation between the affinity of the GR-DBD to each half-site and the amount of interference in the cleavage assay. Indeed, we found that in the presence of saturating levels of GR-DBD a slightly greater reduction in rate was seen for damage to the higher affinity RH (5- or 13-fold) than to the lower affinity LH (3.8- or 10-fold) regardless of the UDG source (E. coli or human, respectively). Furthermore, the strongest effect by GR-DBD binding is within the confines of the 15-nucleotide GRE as little difference was seen in cleavage on substrates containing damage just outside this region. The slight decrease seen in DS19 may reflect a change in DNA secondary structure at this site upon GR-DBD binding or nonspecific binding by GR-DBD. (We note that nonspecific binding of GR-DBD is salt-dependent, being partially alleviated at concentrations above 70 mM salt, and the total salt concentration in the GR-DBD binding buffer used was 72.5 mM (32)).

We and others have shown that the activity of pol $\beta$ is suppressed or inhibited when DNA is in a nucleosome (13, 33). The co-crystal structure of pol $\beta$ shows that protein-DNA contacts are extensive, and binding results in an $\sim 90^\circ$ kink in the DNA (14). Therefore, because DNA wrapped around the histone octamer has reduced torsional flexibility, it is not surprising that pol $\beta$ function is impeded. Here we used naked DNA $\pm$ GR-DBD to examine whether a regulatory protein interferes with pol $\beta$ activity similarly to the histone octamer. Indeed,

### Figures

**Figure 4.** Pol $\beta$ activity on dU-damaged MMTV DNA with and without bound GR-DBD. **A** and **B**, unlabeled MMTV DNA fragments were initially treated with UDG and Ape1 and then exposed to 1 $\mu$m GR-DBD prior to the addition of 0.05 mM pol $\beta$ and [32P]dCTP. **A**, pol $\beta$ activity on the RH substrate. The panel shows the percentage of incorporation versus time without ($-GR$; solid lines) or with ($+GR$; dotted lines) bound GR-DBD where values were normalized to the 20-min value for the $-GR$ samples. Values were determined from scans of denaturing polyacrylamide gels showing the accumulation of radiolabeled product over time (inset). Each data point represents the mean $\pm$ S.D. of at least three independent experiments. **B**, comparison of pol $\beta$ incorporation (Incorp.) rates for the different damaged DNAs. Each column represents the mean $\pm$ S.D. of at least three independent experiments measuring the incorporation rates without (solid bars) and with (open bars) GR-DBD. Statistically significant differences were seen for samples RH and DS6 ($p < 0.05$).

**Figure 5.** DNA ligase I activity on dU-damaged MMTV DNA with and without bound GR-DBD. Unlabeled substrate was treated with a 10 nM concentration of both UDG and Ape1 followed by treatment with radiolabeled dCTP and pol $\beta$. Singly labeled substrate was phenol:chloroform-extracted and ethanol-precipitated to remove all enzymes. Substrates (4 pmol) were then treated with 100 nM DNA ligase I over the course of 1 h. Time points were taken every 15 min, and products were run on denaturing polyacrylamide gels. Values were determined from scans of the gels showing FL and cleaved (C) products.
based on the reduced rate of cleavage by UDG/Ape1 seen with substrates containing damage in the GRE, we expected a similar trend for pol β. In contrast to our results with nucleosomes, however, we were surprised to see no reduction in pol β activity in the presence of GR-DBD. Furthermore, there was a small enhancement in activity on substrates RH and DS6. If so, this could explain the enhancement seen only within the closest proximity to GR-DBD binding.

DNA ligase I has been shown to form a complex with pol β both in vivo (24) and in vitro (36), forming a multiprotein complex that is responsible for sealing the nick after repair of a damaged nucleotide. This close association presumably results from an easier access by pol β. If so, the slight enhancement seen for pol β would proceed unheeded. As for the slight enhancement, the intrinsic properties of the DNA fragment that are changed by GR-DBD binding (e.g., the predicted 35° bend in the helical axis (34)) may enhance pol β activity. Solution structure data of the GR-DBD-GRE complex shows that amino acids 510–517 of the GR-DBD induce DNA bending and unwinding (35), events that might position the abasic site for an easier access by pol β. Between the location of damage and the effect on the different BER steps, it appears that subsequent steps are much less affected by the presence of GR-DBD. Interestingly, the slight enhancement seen for pol β did not correlate with DNA ligase I activity.

Comparison of the results for UDG/Ape1 cleavage and pol β incorporation (Fig. 6) allows one to examine the relationship of protein binding, depends upon the binding affinity of each protein. Initially, we predicted that GR-DBD would physically block access of the repair proteins to the damaged nucleotide. Although this occurred for UDG/Ape1, it was not the case for pol β or DNA ligase I. Importantly, the GR-DBD complex did not create the same block to BER enzymes as the histone octamer. Our results indicate that the ability of BER proteins to access damaged nucleotides is highly dependent on the DNA-protein interactions in place around the damage site. Clearly, it will be of interest to compare the repair efficiency and mutation rates within different locations of chromatin of intact cells and under different regulatory conditions.

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FIGURE 6. Summary of relative BER enzyme activity (+GR/−GR) at various locations of dU damage in MMTV promoter. Each column represents the mean ± 1 S.D. of the -fold change between +GR and −GR samples for at least three independent experiments. A value of 1 (dotted line) denotes no difference in UDG/Ape1 cleavage rates (solid bars) or pol β incorporation rates (gray bars) when GR-DBD is bound.
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