Uracil-DNA glycosylase as a probe for protein – DNA interactions

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ABSTRACT

The DNA repair enzyme Uracil-DNA Glycosylase (UDG) can be used to investigate three different features of protein – DNA interactions. Complexes can be probed by simple protection experiments ('footprinting') or by two kinds of interference assays: a missing thymine site (MT-site) experiment and a missing thymine methyl site (MTM-site) experiment. The three probing methods are assessed using the well-characterized *in vitro* systems of λ repressor and *lac* repressor binding to their respective operator sites. The results obtained with UDG probing agree well with previous probing experiments on the same systems and, in certain cases, extend previous interpretations: for example, comparison of the results obtained with the two interference assays shows that formation of the *lac* repressor – operator complex requires interactions with the methyl group of one particular thymine residue (T-13) in the operator but also requires interactions with other parts of the thymine base at operator positions 7, 8, 9, 21, 23 and 24. Overall, the properties of UDG recommend it as a versatile and convenient method to investigate DNA – protein interactions both *in vitro* and possibly *in vivo*.

INTRODUCTION

Interactions between proteins and their specific DNA binding sites have been probed by a variety of reagents (1). A partial list of currently used probes would include nucleases such as DNase I (2) and micrococcal nuclease (3), as well as chemical reagents such as dimethyl sulphate (4), hydroxyl radical (5), ethylnitroso urea (6) and KMnO₄ (7). Probing experiments are designed either as 'protection' assays, in which the reagent is allowed to react with preformed protein–DNA complexes, or as 'interference' assays, in which the reagent is allowed to react with uncomplexed DNA and the ability of the modified DNA to complex with protein is subsequently assayed by a technique such as electrophoretic mobility shift. Both types of experiment can provide detailed information about the proximity of protein

residues to individual DNA groups in the binding site. Results have usually agreed well with X-ray structures of the complex, where these are available (8,9).

A more recent direction has been to use DNA probes to assess protein binding in the natural chromosomal context inside the cell. For example, KMnO₄, dimethyl sulphate and a prokaryotic methylase have been used to look at transcription complexes in *E.coli* (10), at binding sites of important regulatory proteins such as muscle creatine kinase in mouse muscle cells (11), and at DNA accessibility in *S.cerevisiae* (12), respectively. Such *in vivo* probing experiments are in their early stages and there would seem to be considerable scope for the development of new reagents for probing DNA conformation and DNA–protein interactions *in vivo*. We suggest that the enzyme Uracil-DNA-Glycosylase (UDG) is a potential candidate for such an *in vivo* reagent.

The *E.coli* UDG is a well-characterized small (25.6 kDa) stable DNA repair enzyme (13). UDG catalyses the excision of uracil from DNA by cleaving the N-glycosylic bond between the base and the sugar, thereby producing an apyrimidinic (Ap) site. The phosphodiester backbone at the Ap site remains intact but can be subsequently cleaved by hot alkali (14).

Figure 1 diagrams three different ways in which UDG can be used to probe a DNA – protein complex: (1) UDG-protection ('footprinting'), which measures the ability of UDG to excise uracil residues from a binding site in the presence of binding protein; (2) Missing thymine site (MT-site) interference assay to identify specific thymine bases needed for complex formation, and; (3) Missing thymine methyl site (MTM-site) interference assay to identify interactions involving only the methyl group on the thymine base.

The present paper describes the *in vitro* use of UDG to probe two well-characterized systems of DNA – protein interactions: the *lac* and λ -repressor proteins binding to their respective operator sites into which uracil has been previously incorporated by PCR. A 'uracil interference' assay similar to the MTM-site assay described here, has recently been used by Pu and Struhl (15) to identify important thymine methyl groups in the DNA binding site of the yeast GCN4 protein.

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Figure 1. Three methods by which UDG can be used to probe DNA-protein interactions. End-labelled uracil-containing DNA is prepared by PCR. In UDG Protection Experiments (left), protein-DNA complexes are subjected to UDG cleavage, resulting in the formation of Ap sites at accessible uracil residues. After deproteinisation, the Ap sites are cleaved with alkali and the reactions analysed on a sequencing gel. The UDG reactions with and without protein are compared to reveal the footprint of the protein on the DNA. In Missing Thymine Site (MT-site) Experiments (middle), the end-labelled uracil-containing DNA is first subjected to UDG treatment to form Ap sites. The modified DNA is then incubated with protein and the mixture is resolved on a native gel. DNA is eluted from the 'Bound' (B) and 'Free' (F) bands, Ap sites cleaved with alkali and the products analysed on a sequencing gel. The T residues essential for protein-DNA complex formation are absent from the B fraction and enriched in the F fraction. In the Missing Thymine Methyl Site (MTM-site) Experiments (right), a bandshift reaction is performed with protein and uracil-containing DNA. The DNA is eluted from the B and F fractions, treated with UDG and alkali and the cleavage products analysed on a sequencing gel. Thymine methyl groups essential for protein-DNA interaction are absent from the B fraction and enriched in the F fraction.

MATERIALS AND METHODS

The λ OR operator sequences, contained in a 194 bp Alu fragment cloned into the SmaI site of pUC18, were amplified using the KpnI primer and the Reverse primer. A 245 bp fragment containing the *lac* operator sequences was amplified directly from Bluescript KS (Stratagene) using the SK primer and a custom primer (ACCTCACTCATTAGGCAC) complementary to upstream sequences on the Bluescript vector. λ Repressor was purchased from BRL. Purified *lac* repressor was kindly provided by Dr Tom Record, University of Wisconsin. UDG was purified as previously described (16) and is now commercially available from several sources.

Polymerase Chain Reactions (PCR) contained, in a total volume of 50 μ l, 25 pmoles of each primer (in most experiments, one of the primers was 5'-end-labelled with 32 P), 0.5 μ g of DNA,

Figure 2. A. Relation between input and output mole fractions ($U/(U+T)$). The input mole fraction ($dUTP/(dUTP + dTTP)$) was varied from 0 to 1 during the PCR amplification. Uracil incorporation was measured as described in the Methods Section. The theoretical curves are calculated based on the following model: when the Taq DNA polymerase encounters an 'A' residue in the template, it makes one of two choices: either it binds dUTP and incorporates a 'U' residue, or it binds dTTP and incorporates a 'T' residue. The assumption is that this equilibrium binding step controls the relative incorporation of dUTP and dTTP. Curves are calculated for cases in which the equilibrium association constant for binding dTTP is 30, 50 or 100 fold higher than for binding dUTP. B. Uniformity of uracil incorporation by Taq DNA polymerase. λ operator DNA is prepared by PCR amplification using varying input $U/(U+T)$ amounts and an upper-strand end-labelled primer. The gel isolated PCR products are treated with UDG and alkali and then electrophoresed on an 8% sequencing gel. Cleavage sites correspond to positions of T in the DNA sequence.

0.2 mM each of dATP, dGTP, dCTP and (dUTP+TTP) and 2 units of Taq DNA Polymerase; the buffer contained 50mM KCl, 1.5mM $MgCl_2$, 10mM Tris, pH 8.3, 0.05% NP-40 and 0.05% Tween-20; amplifications were for 40 cycles (94°C for

Figure 3. UDG protection experiments. The λ right operator sequence, amplified by PCR using one end-labelled primer, one unlabelled primer and a 5% U/(U+T) input ratio, was mixed with λ repressor and treated with UDG followed by hot alkali. UDG footprints on the upper (A) and lower (B) strands are shown; C. The UDG footprint shows little dependence on the input levels of (U/U+T) in the PCR amplification; D. The UDG footprint is insensitive to cleavage kinetics.

30 secs, 45°C for 1 min, 72°C for 1 min). The reaction products were separated on a 6% native polyacrylamide gel and detected by autoradiography. The full length amplification products were excised, embedded into a 1% agarose gel and electrophoresed onto Schleicher and Schuell NA45 DEAE membrane. The DEAE membrane was washed in TBE buffer (90 mM Tris-Borate, 1mM EDTA pH 8.0) and the DNA eluted twice using 200 μ l of high salt buffer (1.0 M NaCl, 0.1 mM EDTA and 20 mM Tris-HCl, pH 8.0) at 55°C. After ethanol precipitation, the DNA was washed twice with 70% ethanol and resuspended in water to give 20,000 cpm/ μ l (approximately 3 ng DNA/ μ l).

In the dual-labelling experiment (Fig. 2A), trace amounts of [2-¹⁴C] Thymidine 5' triphosphate (ICN, 47.5 mCi/mmol) and deoxy[5-³H]Uridine 5' triphosphate (Amersham, 1.5 Ci/mmol) were added to the PCR reaction. In this case, the full length amplification product was detected by ethidium bromide staining, eluted from the gel and the amounts of uracil and thymine incorporated into the DNA were determined by liquid scintillation counting.

Lambda repressor DNase I footprints

Lambda repressor was incubated in 50 μ l of binding buffer (10 mM Tris, pH 7.0, 2 mM CaCl₂, 50 mM KCl, 2.5 μ g/ml BSA and 5% glycerol) with 1 μ g of poly(dI-dC) for 20 min at room temperature. End-labelled DNA (3 ng) was added and incubation continued for a further 10 min. Fifty μ l of DNase I reaction buffer (25 mM NaCl, 10 mM HEPES, pH 7.8, 5 mM MgCl₂, 1 mM CaCl₂) and 2 units of DNase I (BRL) were added; after 2 min the reaction was stopped by addition of 10 μ l Stop Buffer (100 mM MES, pH 6.0, 150 mM EDTA, 5% SDS, 250 μ g/ml herring sperm DNA). Following a phenol/chloroform extraction, the DNA was ethanol precipitated, washed

thoroughly with 70% EtOH, dried *in vacuo*, resuspended in formamide loading buffer and electrophoresed on an 8% sequencing gel.

UDG footprints

Protein and DNA were incubated in 50 μ l of binding buffer as described above. Five ng of UDG was added and the reaction later terminated with 5 μ l of Stop Buffer; as discussed in the text (Figure 3D), as long as the UDG reaction is allowed to continue for at least two minutes, the precise reaction time is not critical. After phenol/chloroform extraction, ethanol precipitation and 70% ethanol washes, the DNA pellet was resuspended in 5 μ l of 50 mM NaOH, 5 mM EDTA and heated at 90°C for 2–5 min. After cooling, the DNA was dried *in vacuo*, resuspended in formamide loading dye, heated at 90°C for a further 3 min and electrophoresed on an 8% sequencing gel. For the UDG 'ladders', DNA (3 ng) was incubated with 0.5 ng of UDG for 2 min at 37°C, prior to alkali treatment and electrophoresis.

Missing thymine site (MT-Site) interference assay

UDG (2.5 ng) was added to 50 ng of DNA in 50 μ l of *lac* binding buffer (10 mM Tris, pH 7.9, 125 mM KCl, 10 mM MgCl₂, 10 mM CaCl₂, 0.1 mM DTT and 25% glycerol) and the reaction was incubated at 37°C for 10 min to form Ap sites. One μ g of *lac* repressor protein was added and the reaction was incubated at room temperature for 20 min. After electrophoresing the mixture on a 6% native acrylamide gel, the 'bound' and 'free' bands were excised and eluted using DEAE membrane as described above. The DNA was resuspended in 10 μ l of 50 mM NaOH, 5 mM EDTA, heated at 90°C for 5 min, dried *in vacuo*, resuspended in 10 μ l of formamide buffer, heated at 90°C for a further 3 min and electrophoresed on an 8% sequencing gel.

Missing thymine methyl site (MTM-Site) interference assay

50 ng DNA, 1 μ g of *lac* repressor protein and 50 μ l of *lac* binding buffer were incubated at room temperature for 20 min and the mixture then electrophoresed on a 6% native acrylamide gel at 4°C. The 'bound' and 'free' DNA species were excised and eluted as described above. The DNA pellets were then resuspended in 10 μ l of TE buffer (10 mM Tris, pH 7.8, 1 mM EDTA) with 2.5 ng of UDG and incubated at 37°C for 10 min. Alkali cleavage was performed by adding 2 μ l of 0.2 M NaOH, 20 mM EDTA and heating at 90°C for 5 min. The DNA was dried, resuspended in 10 μ l of formamide buffer, heated at 90°C for 3 min and electrophoresed on an 8% sequencing gel.

Figure 4. Effect of uracil on the binding of λ repressor to the OR operator. The λ operator (upper strand labelled) was amplified by PCR using 0% and 5% U/(U+T) input ratios, mixed with λ repressor protein and the mixture digested with DNase I. The λ repressor shows decreased binding at the uracil-containing OR3 site.

RESULTS AND DISCUSSION**PCR incorporation of uracil residues into DNA binding sites**

A single preparation of uracil-containing DNA can be used for all three of the UDG-based probing methods diagrammed in Figure 1. The DNA must be labelled at a unique position and must contain, on average, fewer than one uracil residue per DNA strand. Since the final excision/cleavage products of the probing experiment are separated on standard sequencing gels (which resolve fragments from 40 to 400 nucleotides in length), uracil residues must replace roughly 1–10% of the thymine residues in the normal DNA binding site. Thus, the first step in developing a UDG-based footprinting system is to establish conditions for incorporating uracil residues at the required level into binding site DNA.

Binding sites for the protein of interest are inserted into the multiple cloning site of a standard plasmid such as pBluescript. The insert is then amplified using Taq DNA polymerase, standard sequencing primers and the cycling parameters described in the methods section. Initial experiments investigated the relation between input and output mole fraction of uracil in the PCR reaction, although in general, DNA polymerases do not strongly discriminate between dUTP and dTTP in DNA synthesis (17). PCR primers were unlabelled while dUTP and dTTP were labelled with ^3H and ^{14}C respectively. The total dUTP + dTTP concentration was held constant at 0.2 mM but the input mole fraction U/(U+T) varied from 0 to 100%. For each input ratio, full length amplification product was gel-purified and the ratio of incorporated isotopes was determined by scintillation counting. Figure 2A plots the measured level of incorporated uracil as a function of the level of input uracil (as dUTP). As can be seen, the incorporated mole fraction of uracil increases from 0 to 10% as the input mole fraction increases from 0 to 90%. Quantitatively, this is the behaviour expected if the only difference between uracil and thymine incorporation is that dUTP binds 30–100 fold less tightly than dTTP to the Taq DNA polymerase (see theoretical curves in Figure 2A). Overall, the input mole fraction of dUTP is conveniently non-critical for incorporating the necessary one uracil for every 40–400 base pairs of binding site.

A second requirement for the use of UDG as a footprinting probe is that each potential cleavage site (corresponding to T residues in the DNA sequence) be represented more-or-less equally in the final UDG cleavage products. This is demonstrated in Figure 2B, in which the λ right operator (i.e. containing the three λ repressor binding sites: OR1, OR2 and OR3) was amplified with one ^{32}P end-labelled primer, one unlabelled primer and with an input mole fraction (U/(U+T)) ranging from 0 to 15%. Amplified products were gel-isolated, incorporated uracil bases were excised by reaction to completion with UDG, the phospho-diester backbone at the resulting Ap sites cleaved

Figure 5. Summary of UDG footprinting with the λ repressor-operator system. The UDG footprints are represented by the bold outer lines. The DNase I footprints are marked on each strand by the thin lines. The (genetically-defined) 17 bp operator sequences are marked by a line between the two DNA strands.

with alkali and the cleavage products displayed on a sequencing gel (Figure 2B). As expected, cleavage sites correspond only to T residues in the original template sequence and all such sites are represented. Overall, there are no major differences in band intensity at the various sites. Although the intensity of a few specific bands does appear to decrease at high U levels, this has no effect on our footprinting data. Any differences that do occur reflect incorporation preference by the Taq DNA polymerase i.e. the UDG reactions were performed to completion (18).

UDG protection assay

We first consider the use of the UDG enzyme in a simple protection or 'footprinting' assay. Saturating amounts of protein (in the present case, purified λ -repressor) are allowed to bind to end-labelled uracil-containing DNA corresponding to the λ OR1, OR2 and OR3 binding sites. UDG is then added to the complex to excise any accessible uracil residues from the binding site. The reaction is stopped, the protein is removed, the phosphodiester backbone is cleaved by alkali at Ap sites produced by the UDG reaction and finally, the cleavage products are displayed on a standard sequencing gel. As shown in Figure 3,

there are indeed positions in the λ repressor binding site from which uracil residues can not be excised by UDG in the presence of the repressor protein. These protected uracils fall within the previously defined binding sites OR1 and OR2 in both the upper and the lower strand (Figure 3A and 3B respectively). Protection of uracil residues in OR3 is less evident, especially in the lower strand; the implications of this weaker protection will be discussed below.

The UDG footprinting assay turns out to be both convenient and robust. For example, repressor footprints are essentially unchanged over a considerable range of input dUTP concentration (Figure 3C). Moreover, footprints remain essentially unchanged in the presence of excess amounts of UDG and for reaction times from 2–30 minutes or longer (Figure 3D); in other words, the extent of cleavage is dominated by the initial uracil incorporation levels and not by the reaction kinetics. This is in contrast to probes such as DNase I, which can in principle cleave at all residues in the binding site and whose kinetics must consequently be tightly controlled. Finally, UDG is a very stable enzyme and footprints can be obtained under a wide range of solution conditions (data not shown), providing practical advantages over probes such as DNase I, which requires divalent ions, or hydroxyl radicals, which demand the strict exclusion of free radical scavengers (19).

We now return to the weak or missing UDG-footprint corresponding to the λ OR3 site. As noted above, if a uracil residue in the binding site is not removed when UDG is used to probe the protein–DNA complex, it implies that this particular uracil residue is protected by the binding of the protein. However, if a uracil residue at a particular position in the binding site is excised, there are two possibilities: (i) the residue is not normally protected by bound protein, or; (ii) the protein is prevented from binding by the presence of the uracil residue, i.e. binding requires the thymine methyl group. We show below, for the case of the *lac* repressor–operator system, how the missing thymine methyl site (MTM-site) interference assay can be used to distinguish between these two possibilities. However, to establish the principle by a method independent of UDG, λ operator sites were prepared with or without incorporated uracil residues, mixed with λ repressor and the resulting complexes were probed with DNase I in a conventional footprinting assay. The results (Figure 4) show clearly that the presence of uracil in the binding site has no apparent effect on protection of OR1 and OR2. However, uracil incorporation substantially weakens the footprint seen with OR3.

Figure 6. MT-site (A,C) and MTM-site (B,D) interference assays on the upper (A,B) and lower (C,D) DNA strands of the *lac* operator. The arrows point to bands that are absent in the bound fraction and enriched in the free fraction. MT-site assays identify two thymine bases on the upper strand (A) and five on the lower strand (C) that are essential for protein–DNA complex formation. The MTM-site assays show that only one of these involves interactions with a thymine methyl group in the lower strand (D). No thymine methyl groups in the upper strand are essential (B). The site in D marked with an asterisk is not seen under all conditions.

Figure 7. Summary of MT-sites and MTM-sites interference assays on the *lac* repressor–operator system. The operator positions are numbered according to reference 20. Lines between strands represent residues that crosslink with the *lac* repressor in UV-protection experiments (19). The arrow marks the thymine residue (position 13) at which the methyl group is essential for protein–DNA complex formation (MTM-site assay). Arrowheads indicate interactions with thymine bases (positions 7, 8, 9, 21, 23 and 24) that occur at moieties other than the methyl group.

In other words, the absence of a T methyl group at certain positions within OR3 lowers repressor affinity. Indeed, the crystal structure of λ repressor-operator complex points to several potential interactions between protein side chains and thymine methyl groups in the binding site (20). Presumably, the influence of the missing methyl group only becomes apparent with the weakest binding site, OR3.

Our results obtained from the UDG and DNase I footprinting assays are aligned with the sequence of the λ right operator in Figure 5. The footprints are in general agreement with the genetically defined operator binding sites. The UDG footprint is smaller than the DNase I footprint, possibly reflecting the smaller size of the UDG enzyme or the proximity of the UDG active site to the physical boundaries of the protein.

Missing thymine site (MT-Site) and missing thymine methyl site (MTM-site) interference assays

We tested both types of interference assay (see Figure 1) with the complex formed between the *lac* repressor and its operator site.

In the MT-site interference assay, the end-labelled uracil-containing DNA of the binding site is pre-treated with UDG to excise the incorporated uracil residues. The DNA, now containing Ap sites, is mixed with excess *lac* repressor protein and the 'bound' and 'free' species separated on a native acrylamide gel. DNA is extracted from the gel, the phosphodiester backbone at Ap sites is cleaved with alkali and the cleavage products are analyzed on a sequencing gel. Our expectation is as follows: if repressor binding requires that there be a base (either thymine or uracil) at a particular position in the binding site, then a product corresponding to cleavage at this site will be depleted from the 'bound' fraction but will be enriched in the 'free' fraction. The results of the MT-site assay are shown in Figure 6A and 6C for the upper and the lower strand, respectively, of the *lac* operator. The arrows point to two positions in the upper strand and five positions in the lower strand that are absent from the bound fraction but appear relatively enriched in the free. The implication is that the *lac* repressor must form an important contact with a residue at these positions.

The MTM-site assay (Pu and Struhl's uracil interference assay (15)) can be used to classify the interactions with the thymine base into interactions that occur with the 5-methyl group and interactions that involve some other group on the base. A bandshift reaction is performed on the mixture of *lac* repressor and uracil-containing binding site. The expectation is that residues at which the thymine methyl group is crucial for *lac* repressor-operator complex formation will be absent from the shifted fraction but will be enriched in the free DNA fraction. In the upper strand of the *lac* operator (Figure 6B), we could not detect any position at which the lack of a thymine methyl group prevented *lac* repressor binding. However, in the lower strand (Figure 6D), there is reproducibly one site (arrow) that requires the presence of a methyl group for repressor binding; (a second site, indicated by the asterisk, is less reproducible and hence is not emphasized). As required, the site that requires the methyl group for binding corresponds to one of the thymine residues identified in the MT-site assay.

The results of the MT-site and MTM-site interference assays on the *lac* repressor-operator system are summarised in Figure 7. By and large, our results agree very well with results from probing experiments done over the past fifteen years. For

example, the current MT-site experiments identify seven thymine sites (two in the upper strand, five in the lower strand) that are important for formation of the *lac* repressor-operator complex. These are all sites that, when replaced with bromodeoxyuridine residues, can be crosslinked to the repressor protein (21). The MTM-site interference assay shows that on one specific thymine (thymine 13), it is the 5-methyl group that is essential for complex formation. Goeddel *et al* (22) had previously found that uridine substitution at this site did indeed decrease the stability of the repressor-operator complex. Comparison of the results obtained with the two interference assays indicates that interactions must also occur with the other six thymine residues at points other than the 5-methyl group. This suggestion fits with recent studies using molecular dynamics (23) and NMR (24), which indicate possible interactions between His-29 of the repressor and the C6H of thymines 8 and 9.

SUMMARY AND CONCLUSIONS

We have described the use of the DNA repair enzyme UDG as a probe of DNA-protein interactions. Simple variations in the experimental design can be used to evaluate three quite different structural determinants of sequence-specific binding (see Figure 1). These three experimental approaches have been tested using the well-described *lac* and λ repressor-operator systems. Overall, results from the current experiments agree well with previous findings and provide confidence for applying UDG to probe less-studied examples of DNA-protein interactions.

Taq DNA polymerase can incorporate many nucleotide analogues into DNA (25), (26). Various base-protein interactions in protein-DNA complexes could potentially be investigated by extending the experimental approaches described in this paper to other DNA glycosylase enzymes.

Our ultimate goal is to use UDG as an *in vivo* probe of DNA conformation and gene activity. Uracil can be readily incorporated into genomic DNA (27) and UDG can be placed under inducible control (unpublished results). The results of the current study provide the necessary *in vitro* calibration for the use of UDG *in vivo*.

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