Accelerated Publications

The XPC−HR23B Complex Displays High Affinity and Specificity for Damaged DNA in a True-Equilibrium Fluorescence Assay†

Thomas Hey,‡ Georg Lipps,‡ Kaoru Sugasawa,§ Shigenori Iwai,|| Fumio Hanaoka,§,⊥ and Gerhard Krauss*,‡

Lehrstuhl für Biochemie, Universität Bayreuth, Universitätsstrasse 30, 95447 Bayreuth, Germany, RIKEN, The Institute of Physical and Chemical Research, and CREST, Japan Science and Technology Corporation, Wako-shi, Saitama 351-0198, Japan, Biomolecular Engineering Research Institute, Osaka 565-0874, Japan, and Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamada-Oka, Suita, Osaka 565-0871, Japan

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ABSTRACT: The XPC−HR23B complex is a prime candidate for the initial damage recognition step during global genome nucleotide excision repair. A specific interaction between the XPC−HR23B complex and various types of damaged DNA substrates has been demonstrated in recent work by electrophoretic mobility shift assays or immunoprecipitation. Although these studies allowed the estimation of relative binding affinities for the different types of lesions, the presence of large amounts of competitor DNA or the need for glutaraldehyde fixation prevented the quantification of equilibrium constants. We have performed a quantitative study on the binding of XPC to damaged DNA using fluorescence anisotropy measurements. The XPC−HR23B complex binds with high affinity ($K_D \sim 1-3$ nM) to fluorescent 36 bp DNA fragments containing a single cisplatin 1,3-intrastrand adduct or a six-nucleotide mispaired region. From stoichiometric titration experiments, it is concluded that ∼70% of the XPC−HR23B preparation is active in DNA binding. Binding experiments employing fluorescent probes with a single defined photoproduct reveal a 30-fold preference of XPC for 6,4-photoproducts as compared to a cyclobutane dimer. Competition experiments with undamaged and damaged plasmid DNA indicate that the XPC−HR23B complex discriminates between damaged and undamaged sites with high specificity. The specificity factor is between 100 and 3000, depending on the number of nonspecific sites considered in the calculations. Upon addition of XPA to the XPC binding reaction mixtures, it was not possible to detect cooperative ternary complex formation on the platinated 36 bp probe.

Nucleotide excision repair (NER)[1] is a major pathway for the removal of DNA lesions such as UV-induced DNA photoproducts and helix-distorting adducts caused by carcinogens (*1*, *2*). Impaired NER activity is associated with several human disorders, including xeroderma pigmentosum for which seven NER-deficient complementation groups and the genes involved have been identified (XP-A−XP-G). In eukaryotes, two distinct NER subpathways are operative, namely, transcription-coupled repair (TCR) and global genome repair (GGR), which employ a different subset of NER proteins. Among the XP gene products, the XP-C complementing protein (XPC) is specifically required for GGR.

Although the essential components of human NER have been identified and reconstitution of NER in vitro from purified recombinant repair components has been achieved recently (*3*), the specific functions of some of the NER components, namely, XPA, replication protein A (RPA), and XPC, are still not fully understood. Furthermore, the coordination and spatial organization of the various reactions of NER remain to be determined. For instance, the mechanistically important question of the initial damage recognition during NER is still a matter of debate (*4*). The main candidates for damage sensor proteins are the RPA−XPA complex and the XPC gene product. The XPC protein is a 940-amino acid protein which is found in a tight complex with the HR23B protein, the human homologue of the yeast RAD23 protein (*5*, *6*). A role of the XPC−HR23B complex in damage recognition has been suggested from kinetic repair assays where XPC was found to be the primary damage sensor (*7*). Conflicting results have been reported on this

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* To whom correspondence should be addressed. Phone: +0049-(0)921-552428. Fax: +0049(0)921-552429. E-mail: gerhard.krauss@uni-bayreuth.de.

‡ Universität Bayreuth.

§ RIKEN, The Institute of Physical and Chemical Research, and CREST, Japan Science and Technology Corporation.

|| Biomolecular Engineering Research Institute.

⊥ Osaka University.

[1] Abbreviations: XP, xeroderma pigmentosum; NER, nucleotide excision repair; UV, ultraviolet light; CPD, cyclobutane pyrimidine dimer; 6,4-PP, pyrimidine(6,4)pyrimidone photoproduct; CPt, cisplatin; GGR, global genome repair; ss, single-stranded.

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issue (*8*), and the point of entry of XPC into the repair complex is still a matter of controversy. Nevertheless, the high-affinity binding of XPC to damaged DNA shown by various methods such as gel mobility shift, immunoprecipitation, and footprinting (*9*, *10*) strongly suggests a function of XPC in the damage recognition step. However, quantitative data on the specificity of the XPC−HR23B complex binding to damaged DNA are lacking, and it is unclear how its ability to discriminate damaged DNA from undamaged DNA compares to that of RPA and the XPA−RPA complex. This is mainly due to problems with the solubility of the purified XPC protein requiring addition of nonspecific DNA to stabilize the protein. Furthermore, the presence of protein aggregates precluded a quantitative determination of binding constants.

MATERIALS AND METHODS

Preparation of Proteins. XPC and HR23B were separately expressed and purified following published protocols (*6*, *11*). Both proteins were combined to reconstitute the XPC−HR23B complex, and the latter was further purified by subsequent chromatographic steps on HiTrap-heparin and chelating columns (*9*). XPA was expressed as a His-tagged protein in *Escherichia coli* BL21(DE3) pLysS using the plasmid pET15b-XPAC (*12*) generously provided by R. Wood and purified by metal chelate chromatography using Ni−NTA fast-flow Sepharose (Qiagen) as described previously (*13*).

Preparation of Damaged DNA Substrates. The CPD and 6,4-PP probes were obtained by chemical synthesis as described previously (*14*, *15*), and probes containing the cisplatin 1,3-d(GTG) intrastrand adduct were prepared by incubation with cisplatin for 16 h at 37 °C in the dark according to the method of Moggs et al. (*16*). The degree of platination was checked by denaturing PAGE and restriction analysis using *Apa*LI. For the preparation of double-stranded DNA, equimolar amounts of single-stranded damaged oligonucleotide probes and the fluorescently labeled complementary strands were hybridized in buffer NEB4 (New England Biolabs), and these reactions were tested for quantitative hybridization by native PAGE after radioactive 5′-labeling with T4-PNK and [γ-^{32}P]ATP. All probes used in this study contained <5% single-stranded DNA.

Plasmid DNA pUC19-ARS1$_{16}$ (6.4 kb; pUC19 containing 16 copies of the *Saccharomyces cerevisiae* ARS1 element) was purified using the Plasmid Maxi Kit from Qiagen. UV-damaged plasmid DNA was obtained by irradiation with germicidal lamps (G8T5) at 254 nm and a fluence rate of 10 J s^{-1} m^{-2} for 20 min. According to Haseltine et al. (*17*), no significant fragmentation of the DNA occurs under these conditions which was also verified by agarose gel electrophoresis.

Fluorescence Depolarization Measurements. All binding experiments in this study were carried out in XPC assay buffer [20 mM sodium phosphate (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 0.01% (v/v) Triton X-100, and 100 μg/mL BSA] in 10 mm × 2 mm microcuvettes with an initial sample volume of 120 μL. An LS50B spectrofluorometer (Perkin-Elmer) equipped with a polarization device and a thermostated jacket at 30 °C was used. Excitation and emission bandwidths were adjusted to 12 and 18 nm, respectively. Fluorescence titrations were performed at an excitation wavelength of 495 nm with a vertical polarizing filter and monitored at an emission wavelength of 525 nm using a 515 nm cutoff filter. Under these conditions, a fluorescent probe concentration of 5−10 nM was required for measurements with an acceptable signal-to-noise ratio. At least 10 data points with an integration time of 5 s were collected for each titration point. With the exception of Figure 2A showing a typical binding curve, the corresponding error bars have been omitted for clarity. The resulting mean values were least-squares fitted to a simple one-site binding model as described previously (*18*) using Origin 5.0 (Microcal, Northampton, MA). K_Ds and errors given in Table 1 represent the values obtained from this fit. The K_D for the "bubble" DNA was reproducibly determined by three independent competition titrations within the error range of the fit. The small amount of the XPC−HR23B complex that was available prevented the performance of triplicate experiments for all substrates.

FIGURE 1: Oligonucleotides used for fluorescence anisotropy measurements. Unpaired or adducted bases are printed in bold; **F** represents a fluorescein group attached to a 3′-end of DNA.

For the competition titrations, the mixture for the binding reaction from Figure 2A was diluted 3-fold with 10 nM bubble DNA in XPC assay buffer after data collection for the last titration point. After the mixture had been split into three fractions, the XPC−bubble DNA interaction competed with single-stranded oligonucleotides or plasmid DNA, either undamaged or damaged after UV treatment.

RESULTS AND DISCUSSION

In this study, we have used fluorescence anisotropy to measure the affinity of the XPC−HR23B complex for model oligonucleotides containing defined DNA lesions (Figure 1). The oligonucleotides carried a terminal fluorescein modification located distant from the damaged site that served as an indicator for complex formation with the XPC−HR23B complex. The fluorescence anisotropy method (*19*) is based on the increase in the extent of fluorescence polarization upon binding of the small fluorescently labeled damaged oligonucleotide to the large XPC−HR23B complex. We have applied this method recently for the characterization of the binding of XPA and RPA to various damaged DNA substrates (*18*). In contrast to the aforementioned methods used for the study of XPC−DNA interactions, the fluorescence anisotropy measurements are performed under true-equilibrium conditions, and quantitative information about the strength of complex formation between the repair proteins and the damaged DNA probes can be obtained within a broad range of buffer and salt conditions.

XPC used in this study was expressed in insect cells and combined with bacterially expressed HR23B to yield the

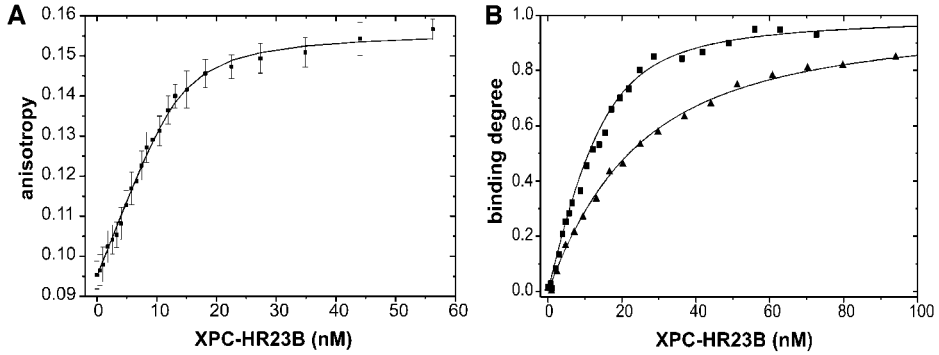

FIGURE 2: Fluorescence anisotropy measurements of binding of the XPC−HR23B complex to damaged DNA. (A) Titration of the six-nucleotide bubble probe (10 nM) with the XPC−HR23B complex yields a stoichiometric binding curve. (B) The XPC−HR23B complex binding to a nondamaged (▲) or cisplatin 1,3-intrastrand adducted (■) 36 bp fluorescent probe (10 nM). The concentrations of XPC given have not been corrected for the DNA binding activity determined from the stoichiometric titration (Figure 2A).

Table 1: Binding Affinity and Specificity of Binding of the XPC−HR23B Complex to Various Damaged and Undamaged DNA Substrates

DNA substrate (see Figure 1)	Affinity K_D[a] (nM)
damaged DNA	
six-nucleotide bubble	1.1 ± 0.3
6,4-PP	0.5 ± 0.2
CPt	3 ± 1
CPD	16 ± 2
controls	
undamaged ds36mer	10 ± 3
undamaged ss36mer	14 ± 3[b]
Specificity	
	specificity factor[c]
damaged vs UV-damaged plasmid	91 ± 7[d]
	3100 ± 250[e]

[a] Corrected for the protein activity of two batches. [b] Data obtained from competition titrations. [c] Ratio of the binding affinities of plasmid DNA obtained from competition experiments. [d] Model of nonoverlapping binding sites. [e] Model of overlapping nonspecific binding sites; see the text for details.

XPC−HR23B complex as described previously (*9*). Only limited amounts of pure, highly active complex were available, and the following experiments with the DNA substrates listed in Figure 1 were performed with ~20 μg of the XPC−HR23B complex. Figure 2 shows typical titration curves. All binding curves could be fitted satisfactorily using a simple 1:1 model (DNA + XPC−HR23B ↔ XPC−HR23B−DNA). In the experiment of Figure 2A, the fluorescently labeled bubble duplex at a concentration of 10 nM was titrated with the XPC−HR23B complex. The fluorescence anisotropy of the damaged DNA probe increased from 0.095 for the free probe to ~0.155 for the complex. We did not observe a significant change in the fluorescence intensity of the fluorescein moiety upon formation of the XPC−HR23B−DNA complex, which facilitated evaluation of the binding curves. Binding is nearly stoichiometric with a dissociation constant of ~1 nM (Table 1). The fit also reveals that ~70% of the XPC−HR23B complex is active in DNA binding. This is an important issue since the specific DNA binding activity of XPC−HR23B preparations may be reduced due to the protein's tendency to aggregate.

Our experiments do not indicate significant aggregation of the XPC−HR23B complex on the DNA probes even at a large excess of protein as reported earlier by Batty et al. (*20*), which points to the high quality of our XPC−HR23B preparation. The binding equilibrium was established within short times (<1 min), and the binding curves for all substrates could be fitted using the 1:1 model.

An issue of primary interest in all studies on the function of the XPC−HR23B complex has been its damage recognition ability. We have addressed this question by employing titration experiments with a 36 bp DNA probe containing a 1,3-intrastrand cisplatin adduct and the undamaged control (Figure 1). This substrate should provide sufficient contact points for binding of the XPC−HR23B complex which has been shown to protect ~30 nucleotides on a damaged double-stranded DNA probe (*10*). The XPC−HR23B complex binds to the platinated oligonucleotide with a K_D of ~3 nM. The undamaged probe is bound only about 3 times weaker (Figure 2B). A similar observation has been reported by Wakasugi and Sancar (*8*) using gel mobility shift experiments on the binding of a short DNA probe with a single photoproduct. The relatively strong binding of the undamaged probe may be explained by a preferential binding of the XPC−HR23B complex to DNA ends.

To exclude nonspecific end effects, we performed competition titrations where the preformed XPC−HR23B−bubble complex was challenged with plasmid DNA, either undamaged or damaged after UV irradiation (Figure 3A). In these experiments, XPC exhibits much stronger discrimination between damaged and undamaged DNA. Whereas UV-damaged plasmid DNA is a strong competitor for the binding of the bubble probe, undamaged DNA is bound with much weaker affinity.

A crucial point in the evaluation of the competition data is the model used for the calculation of the number of damaged and nondamaged binding sites on the long DNA. On the basis of data published by Yeung et al. (*21*), we expect ~200 photoproducts in the 6.4 kb plasmid DNA for the UV dose that is applied. If a random distribution is assumed, a damaged site should be found every 30−40 nucleotides, which is slightly larger than the region covered by XPC in footprinting experiments (*7*) and corresponds to the length of the fluorescent probe.

Therefore, the competitor concentration is expressed as the number of equivalents of the bubble probe size in Figure 3A. Using a nonoverlapping binding site model based on these simplifications, the fit yields a K_D value for photoproduct binding which is similar to the one determined for the bubble substrate. When the same model is applied to

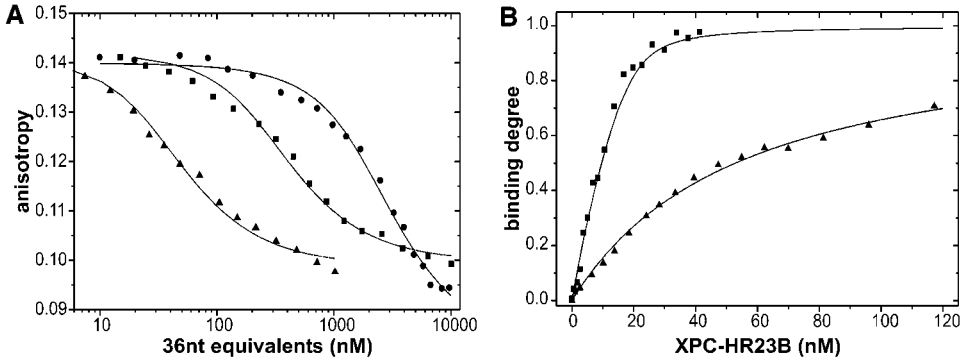

FIGURE 3: Binding of the XPC−HR23B complex to single-stranded DNA and DNA-containing photoproducts. (A) Competition of the binding of the XPC−HR23B complex to the fluorescent bubble DNA probe (10 nM) by addition of the single-stranded oligonucleotide single-stranded 36mer (■), UV-irradiated plasmid DNA (▲), and the undamaged plasmid (●). (B) XPC binding to various types of photoproducts. The 30 bp fluorescent probes (7.5 nM) containing either a TT-6,4-PP (■) or a CPD (▲) were used for titration experiments with XPC (see Figure 1). The specific binding activity of this XPC sample was ~2-fold lower as in the other experiments.

the nondamaged plasmid DNA, a 90-fold higher K_D is obtained, indicating a specificity factor of approximately 90. In this model, however, the number of competitor sites and accordingly the specificity factor are underestimated. A model of overlapping binding sites is better suited to describing the binding behavior because the number of potential nonspecific binding sites is in large excess over the number of protein molecules. Specifically, the number of binding sites corresponds to the total number of base pairs of plasmid DNA (*22*). Compared to the nonoverlapping binding site model, this yields an additional factor of ~30 (6400 nondamaged sites vs ca. 200 photoproducts), and a final specificity factor of approximately 3100 results. These estimates still contain unknowns, e.g., the ratio of cyclobutane dimers to 6,4-photoproducts, which are preferentially bound by XPC (see below). A realistic specificity value is certainly between the extremes of 90 and 3100. The high specificity determined from the use of long DNA as a competitor indicates that the damage recognition ability of XPC may be underestimated with short DNA probes because of binding to DNA ends. The high affinity of the XPC−HR23B complex for the bubble DNA might be due to a preferential binding to single-stranded regions which has been observed for many DNA repair proteins and is often used for their purification (*11*, *12*). The competition titration indicates an approximately 13-fold weaker affinity for a single-stranded probe of mixed purine-pyrimidine composition versus the bubble probe, which is in the same range as the binding of the undamaged 36mer double strand. Clearly, structural determinants other than single-stranded regions are required for damage recognition by the XPC−HR23B complex.

From gel mobility shifts, it has been concluded that the XPC−HR23B complex forms highly stable complexes with damaged DNA that are characterized by a fast association and a very slow dissociation (*20*). Accordingly, quite long reaction times and elevated temperatures of 37 °C were required to reach the binding equilibrium in competition experiments. Contrary to these reports, the binding equilibrium is established in our experiments within a few minutes after addition of the competitor even at 30 °C. The conflicting results may be explained by differences in the quality of the protein preparations. The protein used in this study has been purified by glycerol gradient centrifugation and does not contain aggregates. Furthermore, the fluorescence anisotropy assay allows the use of salt conditions ensuring high solubility of the XPC−HR23B complex.

Recent studies on the interaction of the XPC−HR23B complex with DNA probes containing single photoadducts indicated a strong preference for 6,4-photoproducts (6,4-PP) compared to cyclobutane pyrimidine dimers (CPD) (*9*). A similar observation has been made in photoreactivation experiments on UV-treated DNA fragments (*20*). Our fluorescence titrations confirm these results. As shown in Figure 3B, binding of the XPC−HR23B complex to 6,4-PP occurs in a nearly stoichiometric manner, as has been observed for the bubble DNA. The quality of the binding curve did not allow an accurate quantification of the amount of active protein in this case. Clearly, the protein aliquot used for these experiments was less active in DNA binding. Nevertheless, a K_D in the sub-nanomolar range is obtained. The CPD probe is bound with nearly 30-fold lower affinity, and the K_D is in the same range determined for the undamaged double-stranded probe and the single-stranded probe. XPC apparently is not able to distinguish CPDs from unmodified bases in our assay. In accordance with this finding, CPD containing DNA is poorly repaired in in vitro NER assays (*9*). There is increasing evidence in the literature that CPDs are recognized via damage-sensing proteins distinct from XPC. Candidates for this function include the XPA−RPA complex and the damaged DNA-binding protein (UV-DDB) (*23*). It can be also hypothesized that the recognition of CPDs by XPC requires the cooperation of other protein factors. An enhancement of XPC binding to 6,4-PP containing DNA fragments by addition of XPA has been shown by Wakasugi et al. (*8*). When we investigated the influence of XPA on the binding of the XPC−HR23B complex to the cisplatin-modified probe, we could not observe any cooperative effect. The same is true for the bubble DNA. Addition of XPA to an XPC binding reaction with half-maximal saturation of the damaged probe did not result in a change in anisotropy values (data not shown). Possibly, other proteins (e.g., TFIIH) are required to mediate an interaction between XPC and the XPA−RPA complex.

In Table 1, we have summarized our titration data which are in line with previous results obtained with the same protein preparation using semiquantitative methods such as gel mobility shift assays and immunoprecipitation (*9*, *10*). Significantly weaker binding affinities for the binding of the XPC−HR23B complex to damaged DNA have been reported

by Batty et al. (*20*) which may be ascribed to a lower specific binding activity of their protein preparation. Nevertheless, these authors could show a 400-fold preference of the XPC−HR23B complex for UV-damaged versus undamaged DNA. Our data show that the XPC−HR23B complex binds with the highest affinity to the bubble and 6,4-PP lesions. The interaction with the cisplatin probe is slightly weaker. Undamaged double-stranded and single-stranded oligonucleotides as well as a CPD oligonucleotide are bound with significantly lower affinity. A much higher damage specificity of the XPC−HR23B complex is revealed by the competition experiments with undamaged plasmid DNA, which point to a strong damage discrimination when interference by end effects is excluded. Our data are in accordance with models where the XPC−HR23B complex alone is able to detect strongly helix-distorting lesions with high efficiency. The recognition of lesions that do not significantly alter the geometry of the DNA helix may require the participation of other proteins acting alone or in cooperation with XPC (*10*).

The results presented here demonstrate the power of the fluorescence anisotropy assay for the study of protein−DNA interactions in repair processes. With only a small amount of protein available, we were able to determine equilibrium constants for various damaged DNA substrates and to derive specificity factors.

REFERENCES

1. de Laat, W. L., Jaspers, N. G., and Hoeijmakers, J. H. (1999) *Genes Dev. 13*, 768−785.
2. Wood, R. D. (1997) *J. Biol. Chem. 272*, 23465−23468.
3. Araujo, S. J., Nigg, E. A., and Wood, R. D. (2001) *Mol. Cell. Biol. 21*, 2281−2291.
4. Batty, D. P., and Wood, R. D. (2000) *Gene 241*, 193−204.
5. Masutani, C., Sugasawa, K., Yanagisawa, J., Sonoyama, T., Ui, M., Enomoto, T., Takio, K., Tanaka, K., van der Spek, P. J., Bootsma, D., Hoeijmakers, J. H., and Hanaoka, F. (1994) *EMBO J. 13*, 1831−1843.
6. Masutani, C., Araki, M., Sugasawa, K., van der Spek, P. J., Yamada, A., Uchida, A., Maekawa, T., Bootsma, D., Hoeijmakers, J. H., and Hanaoka, F. (1997) *Mol. Cell. Biol. 17*, 6915−6923.
7. Sugasawa, K., Ng, J. M., Masutani, C., Iwai, S., van der Spek, P. J., Eker, A. P., Hanaoka, F., Bootsma, D., and Hoeijmakers, J. H. (1998) *Mol. Cell 2*, 223−232.
8. Wakasugi, M., and Sancar, A. (1999) *J. Biol. Chem. 274*, 18759−18768.
9. Sugasawa, K., Okamoto, T., Shimizu, Y., Masutani, C., Iwai, S., and Hanaoka, F. (2001) *Genes Dev. 15*, 507−521.
10. Kusumoto, R., Masutani, C., Sugasawa, K., Iwai, S., Araki, M., Uchida, A., Mizukoshi, T., and Hanaoka, F. (2001) *Mutat. Res. 485*, 219−227.
11. Sugasawa, K., Masutani, C., Uchida, A., Maekawa, T., van der Spek, P. J., Bootsma, D., Hoeijmakers, J. H., and Hanaoka, F. (1996) *Mol. Cell. Biol. 16*, 4852−4861.
12. Jones, C. J., and Wood, R. D. (1993) *Biochemistry 32*, 12096−12104.
13. Kuraoka, I., Morita, E. H., Saijo, M., Matsuda, T., Morikawa, K., Shirakawa, M., and Tanaka, K. (1996) *Mutat. Res. 362*, 87−95.
14. Murata, T., Iwai, S., and Ohtsuka, E. (1990) *Nucleic Acids Res. 18*, 7279−7286.
15. Iwai, S., Shimizu, M., Kamiya, H., and Ohtsuka, E. (1996) *J. Am. Chem. Soc. 118*, 7642−7643.
16. Moggs, J. G., Yarema, K. J., Essigmann, J. M., and Wood, R. D. (1996) *J. Biol. Chem. 271*, 7177−7186.
17. Haseltine, W. A., Gordon, L. K., Lindan, C. P., Grafstrom, R. H., Shaper, N. L., and Grossman, L. (1980) *Nature 285*, 634−641.
18. Hey, T., Lipps, G., and Krauss, G. (2001) *Biochemistry 40*, 2901−2910.
19. Lundblad, J. R., Laurance, M., and Goodman, R. H. (1996) *Mol. Endocrinol. 10*, 607−612.
20. Batty, D., Rapic'-Otrin, V., Levine, A. S., and Wood, R. D. (2000) *J. Mol. Biol. 300*, 275−290.
21. Yeung, A. T., Mattes, W. B., and Grossman, L. (1986) *Nucleic Acids Res. 14*, 2567−2582.
22. von Hippel, P. H., and Berg, O. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1608−1612.
23. Wakasugi, M., Shimizu, M., Morioka, H., Linn, S., Nikaido, O., and Matsunaga, T. (2001) *J. Biol. Chem. 276*, 15434−15440.

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