The relationship between DNA repair efficiency at specific locations in the binding site of the nine-zinc finger protein transcription factor IIIA (TFIIIA) and binding of its individual zinc fingers was studied. Homogeneously damaged oligonucleotides, which contained a single cis-syn cyclobutane thymine dimer (CTD) at one of six different sites in the internal control region (ICR) of the 5 S rRNA gene to generate a series of damaged DNA substrates, were prepared by chemical synthesis. Binding of TFIIIA to the substrates was assayed by measurement of dissociation constants ($K_d$), dissociation rates ($k_{off}$), and protein-DNA contacts. The results indicated that a single CTD in the ICR does not significantly affect the $K_d$ of TFIIIA. In contrast, CTDs at positions +55 and +72 (from the transcription start site) in the ICR markedly enhanced $k_{off}$ of TFIIIA from the complex. In addition, CTDs in these two sites increased methylation of the N7 of guanines (by dimethyl sulfate) in the zinc finger contacts of the ICR-TFIIIA complex. Furthermore CTDs at +55 and +72 were more efficiently removed from the complex than CTDs at other sites in the ICR by Xenopus oocyte nuclear extracts. This suggests that repair of CTDs closely correlates with changes in the binding of individual zinc fingers of the ICR-TFIIIA complex. These results have implications for the mechanism of DNA damage recognition and repair in protein-DNA complexes.

Ultraviolet radiation can induce a variety of negative biological effects in cells including necrosis, apoptosis, mutagenesis, and carcinogenesis. These diverse effects result from the generation of stable UV photoproducts, such as cis-syn cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) dimers, which contain covalent bonds connecting two adjacent pyrimidines (1, 2). These lesions disrupt base stacking in the DNA helix and weaken interactions between base pairs, significantly altering the local structure of the DNA helix (3, 4). As a consequence, UV photoproducts can impede binding of transcription factors (5, 6) and progression of polymerases on DNA templates during transcription or replication (7, 8).

Stable UV photoproducts are repaired by nucleotide excision repair (NER), replacing 24–32 nucleotides surrounding a lesion with newly synthesized DNA (9). NER targets a broad spectrum of bulky lesions, which distort the DNA helix and modify DNA chemistry (10). Bulky lesions occurring throughout the genome are repaired by global genome repair. The process is initiated by specific binding of damage recognition proteins to the lesions. In the case of pyrimidine-pyrimidone (6-4) dimers and cisplatin adducts, the XPC-HR23B complex is believed to be the recognition proteins (9). In contrast, damaged DNA-binding protein has been proposed to act as a recognition factor for CPDs prior to the binding of the XPC-HR23B complex (11, 12).

Several factors are known to influence repair of UV photoproducts. First, the degree of helical distortion induced by a lesion may be related to its repair rate, suggesting that recognition of damage is a rate-limiting step for NER (13). In addition, the position of a lesion is one of the determinants of repair rates. Efficient repair in the transcribed strand of an active gene, called transcription-coupled repair, has been found in both prokaryotic and eukaryotic cells (14–16). It has been postulated that transcription-coupled repair is initiated by a stalled RNA polymerase and requires transcription-repair coupling factor in Escherichia coli or CSA, CSB, TFIIH, and XPG in mammalian cells (17, 18). In contrast, preferential repair of the transcribed region, low NER rates have been observed at upstream promoter sites in human JUN, CDC2, and PGK genes (19–21). Since the slow repair regions correspond to transcription factor binding sites, it has been suggested that the assembly of transcription factors around the promoters inhibits NER by shielding lesions from the NER machinery. Furthermore inhibition of NER by a transcription factor has been demonstrated with the TFIIIA-5 S rDNA complex in vitro where CPD removal is markedly decreased by TFIIIA binding (22).

Xenopus TFIIIA is a prototype for zinc finger proteins, consisting of nine tandemly repeated C$_2$H$_2$-type zinc fingers and a C-terminal domain. High affinity binding ($K_p$ = 0.4–2 nM) of TFIIIA for the ICR of 5 S rRNA results from the sequence-specific interactions between the nine fingers and three separate promoter regions in the ICR (Fig. 1): the A-box (+50 to +64), intermediate element (IE, +67 to +72), and B-box (+82 to +92) (23–25). The first three zinc fingers (zf1–zf3) and the last three (zf7–zf9) make contacts with bases in the major groove of the C-box and A-box, respectively, and zfs interact with bases in the IE. The three segments are connected by zf4 and zf6 crossing the minor groove (25). Inhibition of UV photoproducts by TFIIIA binding to 5 S rRNA gene; ICR, internal control region; IE, intermediate element; CTD, cis-syn cyclobutane thymine dimer; pyrimidine-pyrimidone (6-4), 6-(1,2)-dihydro-2-oxo-4-pyrimidinyl-5-methyl-2-(1H,5H)-pyrimidinediones; NER, nucleotide excision repair; dmg, damaged substrate; RNAP, RNA polymerase; DMS, dimethyl sulfate; endo, endonuclease.

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1 The abbreviations used are: CPD, cis-syn cyclobutane pyrimidine dimers (CPDs); pyrimidine-pyrimidone (6-4) dimers, which contain covalent bonds connecting two adjacent pyrimidines; TFIIH, transcription factors (5, 6) and progression of polymerases on DNA templates during transcription or replication (7, 8).
rDNA is observed using UV-irradiated DNA fragments as a binding substrate (6). However, since UV irradiation of homogenous DNA fragments produces a mixed population of fragments with various photoproducts at different positions and different yields, it has remained unresolved which CPD sites in the 5 S rDNA may reduce the DNA binding affinity of TFIIIA. Moreover analysis of in vitro repair of UV-irradiated DNA in the TFIIIA-DNA complex has similar limitations due to the heterogeneity of the DNA substrates.

To overcome these limitations, DNA fragments containing CTDs at specific positions in 5 S rDNA were prepared from chemically synthesized DNA oligonucleotides (Fig. 2A). The results of TFIIIA binding demonstrate that a single CTD in the ICR only slightly modulates the DNA binding affinity of TFIIIA, depending on the position of the CTD. However, CTDs at positions +55 and +72 in the ICR noticeably enhanced dissociation of the TFIIIA-DNA complex and modulated the TFIIIA contacts around the CTD sites. Furthermore in vitro repair of these substrates showed that NER was more efficient at the two sites (+55 and +72) where significant increases in off-rates occurred. Conversely TFIIIA binding inhibited repair of CTDs in the ICR where no significant change in binding occurred. Therefore, these results suggest that modulation of TFIIIA binding by CTDs leads to the heterogeneity of repair rates in the ICR.

MATERIALS AND METHODS

Construction of 5 S rDNA Oligonucleotides Containing CTDs at Specific Sites—Oligonucleotides were synthesized on an ABI 380B automated DNA synthesizer using phosphoramidite chemistry. The 5'-dimethoxytrityl CTD phosphoramidites were synthesized and incorporated into specific sites (Fig. 2A) during synthesis as described previously (26). After synthesis and high pressure liquid chromatography purification, the final single strand oligonucleotides were dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and quantified by UV absorbance at 260 nm.

Double-stranded DNA fragments were prepared by mixing the complementary sequences in equal molar ratio and slow cooling from 90 °C to 4 °C. The annealed fragments were separated from single-stranded oligonucleotides on 10% polyacrylamide native gels in TBE buffer (90 mM Tris base, 90 mM borate, 2 mM EDTA, pH 8.3). The double-stranded DNA band was identified by exposing the wet gel to long wavelength UV (365 nm) on a PhosphorImager screen (Amersham Biosciences) and locating the band by its "shadow" in the phosphorimage (27). DNA was extracted from the gel by elution in TE buffer at 37 °C for 8 h. The solution was filtered with a 0.45-μm centrifuge filter to remove the gel particles, and the DNA was precipitated with ethanol. For strand-specific 5'-end labeling, the appropriate single strand oligonucleotide was phosphorylated with T4 kinase ( Invitrogen) and [γ-32P]ATP.
with unlabeled ATP. Then oligonucleotides were annealed to their complementary sequences to generate 5- or 6-base overhangs on the 3'-end. The resulting DNA fragments with +38 to +104 of 5 S rDNA were ligated to the sequence of −20 to +37 to produce a 125-bp (−21 to +104) DNA fragment with CTDs at +50 (dmg_A1) or +55 (dmg_A2). Similarly a 101-bp (+38 to +144) 5 S rDNA fragment with CTDs at +88 (dmg_C2), +90 (dmg_C3), and +101 (dmg_OC) were prepared by ligation of the 67-bp damaged fragment to the sequence of +105 to +144. Standard ligation reactions were carried out using 1 unit of T4 DNA ligase (Invitrogen) in 25 μl of reaction buffer at 22°C for 1 h. The ligated products were isolated on a 7 M urea, 8% polyacrylamide gel and used for in vitro repair experiments.

Preparation of Recombinant TFIIA—Recombinant Xenopus laevis TFIIA was expressed and purified from E. coli BL21(DE3) containing plasmid pTA-102 as described by Del Rio and Setzer (28). Briefly, TFIIA expression was induced with 1 mM of isopropylthio-β-D-galactoside and 100 μM ZnSO4. After cell lysis, the inclusion body of TFIIA was suspended in buffer A (20 mM HEPES, pH 7.4, 5 mM MgCl2, 5 mM dithiothreitol, 50 μM ZnSO4, 10% glycerol) with 250 mM NaCl, 5 M urea, and 1 M phenylmethanesulfonyl fluoride. Final purification was carried out using a phenyl column (Amersham Biosciences) and a 100-ml gradient from 1.2 to 0.5 M (NH4)2SO4. Protein concentration was measured using the Bradford method (29), and the purified TFIIA solution (about 500 μM) was stored at −70°C. The stock solution was diluted to an appropriate concentration with TFIIA binding buffer (see below) before use.

Measurement of Dissociation Constants (Kd) of TFIIA-5 S rDNA Complexes—The 67-bp DNA substrates were labeled with [γ-32P]ATP by T4 kinase as described above. The labeled DNA fragments were incubated with TFIIA in 20 μl of TFIIA binding buffer (20 mM Tris, pH 7.5, 7 mM MgCl2, 100 μg/ml bovine serum albumin, 70 mM KCl, 0.1% Nonidet P-40, 10% glycerol, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol, 10 μg/ml poly(dI-dC), and 10 μM ZnCl2) at room temperature for 1 h (28). The TFIIA and DNA substrates were incubated at varying concentrations as indicated in each figure. The binding mixtures were loaded onto native gels (10% polyacrylamide, 25 mM Tris base, 200 mM glycine, 5% glycerol) and run in 25 mM Tris base, 200 mM glycine buffer at 10 V/cm for about 2 h to obtain sufficient separation of free and bound DNA. Subsequently the gels were vacuum-dried and exposed to a PhosphorImager (Amersham Biosciences, Model 445-P90). Intensities of DNA bands were quantified by integration of peak intensities using ImageQuant NT (Amersham Biosciences) and peakint 4.0 (SPSS Inc.) as described in Li et al. (30). Bound and free DNA concentrations were calculated from the intensities and total concentration of DNA using the following equations,

\[
[\text{[bound DNA]}] = \frac{I_{\text{bound}}}{I_{\text{bound}} + I_{\text{free}}} \times [\text{total DNA}]
\]  
(Eq. 1)

\[
[\text{[free DNA]}] = \frac{I_{\text{free}}}{I_{\text{bound}} + I_{\text{free}}} \times [\text{total DNA}]
\]  
(Eq. 2)

where \(I_{\text{bound}}\) and \(I_{\text{free}}\) are the intensities of the integrated bound and free DNA, respectively. The ratio of bound and free DNA versus the concentration of bound DNA was plotted and fit to a least-squares linear regression to obtain a Kd value using the following equation (31).

\[
\frac{[\text{bound}]}{[\text{free}]} = \left(\frac{[\text{TFIIA}]_{\text{total}} - [\text{bound}] \times 1}{K_d}\right)
\]  
(Eq. 3)

The Standard Gibbs free energy (\(\Delta G^0\)) was calculated from the following equation.

\[
\Delta G^0 = -RT \ln \frac{1}{K_d}
\]  
(Eq. 4)

Dissociation of TFIIA-5 S rDNA Complexes—End-labeled 67-bp DNA substrates (0.24 pmol) were incubated with about 0.8 pmol of TFIIA was 50 μl of TFIIA binding buffer for 1 h. After incubation, the mixture was divided into eight aliquots of 9 μl, and the dissociation reaction was initiated by addition of unlabeled competitor (67-bp undamaged 5 S rDNA) to the aliquots. (The times of addition of competitor were adjusted such that samples having the desired incubation times

![Diagram](http://www.jbc.org/Downloaded from July 25, 2018)
could be loaded simultaneously onto the gel.) The mixtures containing competitor and TFIIIA-DNA complex were incubated at 22 °C for various times (0, 7.5, 15, 22.5, 30, 60, 90, and 120 min) and loaded on 10% native polyacrylamide gels to separate bound and free DNA. The bound and free DNA concentrations were quantified as described above, and dissociation time courses were obtained by plotting [bound]/[bound]₀ versus time plotted and fit to a linear regression. The k_on and t_1/2 values were determined from the following equations (31).

\[
\text{ln}\frac{[\text{bound}]}{[\text{bound}]}_0 = -k_{\text{off}} \times t \quad \text{(Eq. 5)}
\]

\[
\text{t}_1/2 = \ln 2 / k_{\text{off}} \quad \text{(Eq. 6)}
\]

**Methylation Protection in TFIIIA-5 S rDNA Complexes—**End-labeled 67-bp DNA substrates (0.3 pmol) were incubated with about 1.5 pmol of TFIIIA in 50 µl of TFIIIA binding buffer. Then 1 µl of 8% dimethyl sulfoxide (DMS) in ethanol was added to the free DNA or complex solution and incubated at room temperature for 2 min. The samples were loaded onto 8% native polyacrylamide gels and run until bound and free DNA were well separated. The DNA was recovered from the gel by elution in 200 µl of TE buffer at 50–60 °C for 5 h followed by extraction using a QIAEX kit (Qiagen). The DNA samples were dried and redissolved in 7 M urea, 10% polyacrylamide, 1× TBE for about 1 h at 15 V/cm. The intensities of resolved bands on a denaturing gel were quantified using ImageQuant and PeakFit 4.0 software (30). The percentage of CTDs remaining was determined at various times using the equation 100 × F_b/F_u, where F_b and F_u are fractional intensities in the CTD band at times t and 0, respectively.

**RESULTS**

**Construction of 5 S rDNA Substrates Containing CTDs at Specific Sites—**As illustrated in Fig. 2A, DNA substrates were designed to contain CTDs in the A-box (dmg_A1 and dmg_A2), IE (dmg_IE), and C-box (dmg_C2 and dmg_C3) and outside of the ICR (dmg_OC). Since synthesis of CC, TC, or CT cyclobutane dimer phosphorimidates has not been established in our laboratory, a C→T mutation was required for insertion of CTDs in the C-box. Therefore, cytosines at +87 (dmg_C2) and +89 (dmg_C3) were chosen as mutation sites, and the mutated sequences were used as controls (Fig. 2A).

The location of CTDs in the DNA substrates was verified by T4 endo V digestion, which produces a single strand break at CPD sites (33). As shown in Fig. 2B, about 80% of the DNA substrates contained a CTD at the designed position (i.e. are cut by T4 endo V). Impurities, represented as uncut bands on T4 endo V (+) lanes, result, in part, from a reverse reaction of CTDs and/or chemical modification of the CTD during oligonucleotide synthesis as described by Kosmoski and Smardon (26, 27).

**Binding Affinity of TFIIIA to the ICR Containing a Single CTD—**The K_d values of TFIIIA-DNA complexes were obtained by titration of a fixed amount of TFIIIA with increasing amounts of 67-bp DNA substrates followed by gel mobility shift assays. Variation in DNA concentration was used since, compared with protein titration, concentrations of DNA are more accurately estimated, and the variation of K_d values was reduced. Representative gels and Scatchard plots are shown in Fig. 3. Consistent with the report by Clemens et al. (31), the undamaged ICR fragment had a K_d of 2.1 nm. On the other hand, the mutated control sequences (control C2, +87C→T; control C3, +89C→T) had a 2-fold lower affinity for TFIIIA with a K_d of 4 nm (Table I), values similar to those obtained by Pieter et al. (34) and Veldhoen et al. (35).

As shown in Table I, a CTD at +50 (number corresponds to the 5' side of the CTD in 5 S rDNA) in dmg_A1 had no significant effect on the binding affinity of TFIIIA (K_d = 2.3 nm). Footprinting and mutation studies indicate that +48p→+51T is located on the edge of the TFIIIA binding region (24, 34). Hence TFIIIA may tolerate the conformational change in DNA induced by a CTD at +50, and the binding affinity is not significantly changed. Similarly, as expected, a CTD at +101 (dmg_OC) had no influence on TFIIIA binding. On the con-
measured by incubation of preformed complexes with a 37-fold molar excess of competitor (67 bp, undamaged DNA) to prevent reassociation of labeled DNA to free TFIIIA. Representative gels (Fig. 4, A and B) indicate that the complexes underwent faster dissociation at early times and slower dissociation at later times. The apparent \( k_{off} \) values were calculated from initial slopes of semilogarithmic plots of \([\text{bound}]_t/\text{bound}_0\) versus time (Fig. 4C). As shown in Table II, the native 5 S rDNA sequence has a \( k_{off} \) of 1 h\(^{-1}\) and a \( t_{1/2} \) of 43 min, whereas the control C2 and C3 sequences showed a 2–3-fold increase in dissociation rate. In the case of CTD-containing sequences, dmg_IE and dmg_A2 had 2–4-fold higher \( k_{off} \) values as compared with undamaged DNA. Thus, their \( t_{1/2} \) values were reduced to 18 and 11 min, respectively, and the DNA substrates were released from the complex more rapidly than the undamaged DNAs. In contrast, CTDs at +88 (dmg_C2) and +90 (dmg_C3) did not change the dissociation rate of the complexes (Table II).

Past reports indicate that the \( k_{off} \) of TFIIIA varies depending on the type of competitor used. For example, a \( t_{1/2} \) of 5 min was observed with single-strand M13 bacteriophage DNA (36), and a \( t_{1/2} \) of 16 min was observed with 5 S rRNA (37). However, Clemens et al. (31) measured a \( t_{1/2} \) of 100 min with the 5 S rDNA in a plasmid. In our experiment, a 67-bp 5 S rDNA fragment was used as the competitor, and a \( t_{1/2} \) of 43 min was obtained. The reason for these differences remains unresolved; however, relative \( k_{off} \) values of individual substrates reflect the effect of CTDs on the dissociation rate, and the results in Table II clearly show that CTDs at +55 and +72 enhanced dissociation of the TFIIIA-DNA complex.

**Protein-DNA Contacts within TFIIIA-5 S rDNA Complexes with Single CTDs**—DMS methylates primarily the N\(^\circ\) of guanines, which is located in the major groove, and the N\(^3\) of adenosines in the minor groove (38). Since DMS reactivity of the N\(^\circ\) of guanines is about 8-fold higher than that of the N\(^3\) of adenosines (38) and the reactivity at these sites can be modulated by DNA-binding proteins (39), DMS has been utilized to probe protein-DNA interactions in the major groove. Based on preliminary experiments, 0.16% DMS was found to be an optimum concentration for this assay. (At higher DMS concentrations, \( k_{off} \) values of individual substrates reflect the effect of CTDs on the dissociation rate, and the results in Table II clearly show that CTDs at +55 and +72 enhanced dissociation of the TFIIIA-DNA complex.)

**Protein-DNA Contacts within TFIIIA-5 S rDNA Complexes**

| Time(min) | 0 | 7.5 | 15 | 22.5 | 30 | 60 | 90 | 120 |
|-----------|---|-----|----|------|----|-----|----|-----|
| Control   | 1.0 ± 0.1 | 1.0 |    |      |    |     |    |     |
| dmg_A1    | 1.7 ± 0.4 | 1.7 |    |      |    |     |    |     |
| dmg_A2    | 3.9 ± 0.9 | 4.0 |    |      |    |     |    |     |
| dmg_IE    | 2.4 ± 0.4 | 2.4 |    |      |    |     |    |     |
| dmg_OC    | 0.8 ± 0.2 | 0.8 |    |      |    |     |    |     |
| Control C2| 2.3 ± 0.2 | 1.0 |    |      |    |     |    |     |
| dmg_C2    | 1.8 ± 0.4 | 0.8 |    |      |    |     |    |     |
| Control C3| 2.8 ± 0.1 | 1.0 |    |      |    |     |    |     |
| dmg_C3    | 2.7 ± 0.1 | 1.0 |    |      |    |     |    |     |

\( ^a k_{off, damaged \text{DNA}/k_{off, control}} \)

Y. Kwon and M. J. Smerdon, unpublished results.

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**Fig. 4. Dissociation of TFIIIA-5 S rDNA complexes.** Representative gels showing the dissociation of undamaged DNA (A) and dmg_A2 DNA (B) from the complexes are shown. Preformed TFIIIA-5 S rDNA complexes were incubated with unlabeled competitor 5 S rDNA for 0, 7.5, 15, 22.5, 30, 60, 90, and 120 min (lanes 1–8) and separated on native gels. C, the fraction of TFIIIA-5 S rDNA complexes remaining ([bound]/[bound]₀) was plotted versus incubation time (0–30 min), and the data were fit by linear regression. The DNA substrates are undamaged 5 S rDNA (●), dmg_OC (▲), dmg_A1 (■), dmg_IE (▲), and dmg_A2 (●). Each data point represents the mean ± 1 S.D. of three independent experiments.

**Dissociation Rates of the TFIIIA-5 S rDNA Complexes with Single CTDs**—Dissociation of the TFIIIA-DNA complexes was

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**TABLE II**

| Dissociation rates and half-lives of TFIIIA-5 S rDNA complexes | \( h^{-1} \) | \( k_{off} \) | Half-life (min) |
|-----------------|--------------|-------------|----------------|
| Control         | 1.0 ± 0.1    | 1.0         | 43 ± 5         |
| dmg_A1          | 1.7 ± 0.4    | 1.7         | 26 ± 6         |
| dmg_A2          | 3.9 ± 0.9    | 4.0         | 11 ± 2         |
| dmg_IE          | 2.4 ± 0.4    | 2.4         | 18 ± 3         |
| dmg_OC          | 0.8 ± 0.2    | 0.8         | 55 ± 14        |
| Control C2      | 2.3 ± 0.2    | 1.0         | 18 ± 1         |
| dmg_C2          | 1.8 ± 0.4    | 0.8         | 24 ± 5         |
| Control C3      | 2.8 ± 0.1    | 1.0         | 15 ± 1         |
| dmg_C3          | 2.7 ± 0.1    | 1.0         | 15 ± 1         |

\( ^a k_{off, damaged \text{DNA}/k_{off, control}} \)
shows that TFIIIA protected guanine residues at +86, +85, +82, +81, +71, and +70 in the non-transcribed strand of the undamaged DNA (lane 3). In addition, TFIIIA-DNA contacts in these regions in dmg_A1 (lane 6) or dmg_A2 (lane 9) were similar to undamaged DNA, indicating CTDs at +50 and +55 did not modulate binding of zf1-zf6 to the C-box and IE. Also the protection in the C-box of dmg_C2 (not shown) and dmg_C3 (lane 17) show that contacts of zf1-zf6 with the damaged substrates were similar to that of undamaged DNA.

In contrast to the C-box and IE, guanine residues in the non-transcribed strand of the A-box (−50G, −55G, and −60G) were only slightly protected by TFIIIA (Fig. 5A). Moreover, when 3'-end-labeled DNA (labeled in the non-transcribed strand) was used for this assay, only marginal protection at positions of +51, +56, +59, and +60 was observed. However, when the 5'-end of the transcribed strand of DNA substrates was labeled, obvious footprints of TFIIIA binding were obtained in the A-box (Fig. 5B). In the presence of TFIIIA, −55G was hypermethylated by DMS, whereas methylation of −50G and −55G was reduced (lane 3). Similar protection patterns were seen with dmg_A1 and dmg_IE, suggesting that the CTDs at positions +50 and +72 did not affect binding of zf7-zf9 to the A-box. However, a CTD at +55 in dmg_A2 markedly altered the contacts of TFIIIA in the A-box. The level of methylation at +52G, −53G, and −57G in the TFIIIA-dmg_A2 complex increased compared with that of undamaged DNA (compare lane 9 with lane 3). Also binding of TFIIIA may promote methylation of +52G and −55G (compare lane 9 with lane 8). This hypermethylation implies that zf7-zf9 partially dissociate from the A-box, and the fingers may adapt a different conformation in the complex due to a CTD at +55, which promotes methylation reactivity in this region.

The footprint of TFIIIA-dmg_IE demonstrates that TFIIIA protected +70G and −70G less in the dmg_IE sequence than in undamaged DNA (Fig. 5A, compare lanes 2 and 3 with lanes 10 and 11). Presumably a CTD at +72 alters the interactions between zf5 and −70G +70G of the IE. However, methylation of the A-box (Fig. 5B, lane 12) and C-box (Fig. 5A, lane 11) of dmg_IE was very similar to that of undamaged DNA (Fig. 5, A and B, lane 3). Therefore, a CTD at +72 might partially disrupt the interaction between zf5 and IE.

**DNA Repair of Single CTDs in the ICR of TFIIIA-5 S rDNA Complexes**—Repair of CTDs in the TFIIIA-5 S rDNA complex was measured by incubation with *Xenopus* oocyte nuclear extracts, which possess a robust NER activity (e.g. over 10^10...
Each DNA band in gels such as those shown in Fig. 6 was quantified as described under "Materials and Methods." The percentage of CTD (%CTD) remaining, relative to the amount of CTDs at zero time, was calculated and plotted for the incubation times shown. Data represent the mean ± 1 S.D. of three independent experiments except for dmg_IE (C), which shows the average of data obtained from two independent experiments.

The percentage of CTD (%CTD) in free DNA was quantified for different experiments, and the time course of repair was calculated for the incubation times shown. Data represent the mean ± 1 S.D. of three independent experiments except for dmg_IE (C), which shows the average of data obtained from two independent experiments.

**DISCUSSION**

We have studied the binding of TFIIIA to its cognate recognition sequence in 5 S rDNA containing UV photoproducts (CTDs) at six different locations and the relationship between binding and DNA repair in vitro. Analysis of TFIIIA binding showed that CTDs at positions +55 and +72 decreased the binding affinity by ~2-fold and increased the $k_{off}$ by ~2-fold. The lower binding affinity was concomitant with the local disruption (or rearrangement) of zinc-finger-DNA contacts around the damaged sites.

Although the crystal structure of the partial complex did not include zf7–zf9 and the A-box (25), biochemical studies suggest that the overall structure of this region is very similar to that of zf1–zf3 and the C-box (23, 24). In the case of position +55, a CTD may inhibit the sequence-specific interactions between zf7–zf9 and the A-box in a manner similar to mutations or base deletions in this region (24, 42). Furthermore electron microcopy and circular permutation results suggest the existence of significant structural changes in the A-box region upon TFIIIA binding (43, 44). Therefore, it is possible that the CTD at +55 is less compatible with the DNA structural change associated with TFIIIA binding and causes displacement of zinc fingers from the complex. In addition, the CTD at +55 had the intriguing feature of an increase in $k_{off}$ (4-fold larger than the control) that was greater than an increase in $K_d$ (2-fold larger than the control). Based on the relationship $K_d = k_{off}/k_{on}$, it is likely that $k_{on}$ (or the association rate) of the damaged DNA is enhanced by the CTD at +55.
From structural studies on the complex, $^{+72\text{T}+73\text{T}}$ is positioned in the linker region where Zf4 crosses the minor groove between the C-box and IE and has a few contacts with the phosphodiester backbone (25, 45). These data indicate that the small decrease in affinity of the TFIIIA-dmg_IE complex may be due to a conformation change in the phosphodiester backbone induced by the CTD, which weakens zinc-finger-DNA interactions. The crystal structure of the complex (25) shows that Arg$^{195}$ and Arg$^{203}$ of Zf5 strongly interact with $^{+70\text{G}+71\text{G}}$ in the ICR. Therefore, distortion of DNA by the CTD at +72 may affect the orientation of Zf5, which influences the strong contacts of $^{+70\text{G}+71\text{G}}$ with the Arg residues. In agreement with this notion, methylation reactivity of these sites is enhanced by the presence of CTDs (Fig. 5A, lane 11).

To incorporate CTDs in the C-box, the sequence at $^{+8\text{C}}$ and $^{+8\text{C}}$ was mutated to thymine (see “Materials and Methods”), and each of these mutations caused a small decrease in binding affinity for TFIIIA (Table I). However, CTDs at +88 and +90 slightly increased binding affinity of TFIIIA compared with the undamaged DNA. Furthermore, as judged by methylation protection, similar protein-DNA contacts exist in the complexes with either damaged or undamaged DNA (Fig. 5A, lanes 12–17). These results indicate that CTDs are compatible with Zf1–Zf3 binding in this region and thus do not cause a decrease in TFIIIA binding. Interestingly the structure at atomic resolution reveals that $^{+88\text{T}}$ is not involved in base-side chain interactions (25, 46). Hence the pyrimidine ring in $^{+88\text{T}}$ is expected to be relatively flexible so as to accommodate a CTD. Moreover TFIIIA bends undamaged DNA in the C-box at +85 and +90 by 24.4° and 18.4°, respectively. Therefore, it is possible that prebending of DNA induced by a CTD can increase binding affinity for TFIIIA (4). Similarly a CTD at +90 in dmg_C3 may also slightly increase the binding affinity of TFIIIA (Table I).

The repair profile that we observed at each CTD site is in good agreement with the previous observations of Conconi et al. (22) that TFIIIA inhibits DNA repair inside the ICR and that the degree of inhibition is heterogeneous. The consistency indicates that the chemically synthesized DNA represents a homogeneous subset of DNA randomly damaged by UV irradiation. However, the previous result contains some ambiguity, which arises from limited resolution of CPD mapping, difference in CPD yields at different sites, and the presence of pyrimidine-pyrimidone (6-4) dimers in a fraction of the fragments. In this study, DNA repair at individual CTD sites can be analyzed without such ambiguities, allowing comparison of TFIIIA binding and repair efficiency at each site. Inhibition of repair of CTDs by TFIIIA binding depends on their location in the ICR sequence. The CTD at position +88 (dmg_C2), which had a relatively high binding affinity for TFIIIA ($K_d = 1.9\text{ nM}$), exhibited the lowest repair efficiency (Fig. 7D). In contrast, repair at position +55 (dmg_A2) was almost unchanged by TFIIIA binding (Fig. 7B). The fast repair and high off-rate ($k_{\text{off}}$) of dmg_A2 suggest that dissociation rate is an important determinant of efficient repair of CTDs in the complex. In addition, the multiple zinc fingers in TFIIIA must allow displacement of some fingers at (or near) the damaged site, while the others remain tightly bound.

This “broken finger” hypothesis is supported by the results of the methylation protection assay (Fig. 5). Reduced binding of zinc fingers at (or near) the CTD at position +55 was indicated by enhanced methylation of $^{+52\text{G}}, ^{+53\text{G}},$ and $^{+54\text{G}}$ when the CTD was present (Fig. 5B). Thus, the damage-induced conformational change in the complex may enhance access of NER proteins to the CTD at +55. Furthermore DNA repair at +72 (dmg_IE) was only slightly inhibited by TFIIIA binding (Fig. 7C), and the methylation protection assay indicated that dmg_IE was not able to make full contact with TFIIIA (Fig. 5A). Therefore, CTDs at these two positions must displace one (or more) of the zinc fingers in the A-box and IE, respectively. However, in the latter case, where changes in TFIIIA-DNA contacts are localized in the IE box, increased accessibility to +72 CTD in the complex may not completely explain the efficient NER at this site. Presumably the more rapid dissociation of dmg_IE from the complex (2.4-fold of undamaged DNA) contributes significantly to the rapid repair. Finally the CTD at +90 (Fig. 7E) was associated with faster repair than the CTD at +88. This may also result from the higher off-rate of dmg_C3 from the complex than that of dmg_C2.

Based on the binding assay, it may be speculated that transcription of the 5 S rRNA gene in the presence of CTDs is affected. First, as shown with dmg_A2 and dmg_IE, CTDs at +55 or +72 may not allow proper orientation of zinc fingers and (or) the C terminus of TFIIIA, which are crucial for sequential recruitment of TFIIIC, TFIIIB, and RNA polymerase (RNP) III (47). Consequently the rate of transcription initiation may decrease. On the other hand, CTDs at +50 and +88 may allow proper formation of the TFIIIA-ICR complex, and consequently the RNP III machinery can be recruited and initiate transcription. If transcription is initiated, it is expected that RNAPIII will be stalled at these sites as indicated by Chen et al. (48). Such a stalled RNAPIII may inhibit DNA repair at positions +50 and +88 increasing mutation frequencies at these sites.

Previous observations that DNA-binding proteins, such as transcription factors, stalled RNAPII, and histones, inhibit NER inside the bound regions (21, 19, 22, 49, 50) suggest that inhibition of NER by DNA-binding proteins is a general phenomenon. Clearly occlusion of DNA lesions by DNA-protein interactions renders the lesion less accessible to NER proteins. However, in the case of nucleosomal DNA, NER might be facilitated by modulation of chromatin structure via histone modification and/or nucleosome remodeling (51–54). On the other hand, considering the diversity of transcription factors and their cognate DNA binding sequences, it is likely that a more general process controls NER efficiency of these complexes. In the present study, we showed that when a CTD enhanced $k_{\text{off}}$ and $K_d$ of the complex, the inhibitory effect of TFIIIA was decreased significantly (e.g. with dmg_A2 and dmg_IE). This suggests that dissociation of complexes occurs during DNA repair, and consequently the “shielding” effect of TFIIIA on NER is abolished. In contrast, dmg_C2, which possessed high binding affinity for TFIIIA, clearly showed slow repair in the presence of TFIIIA.

In summary, these results demonstrate that (a) CTDs in the ICR of the 5 S rRNA gene can increase or decrease binding affinity of DNA for TFIIIA depending on their position, (b) modulation of TFIIIA binding is closely related to the modulation in $k_{\text{off}}$ of the complex, (c) the decrease in binding affinity is accompanied by the loss of TFIIIA-DNA contacts near CTD sites, and (d) NER of CTDs in the TFIIIA-5 S rDNA complex is extremely sensitive to changes in the dissociation rates of the complex.

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