

Exposure of DNA bases induced by the interaction of DNA and calf thymus DNA helix-destabilizing protein

(chloroacetaldehyde/hydrogen-deuterium exchange)

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ABSTRACT The reaction of chloroacetaldehyde with adenine bases in DNA to give a fluorescent product was used to study the availability to intermolecular reaction of positions 1 and 6 of adenine in DNA complexed with calf thymus DNA helix-destabilizing protein. No inhibition of this reaction was observed when heat-denatured DNA was complexed with the protein at a protein/DNA weight ratio of 10:1, compared to free DNA. On the contrary, the same reaction was inhibited markedly for denatured DNA in the presence of calf thymus histone H1 at protein/DNA weight ratio of 2:1. Furthermore, the exchange rate for hydrogens of amino and imide groups of DNA bases in DNA strands with deuterium in the solvent was totally unaffected upon complexing of DNA with the DNA helix-destabilizing protein as examined by stopped-flow ultraviolet spectroscopy. These results indicate that the DNA helix-destabilizing protein forms a complex with single-stranded DNA, leaving DNA bases uncovered by the protein. The fluorescence intensity of DNA pretreated with chloroacetaldehyde was amplified by nearly 3-fold upon addition of the DNA helix-destabilizing protein. The possibility of "unstacking" of DNA bases induced by the protein is discussed.

The DNA helix-destabilizing protein (HD-protein) has been isolated from various organisms by virtue of its strong preferential affinity for single-stranded DNA. It lowers the melting temperatures (t_m s) of double-stranded nucleic acids and stimulates the activity of the homologous DNA polymerase *in vitro* (1-11).

For understanding the *in vivo* role of HD-protein in DNA replication, knowledge of the mechanism of the molecular interaction between the protein and DNA is of primary importance. For an explanation of the stimulative effect of HD-protein on DNA polymerase activity, a "bases-out" model for the DNA-HD-protein complex has been proposed (12, 13). It was assumed that DNA bases of the DNA-HD-protein complex are oriented in such a way that they are easily recognized by the enzyme. It is not yet clear, however, whether or not base residues of DNA are actually exposed and are accessible to intermolecular reactions when single-stranded DNA is fully covered by HD-protein.

In this communication, we report a few pieces of evidence that DNA bases remain uncovered after the binding of HD-protein to DNA. The reaction of chloroacetaldehyde with adenosine and adenylic acid takes place at position 1 (nitrogen) and position 6 (amino group) of the adenine residue (14, 15). The product of this same reaction with DNA, ϵ -DNA, has a fluorescence maximum at 400 nm when excited at 305 nm in aqueous solution at pH 6.8 (16-17). Thus, availability of positions 1 and 6 of the adenine residue in a DNA system can be detected with great sensitivity. For instance, the reaction takes

place only with single-stranded DNA and not with double-stranded DNA purified by treatment with nuclease SI followed by hydroxylapatite chromatography (16-18). We have examined mild conditions for the reaction of chloroacetaldehyde with DNA to avoid inactivation of HD-protein during the reaction and then studied the reactivity of chloroacetaldehyde with adenine bases in poly(dA-dT) and calf thymus heat-denatured DNA in the presence of calf thymus HD-protein (UP1).

To confirm the results obtained from the above experiments, the rate of the hydrogen-deuterium exchange reaction of denatured DNA in the presence and in the absence of UP1 were compared. The exchange of protons of amino and imide hydrogens of DNA bases was monitored by stopped-flow ultraviolet spectroscopy (19). In this system we can eliminate the possible effect of chemicals such as chloroacetaldehyde on the structure of DNA-protein complex. We also explored the possibility that UP1 "unstacks" DNA bases upon binding to DNA by examining the effect of UP1 on the fluorescence intensity of ϵ -DNA (DNA pretreated with chloroacetaldehyde).

MATERIALS AND METHODS

General. Chloroacetaldehyde was purchased from Tokyo Kasei Chemical Co. and was doubly distilled before use. Poly(dA-dT) was obtained from P-L Biochemicals. Calf thymus DNA and pancreatic DNase I were obtained from Worthington. Venom phosphodiesterase was obtained from Boehringer Mannheim. Bovine serum albumin was purchased from Sigma Chemicals. Calf thymus histone H1 purified to a single electrophoretic band was a generous gift from Y. Ohba. HeLa [14 C]DNA (2000 cpm/ μ g) was isolated by the procedure of Marmor (20) followed by a brief ultrasonication. Single-stranded DNA was prepared by boiling for 15 min and rapid cooling in ice. Nitrocellulose filters (HAWP) were purchased from Millipore. Deuterium oxide was obtained from CEACEN-SACLAY.

Calf Thymus HD-Protein. UP1 was isolated by the procedure of Herrick and Alberts (21). The protein was homogeneous to 91% with molecular weight 19,000 as determined by sodium dodecyl sulfate/acrylamide gel electrophoresis in a Tris-HCl/glycine buffer system (22). The protein could stimulate calf thymus DNA polymerase activity with poly(dA-dT) as the template and could lower the melting temperature of poly(dA-dT).

Reaction Conditions for Chloroacetaldehyde. The reaction mixture was prepared by addition of 1.9 M chloroacetaldehyde

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Abbreviations: HD-protein, DNA helix-destabilizing protein; t_m , melting temperature; UP1, calf thymus DNA helix-destabilizing protein with molecular weight of 19,000; ϵ -DNA, DNA pretreated with chloroacetaldehyde.

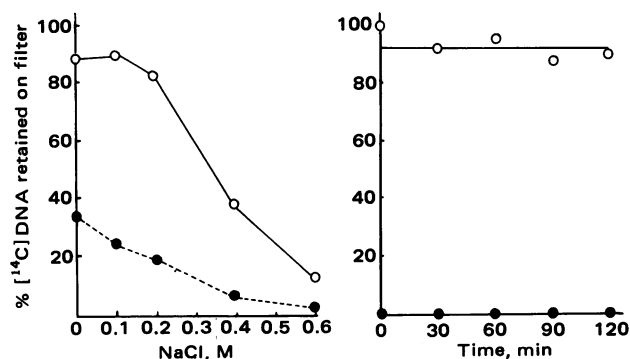


FIG. 1. (Left) Effect of NaCl on binding of UP1 to HeLa [¹⁴C]-DNA. One microgram of either heat-denatured DNA (O) or native DNA (●) was incubated in 0.3 ml of buffer C with varying concentrations of NaCl in the presence of 5.5 μ g of UP1 at 37° for 10 min. The mixture was then passed through Millipore filters and radioactivity trapped on the filters was measured. (Right) Effect of chloroacetaldehyde on binding of UP1 to [¹⁴C]DNA. One microgram of heat-denatured DNA was incubated with 10.6 μ g of UP1 (O) or bovine serum albumin (●) in buffer A containing 0.19 M chloroacetaldehyde at 37° for varying lengths of time. The mixture was then passed through Millipore filters and the radioactivity trapped on the filters was measured.

in doubly distilled water to make a final concentration of 0.19 M in either 20 mM sodium phosphate buffer, pH 6.8/0.15 M NaCl (buffer A) containing the indicated amounts of heat-denatured calf thymus DNA and UP1 or 15 mM potassium phosphate buffer, pH 7.6/10% (vol/vol) glycerol (buffer B) containing the indicated amounts of poly(dA-dT) and UP1. The addition of chloroacetaldehyde was made at 0°, and the reaction mixture was brought to the indicated temperature, either 36.5° or 37°. The reaction was allowed to proceed for 2 hr and was terminated by chilling in an ice-water bath.

Preparation of ϵ -DNA. Heat-denatured calf thymus DNA (400 μ g) in 2 ml of 20 mM sodium phosphate buffer, pH 6.8 (buffer C) containing 0.19 M chloroacetaldehyde was incubated at 37° for 48 hr. The resulting mixture was dialyzed against buffer C to remove chloroacetaldehyde before use.

Analysis of the Product. The fluorescence of the product obtained under the reaction conditions described can be detected only when the DNA is digested into mononucleotides by DNase and phosphodiesterase. After digestion, the fluorescence intensity is amplified by nearly 20-fold (16, 17). However, first the UP1 must be dissociated from the DNA for the enzymes to function. For dissociation of UP1 from DNA quantitatively, either CsCl equilibrium density gradient centrifugation or 5–8 M urea/2 M NaCl gradient centrifugation was used.

CsCl Equilibrium Centrifugation. The sample containing poly(dA-dT) and UP1 after reaction with chloroacetaldehyde was dialyzed against 50 mM Tris-HCl buffer, pH 8.1. The initial density was made 1.64 g·cm⁻³, the buoyant density of poly(dA-dT), by adding 4.23 g of CsCl to the whole dialysate diluted to 3.8 ml with the dialyzing buffer. It was centrifuged in a Hitachi RPS 40T2 rotor at 36,000 rpm for 70 hr at 15°. Thirty fractions were collected from the bottom of the centrifuge tube and the refractive index was determined.

5–8 M Urea/2 M NaCl Gradient Centrifugation. The samples containing heat-denatured calf thymus DNA and UP1 after reaction with chloroacetaldehyde was directly layered onto 4.6 ml of 5–8 M urea gradient in 2 M NaCl. Removal of chloroacetaldehyde from the reaction mixture for centrifugation was found to be unnecessary. The mixture was centrifuged at 40,000 rpm for 14 hr at 15° in a Hitachi RPS 40T2 rotor. Ten fractions were collected from the bottom of the tube.

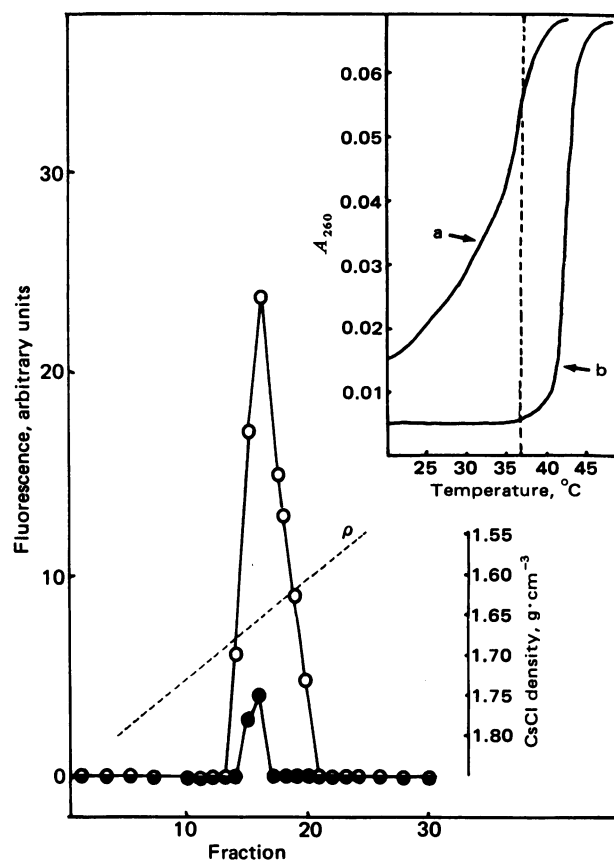


FIG. 2. Reaction of chloroacetaldehyde with adenine residues of UP1-bound poly(dA-dT). (O), Poly(dA-dT) + UP1; (●), poly(dA-dT) only. The melting profiles of poly(dA-dT) (30 μ g) in 3.24 ml of buffer B (curve b) and of poly(dA-dT) (30 μ g) plus UP1 (405 μ g) in 3.24 ml of buffer B (curve a) are shown in the *Inset*; A_{260} nm values shown are after subtraction of arbitrary base line. The rate of temperature rise was 2°/min. Both samples were then chilled in ice, and 360 μ l of 1.9 M chloroacetaldehyde was added slowly to both samples at 0°. The temperature was raised again and the reaction was allowed to proceed for 2 hr at 36.5°. The product was dialyzed against 50 mM Tris-HCl, pH 8.1, and poly(dA-dT) was separated from UP1 by equilibrium centrifugation in CsCl gradient.

Measurement of Fluorescence. Each fraction obtained as described above was extensively dialyzed against buffer C. The volume of the dialyzed fraction was normalized to 0.9 ml with the same buffer. Five micrograms each of pancreatic DNase I and venom phosphodiesterase were added to each dialyzed fraction as well as to a sample containing 10 μ g of ϵ -DNA in the presence of 1 mM MgCl₂ to digest DNA into mononucleotides. Digestion was assumed to be complete when the fluorescence intensity of ϵ -DNA increased by nearly 20-fold as compared to that of undigested ϵ -DNA. The fluorescence (emission wavelength, 400 nm; excitation, 305 nm) was determined for each sample with an MPF4 Hitachi fluorospectrophotometer. The blank for fluorescence was buffer C containing the same amount of the enzymes.

Millipore Filter Binding Assay. The binding conditions for HeLa [¹⁴C]DNA and UP1 are described in the legend to Fig. 1. The mixture was passed through Millipore filters (0.45 μ m) at a flow rate of 300 μ l/min. Each filter was washed three times with 200 μ l of corresponding buffer solution. The Millipore filter was then dissolved in 2 ml of ethylene glycol monoethyl ether and mixed with 6 ml of toluene scintillator. Radioactivity trapped on the Millipore filter was assayed by liquid-scintillation counting.

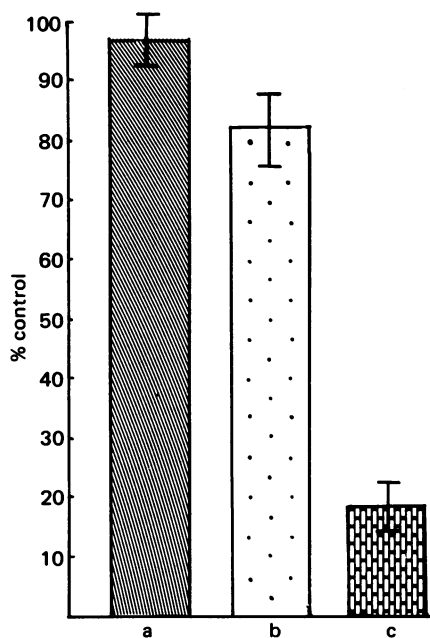


FIG. 3. Effect of UP1 (a), bovine serum albumin (b), or calf thymus histone H1 (c) on the reactivity of chloroacetaldehyde with heat-denatured DNA. The sample contained 20 μ l (20 μ g) of heat-denatured calf thymus DNA in doubly distilled water and 200 μ g of UP1 or albumin or 40 μ g of H1 dissolved in 430 μ l of buffer A. It was incubated at 37° for 10 min to allow binding of protein to DNA before chloroacetaldehyde was added to a final concentration of 0.19 M. The addition of chloroacetaldehyde was made at 0°. The reaction mixture was then incubated at 37° for 2 hr. DNA was dissociated from protein by 5–8 M urea/2 M NaCl gradient centrifugation. The bottom five fractions with fluorescence were then pooled and brought to 6 ml with buffer C, and the total fluorescence was measured. Extent of reaction of chloroacetaldehyde with heat-denatured DNA is expressed as percentage of control, heat-denatured DNA only. Under these reaction conditions, 8% of the total adenine residues in denatured DNA (control) are converted to ϵ -adenine. Results are shown as mean \pm SD of four experiments.

Hydrogen-Deuterium Exchange Rate Determination. The sample for measurement (4 ml) contained 0.6 mg of calf thymus denatured DNA with or without 2.7 mg of UP1 in 5 mM sodium phosphate buffer, pH 6.8/1 mM EDTA/10% glycerol. The sample in $^1\text{H}_2\text{O}$ was rapidly mixed with an equal volume of $^2\text{H}_2\text{O}$ at 26° and the time-dependent decrease in absorbance at 285 nm was recorded with Union Giken stopped-flow spectrophotometer RA 401.

RESULTS

UP1-DNA Binding Assay. It was first confirmed by results of Millipore filter binding assay that UP1 preferentially bound to heat-denatured HeLa [^{14}C]DNA and gradually dissociated from DNA as the NaCl concentration increased (Fig. 1 *left*). Next, the binding ability of UP1 to DNA was examined under the actual reaction conditions for chloroacetaldehyde and DNA used for the following experiments. From the result shown in Fig. 1 *right*, it was concluded that UP1 remained bound to heat-denatured HeLa [^{14}C]DNA at the maximal level through the 2-hr incubation period at 37° in the presence of 0.19 M chloroacetaldehyde. Bovine serum albumin was used as the control protein.

Effect of UP1 on the Reaction of Chloroacetaldehyde with Poly(dA-dT). Poly(dA-dT) is not expected to react with chloroacetaldehyde below its t_m , because then it is double stranded. In the presence of UP1, however, the t_m of poly(dA-dT) is expected to be lower. Actually, it has been found that, at 36.5°, poly(dA-dT) in the presence of UP1 is 80% melted whereas free poly(dA-dT) is still in the double-stranded form (Fig. 2 *inset*). Both samples, poly(dA-dT) in the absence and in the presence of UP1, were chilled in ice and chloroacetaldehyde was added at 0° to each sample so that the final concentration was 0.19 M. The reaction mixtures were then brought to 36.5° again and the reaction was allowed to proceed for 2 hr. The analysis of the product, as shown in Fig. 2, demonstrated clearly that poly(dA-dT) in the presence of UP1 did react with chloroacetaldehyde to a considerable extent whereas free poly(dA-dT) in the absence of UP1 reacted to only a small extent [probably because of structural fluctuation of the poly(dA-dT) molecule at the temperature very close to its t_m]. This result indicates that, in the presence of 0.19 M chloroacetaldehyde, UP1 was still able to induce poly(dA-dT) to form a single strand and to allow chloroacetaldehyde to react with adenine residues as the temperature was raised from 0° to 36.5°. This result, taken together with the result obtained from the previous experiment (Fig. 1 *right*), eliminates the possibility of inactivation of UP1 by chloroacetaldehyde under the reaction conditions used.

Extent of Reaction of Chloroacetaldehyde with Heat-Denatured DNA Complexed with UP1. Heat-denatured DNA, in which the nitrogen and amino group of adenine at positions 1 and 6 do not participate in hydrogen bondings, reacts readily with chloroacetaldehyde. When such DNA is fully complexed with UP1, the reactivity with chloroacetaldehyde is expected to depend on the availability of these reactive sites of adenine residues within the complex. It has been reported

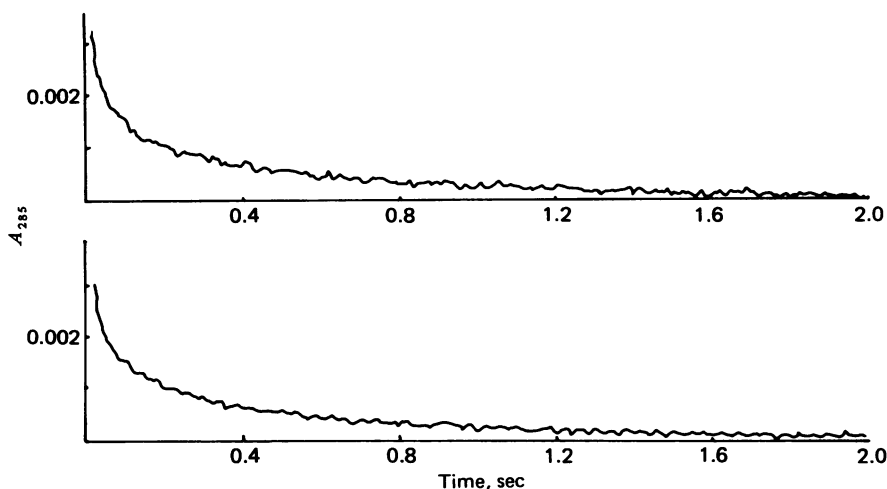


FIG. 4. Stopped-flow ultraviolet absorption studies of the ^1H - ^2H exchange reaction of denatured DNA with (*Upper*) and without (*Lower*) UP1. The same measurement was repeated four times, and the integrated result was recorded.

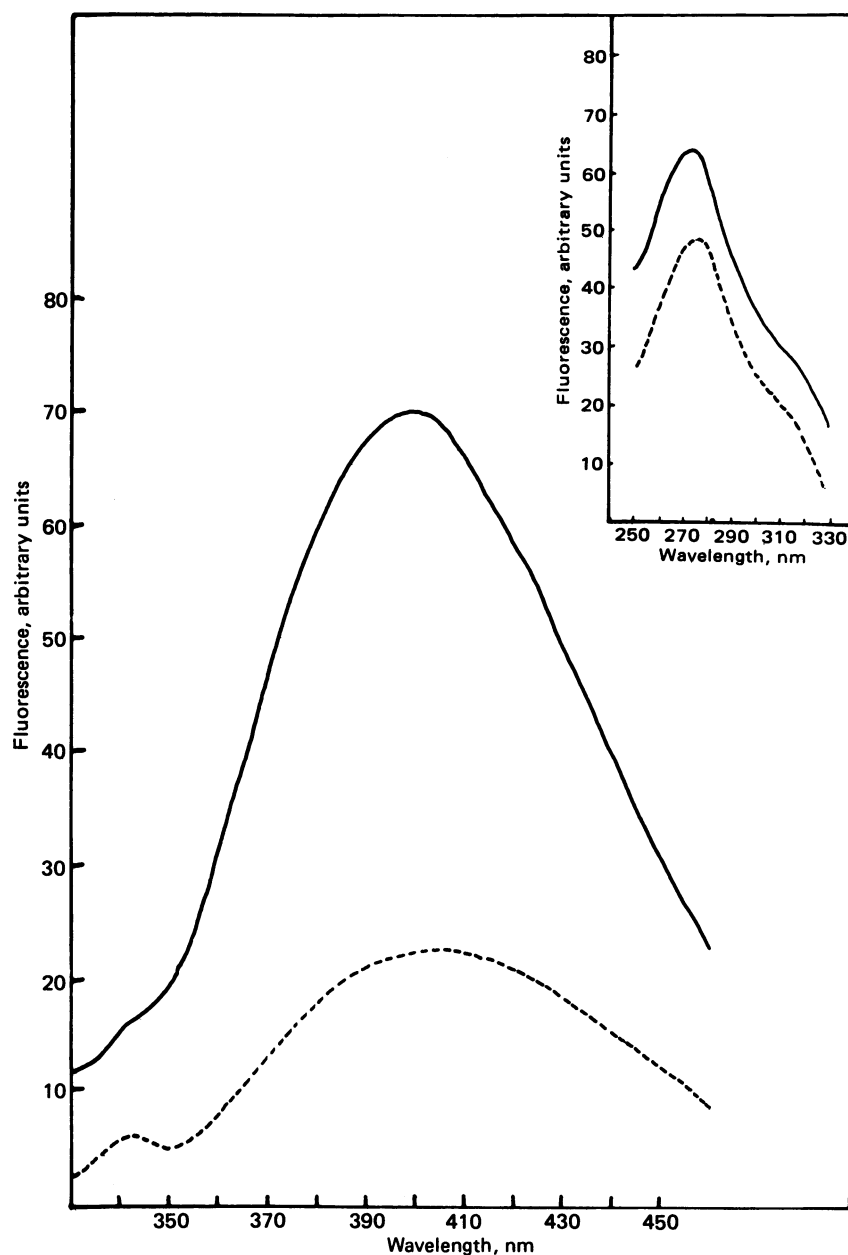


FIG. 5. Effect of UP1 on fluorescence intensity (excitation wavelength 305 nm) of ϵ -DNA. ---, ϵ -DNA (10 μ g) in 500 μ l of buffer; —, ϵ -DNA (10 μ g) + UP1 (80 μ g) in 500 μ l of buffer C. When solid NaCl was added to 0.6 M to the sample containing UP1, the fluorescence intensity peak at 400 nm coincided with that of ϵ -DNA itself. The fluorescence intensity profile for ϵ -DNA (10 μ g) + bovine serum albumin (80 μ g) in 500 μ l of buffer C also coincided with that of ϵ -DNA when background fluorescence due to albumin in this region was subtracted. (Inset) Excitation spectra with emission wavelength 400 nm; those excitation curves are artificially offset.

by Herrick and Alberts (23) that the saturating UP1-DNA complex is reached at the protein/DNA weight ratio of 10:1. For such a complex, either enhanced or the same reactivity with chloroacetaldehyde, compared to free DNA, should be observed only when interaction between DNA and UP1 takes place in such a manner that DNA bases are oriented outward so that they are available for intermolecular recognition.

An experiment was undertaken to study the effect of the presence of UP1 (protein/DNA = 10), histone H1 (protein/DNA = 2), or bovine serum albumin (protein/DNA = 10) on the reaction of chloroacetaldehyde with denatured DNA. The result is shown in Fig. 3. The percentage of incorporation of chloroacetaldehyde into adenine bases of denatured DNA in the presence of UP1 was 98% of that for free DNA. In contrast, H1 inhibited the reaction with chloroacetaldehyde. The extent of chloroacetaldehyde reaction was somewhat less in the presence of bovine serum albumin.

To avoid the sample's turning opaque as a result of mixing H1 and DNA in buffer A, the same experiment was repeated

by adding denatured DNA and H1 in 2 M NaCl and 5 M urea in buffer C followed by stepwise dialysis to remove urea and NaCl in that order. The solution remained essentially clear by this preparation. Both preparations of the complex of H1 and denatured DNA, however, gave similar results for the reaction with chloroacetaldehyde.

^1H - ^2H Exchange Rate for Heat-Denatured DNA Complexed with UP1. The accessibility of base residues in the DNA-UP1 complex to intermolecular reaction was further studied in terms of the exchange rate of hydrogen of DNA bases with deuterium in the solvent. The rate depends on the availability of amino and imide hydrogens of DNA bases. For instance, the rate is slowed by approximately 2 orders of magnitude for native calf thymus DNA compared to heat-denatured calf thymus DNA (19). The rates of decrease in absorbance at 285 nm for denatured DNA with and without UP1 were identical with a half-life of 0.5 sec (Fig. 4). This result supports the previous finding that DNA bases are uncovered by UP1 when complexed with DNA. The binding of UP1 to denatured

DNA in the buffer used in this experiment was checked by Millipore filter binding assay (data not shown).

Effect of UP1 on Fluorescence Intensity of ϵ -DNA. The fluorescence intensity per ϵ -adenine residue of dinucleoside monophosphate (ϵ -A)_p(ϵ -A) was found to be only 5% of that of mononucleotide ϵ -AMP (ref. 24, unpublished data). Furthermore, the fluorescence intensity of ϵ -DNA was amplified by nearly 20-fold after complete digestion of DNA into mononucleotides by pancreatic DNase I and venom phosphodiesterase (16, 17). In both cases, amplification of fluorescence intensity is considered to occur when bases are relieved from stacking. In this context, the possibility for UP1 interaction to unstack the DNA bases was explored. Nearly 3-fold amplification of the fluorescence intensity of ϵ -DNA was observed at a UP1/DNA weight ratio of 8:1 (Fig. 5). UP1 itself did not have fluorescence in this region. A non-DNA-binding protein, such as bovine serum albumin, did not have such an effect. Reversibility of this amplifying effect by UP1 was demonstrated by adding solid NaCl to the sample to make 0.6 M NaCl, at which the protein dissociated from single-stranded DNA (Fig. 1 *left*). Sodium chloride itself did not have a quenching effect at this concentration. It can be concluded that fluorescence amplification is a consequence of the binding of UP1 to DNA. Excitation spectra with emission wavelength 400 nm for ϵ -DNA with and without UP1 were identical (Fig. 5 *inset*).

DISCUSSION

The results of the experiments described in this report are direct evidence of DNA bases being left uncovered when DNA is complexed with UP1. The interaction mode that satisfies such a requirement should be well ordered. Such interaction may be understood by postulating the following model. UP1 binds selectively to single-stranded regions of DNA and the bonding is most likely to be polar in nature because it dissociates from DNA with increasing concentrations of NaCl. This suggests that a phosphate group of DNA is the major site for interaction with UP1. Nevertheless, the mere presence of a phosphate group is not a sufficient condition for bond formation between UP1 and single-stranded DNA because UP1 has a low affinity for double-stranded DNA. This led to an assumption that the structure of DNA must be flexible so that the DNA chain can wind itself around the protein core with the direct binding site (the DNA phosphate group) and orienting DNA bases outward to leave them accessible for intermolecular reaction (possible interaction

with DNA polymerase). By doing so, a stable binding situation will be established. Double-stranded DNA is inadequate for such interaction because of its rigid structure compared to that of single-stranded DNA. As the consequence of such interaction, "DNA base unstacking" may necessarily arise.

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