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Optimal Conditions and Specificity of Interaction of a Distinct Class of Nonhistone Chromosomal Proteins with DNA†

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ABSTRACT: A subclass of nonhistone chromatin proteins with high DNA affinity has been isolated from rat liver. The interaction of the isolated proteins with DNA in vitro was characterized utilizing a nitrocellulose filter binding technique. The temperature, time, concentration, ionic strength, and pH dependence were characterized. Optimal interaction was observed at 0.19 M NaCl, pH 7.5, with a protein to DNA ratio of 13 (w/w). Equilibrium and kinetic competition experiments indicated that these proteins interact optimally with A-T rich and single-stranded DNA. The data also suggest that these proteins might affect the helix-coil transition of DNA.

Interaction of proteins with DNA is a requisite for numerous genome regulatory functions in both bacterial and viral systems (von Hippel and McGhee, 1972). Clearly, such interactions must also be important in the functioning of the eukaryotic chromosome. However, the number and types of interactions may in fact be much larger since the eukaryotic chromosome, or chromatin as it is operationally defined, consists of a supramolecular complex of DNA, RNA, and histone and nonhistone proteins, the latter being termed the NHCP[1]. This complexity and the lack of specific biological and/or biochemical assays have impeded the elucidation of the roles of these macromolecules in chromatin function. Increasingly, however, the DNA binding properties of both histone and NHC proteins have been utilized as a specific probe of possible biological roles for these proteins. In particular, studies have shown that NHCP bind to homologous and heterologous DNA (van den Broeck et al., 1973; Patel and Thomas, 1973), to low and high C_0t DNA (Allfrey et al., 1973), and to intermediate C_0t DNA (Sevall et al., 1975). Also, some of the NHCP exhibit specificity of binding only to DNA from the species from which the NHCP were originally isolated (Kleinsmith et al., 1970; Teng et al., 1971; Kostraba et al., 1975).

We reported previously on the isolation and partial characterization of a subclass of rat liver NHCP which exhibited high affinity for DNA and deoxynucleohistone (Patel, 1972). This group of proteins, referred to as APNH[1], is comprised predominately of two molecular weight species, approximately 19 000 and 16 000 daltons. Initial experiments showed that APNH bound to DNA to a greater extent than other NHP fractions (Patel and Thomas, 1973; Thomas et al., 1973). These experiments also suggested both partial species and single-strand specificity. In this study, the DNA binding properties of APNH were examined in more detail employing a nitrocellulose filtration technique as an assay of DNA-protein complexes (Riggs et al., 1968; Riggs and Bourgeois, 1968; Riggs et al., 1970a). The primary advantages of this technique were its rapidity, sensitivity, and its requirement of very little DNA and protein. This assay was exploited extensively to determine the optimal conditions for the DNA-APNH interaction and to determine various binding parameters which serve as a measure of the specificity of the interaction.

Methods

(*a*) *Buffers and Media.* Luria broth is 1% bactotryptone-0.5% yeast extract-0.5% NaCl, pH 7. K media is M9 buffer (Adams, 1959) which is 1% casamino acids, 1% glucose, 10^{-4}% thiamine, and either 2 μg (K + 2) or 10 μg (K + 10) thymidine per milliliter. SB buffer is 0.19 M NaCl-0.01 M Tris-HCl-0.001 M EDTA, pH 7.5. SSC is 0.15 M NaCl-

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[1] Abbreviations: NHCP, nonhistone chromatin proteins; NHP, nonhistone proteins; APNH, nonhistone chromatin proteins with high DNA affinity; EDTA, ethylenediaminetetraacetate; HAP, hydroxylapatite; PEG, poly(ethylene glycol); Me_2SO, dimethyl sulfoxide.

0.015 M sodium citrate, pH 7.0. PB is sodium phosphate buffer of the indicated pH and molarity.

(*b*) *Protein Isolation and Fractionation.* Isolation of the APNH subfraction of nonhistone proteins was described previously (Patel, 1972; Patel and Thomas, 1973). The procedure depends on the solubility properties and DNA affinity of this particular class of NHCP. All steps up to hydroxylapatite (HAP[1]) chromatography were performed at 4 °C. Briefly, purified rat liver nuclei, washed with buffered 0.14 M NaCl, were dissociated in 2 M NaCl–5 M urea–0.05 M Tris-HCl (pH 7.5) to obtain a soluble chromatin extract. This chromatin solution was then dialyzed vs. 13 volumes of distilled H_2O to reduce the NaCl concentration to 0.14 M. The resulting reconstituted chromatin was collected by centrifugation and was dissociated and reconstituted a second time. This twice-reconstituted chromatin, consisting of histones, DNA, RNA, and APNH, was redissociated in 2 M NaCl–5 M urea–0.001 M PB (pH 8.0) and fractionated by HAP chromatography. The APNH was eluted from the HAP column at 0.05 M PB (pH 8.0). Contaminating basic proteins and nucleic acids in the APNH fraction were removed by chromatography on Bio-Rex 70 and DEAE-Sephadex, respectively (Levy et al., 1972). Proteins thus prepared were dialyzed vs. buffer SB containing 5 M urea and stored at 4 °C until used.

(*c*) *Preparation of Labeled and Unlabeled DNA.* Unlabeled rat liver DNA was purified from nuclei by a modified method of Marmur (Teng et al., 1971) except that isoamyl alcohol was substituted for octanol in the $CHCl_3$ extractions. This DNA was banded in CsCl (ρ_0 = 1.700) at 35 000 rpm for 48–60 hr in a 60 Ti Spinco/Beckman rotor. *Micrococcus luteus* DNA was a gift of Drs. L. Dure and V. Walbot and was similarly banded in CsCl. Poly[d(A-T)·d(A-T)] and poly[d(G-C)·d(G-C)] were obtained from P.L. Biochemicals, Inc. *Escherichia coli* B strain (NB4786) was grown overnight in K + 10 medium and then transferred to 1 l. of Luria broth. The cells were grown to a cell density of 5×10^8 cells/ml and collected by centrifugation. The cells were washed and resuspended to 10^{10} cells/ml in M9 buffer. Spheroplasts were produced by making the solution 6% sucrose–0.063 M EDTA–0.63 mg/ml of lysozyme (Sigma) and incubating at 0 °C for 10 min. The spheroplasts were lysed in 0.2% Sarkosyl at 37 °C. The lysate was extracted with redistilled phenol equilibrated with 0.1 M Tris-HCl (pH 8.0). This DNA was precipitated with 2 volumes of ethanol (0 °C), redissolved in 1 × SSC, and banded in CsCl. Single-strand DNA was prepared prior to use by maintaining a solution of double-strand DNA at 90 °C for 15 min followed by rapid cooling and storage at 0 °C until use. DNAs to be employed in equilibrium and kinetic competition experiments were exhaustively digested with five units[2] of S1 nuclease for 2 h at 37 °C in 0.01 M NaCl–0.05 M sodium acetate–0.001 M $ZnSO_4$–5% glycerol, pH 5.0. This DNA was repurified by banding in CsCl or, in the case of poly[d(G-C)·d(G-C)], in Cs_2SO_4 for 60 h in a 50 Ti rotor at 35 000 rpm.

Rat liver DNA was labeled with ^{125}I by the method of Commerford (1971). [^{125}I]DNA was separated from free ^{125}I by gel filtration on a Sephadex G-50 column (1 × 70 cm) in 0.1 M ammonium acetate buffer (pH 5) or by dialysis vs. buffer SB and was rebanded in CsCl to remove any labeled RNA contaminants. ^3H-Labeled phage T7 DNA was prepared by infecting *E. coli* (NB4786, thy$^-$) at a multiplicity of infection of 0.1–1. The cells were prelabeled for three generations with [^3H]thymidine (New England Nuclear, 9.3mCi/mmol) at 1.5 μCi/ml in K + 2 medium. Lysis occurred within 1.5 h with vigorous aeration. Complete lysis was facilitated by adding 0.5 ml of $CHCl_3$ and aerating for an additional 10 min. Cell debris was removed by centrifugation and phage were concentrated by poly(ethylene glycol) (PEG) precipitation (Yamamoto and Alberts, 1970). The concentrated phage were resuspended in a small volume of 0.2 M Tris-HCl–0.1 M NaCl–0.02 M $MgCl_2$ (pH 8) and extracted with an equal volume of $CHCl_3$. ^3H-Labeled phage T7 was separated from chromosomal DNA by banding the phage in CsCl (ρ_0 = 1.500). ^3H-Labeled T7 DNA was purified from the isolated phage by making the phage solution in 0.15 M NaCl–0.1 M EDTA–0.1 M Tris-HCl (pH 8.0)–0.2% Sarkosyl, incubating at 60 °C for 30 min, and banding the DNA in CsCl (ρ = 1.705). All DNAs were stored over $CHCl_3$ in either 1 × SSC or buffer SB.

(*d*) *Nitrocellulose Filtration Assay for DNA Binding.* The theory and practical aspects of the nitrocellulose filtration technique have been described by Riggs et al. and Bourgeois (Riggs et al., 1970a; Bourgeois, 1972). Essentially, DNA–protein complexes are retained on the filter, while free DNA passes through. Binding is detected by employing radioactively labeled DNA and unlabeled proteins in the binding mixture. The number of DNA–protein complexes is proportional to the amount of radioactive DNA retained on the filter. In most cases, the most useful parameter obtained is the fraction of the total DNA retained on the filter. This parameter is defined as R

$$R = \frac{\text{cpm retained} - \text{cpm background}}{\text{total cpm input}} \quad (1)$$

where R was the parameter optimized in this study. The background radioactivity was defined as the amount of radioactive DNA retained in the absence of protein. This background was primarily a function of DNA purity. Rebanding the DNA in CsCl after in vitro labeling lowered the background considerably, as did presoaking the nitrocellulose filters in 0.4 N KOH for 20 min followed by rinsing and storing the filters in SB buffer (0–4 °C). The inclusion of 5% dimethyl sulfoxide (Me_2SO) in the binding mixture also facilitated lower backgrounds.

A typical DNA binding experiment proceeded as follows. Various amounts of APNH in buffer SB containing 5 M urea were added to a solution of labeled DNA (0.08 μg/ml) in buffer SB plus 5% Me_2SO. This mixture was incubated at 25 °C for a predetermined amount of time and aliquots (three to five replicates) were filtered on Schleicher and Schuell B6 filters at a rate of 0.5–0.7 ml/min. The filters were washed with 1.5 ml of SB buffer plus 5% Me_2SO, dried, and counted for radioactivity. A DNA solution without added protein was routinely filtered simultaneously as a measure of the background retention of the DNA.

In all optimization experiments, the parameter optimized was R (eq 1). For all experiments except the saturation experiments, the DNA and protein concentrations were fixed at 0.08 and 1 μg/ml, respectively. To determine the optimal Na^+, K^+, and Li^+ concentrations, either NaCl, KCl, or LiCl was added to 0.01 M Tris-HCl, pH 7.5, to yield concentrations of 0.01–0.6 M monovalent cation. The optimal pH for the APNH–DNA interaction was measured by adjusting the pH of SB buffer to various pHs in the range of 7 to 9; for the range 6 to 8, 0.01 M PB was substituted for

[2] "One unit" of S1 nuclease is defined as that amount of enzyme required to digest 40 μg of single-strand DNA in 10 min at 37 °C.

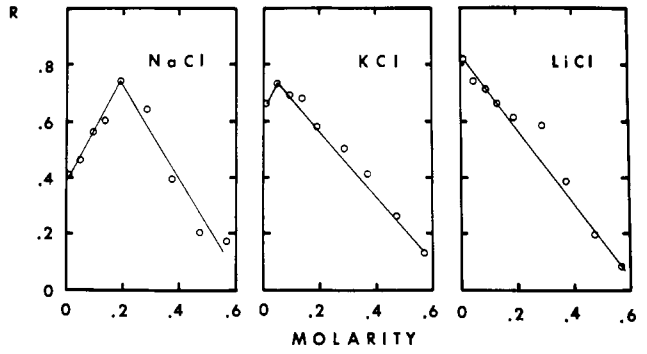

FIGURE 1: Effect of ionic strength and monovalent cations on the DNA-APNH interaction. To 4 ml of 0.01 M Tris-HCl containing varying concentrations of either LiCl, NaCl, or KCl, ^{125}I-labeled rat DNA and Me_2SO were added to a final concentration of 0.08 μg/ml and 5%, respectively. One milliliter was filtered in the absence of protein to determine the background retention of DNA for each salt concentration. APNH was added to the remainder to yield a final protein concentration of 1 μg/ml in 3.1 ml of buffer SB plus 5% Me_2SO. Following a 60-min incubation at 25 °C, 1-ml aliquots were filtered in triplicate on Schleicher and Schuell B6 membrane filters (25 mm) and washed with 1.5 ml of the above buffer. R was calculated from eq 1.

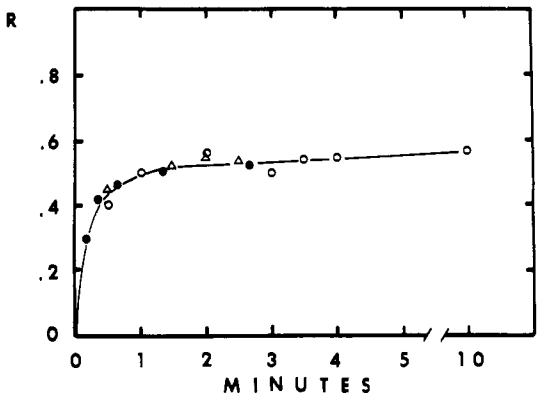

FIGURE 2: Rate of APNH-DNA association. Sufficient APNH to yield a final protein concentration of 0.5 μg/ml was rapidly mixed with 10 ml of [^{125}I]DNA (0.04 μg/ml) in SB buffer plus 5% Me_2SO. Aliquots (0.2 ml) were removed at various intervals, diluted one to five with 4 μg/ml of rat liver DNA in the above buffer, and immediately filtered. R was determined as described in the legend of Figure 1. (○), (●), (△) represent independent experiments.

Tris-HCl in the standard buffer. Effects of sulfhydryl reagents on the DNA-APNH interaction were investigated by adding 2-mercaptoethanol to SB buffer to give concentrations of 0.01–0.3 M mercaptoethanol. The effect of temperature on optimal binding was studied by preincubating the DNA at temperatures of 0 to 70 °C, adding saturating amounts of APNH, and continuing incubation at each temperature for 1 h. The kinetics of the association of DNA and APNH was measured by removing aliquots as a function of time and diluting fivefold with SB buffer plus 5% Me_2SO and 4 μg/ml of rat liver DNA; each aliquot was filtered immediately following dilution.

For saturation binding experiments, either ^{125}I-labeled rat liver DNA or ^{3}H-labeled T7 DNA was mixed with APNH to yield final concentrations of 0.08 μg/ml of DNA and 0.001 to 5 μg/ml of protein in SB buffer plus 5% Me_2SO. Equilibrium competition experiments were performed by mixing ^{3}H-labeled T7 DNA at 0.08 μg/ml with increasing amounts of unlabeled competing DNA prior to the addition of APNH (1 μg/ml) in SB buffer plus Me_2SO. These mixtures were incubated for 1 h and then processed in the normal manner. For kinetic competition experiments, ^{3}H-labeled T7 DNA and APNH were mixed to 0.08 and 0.95 μg/ml, respectively, in SB buffer plus 5% Me_2SO and equilibrated for 1 h. A 50-fold excess of unlabeled competing DNA was added and 0.25-ml aliquots were removed as a function of time and filtered immediately in triplicate.

(*e*) *Analytical Methods.* Single- and double-strand molecular weights of DNA employed in these studies were determined by band sedimentation analysis in the Model E analytical ultracentrifuge. Single-strand $s_{20,w}^0$'s were determined in 0.9 M NaCl–0.1 M NaOH; native $s_{20,w}^0$'s were determined in 1.0 M NaCl. Single- and double-strand molecular weights were calculated from the appropriate $s_{20,w}^0$'s and the equations of Studier (Studier, 1965). DNA concentrations were determined by uv absorption at 260 nm. Protein concentration was determined by uv absorption at 280 and 260 nm (Warburg and Christian, 1941) and/or by the method of Lowry et al. (1951). Dried nitrocellulose filters were counted in a toluene based scintillation fluid. Liquid samples were counted in a triton-toluene based scintillation fluid.

Results

(*a*) *Optimal Conditions for DNA-APNH Interactions.* Initial results with the membrane binding assay suggested that DNA-APNH complexes were retained quantitatively by the filters. Background retention of radioactively labeled DNA in the absence of APNH depended on the filtration conditions and the type and purity of the DNA employed. Conditions were found that yielded constant and low backgrounds. In buffer SB plus 5% Me_2SO and employing filters presoaked in 0.4 N KOH for 20 min, the background retention of ^{125}I-labeled rat DNA was 3–5% and ^{3}H-labeled T7 DNA yielded a background of less than 1% of the input radioactivity. Under similar conditions, 65–75% of the APNH is retained on the filter in the absence of DNA. Upon interaction with APNH, as much as 100% of the DNA could be retained on the filter.

Optimal conditions for the DNA-APNH interaction were determined by varying one parameter at a time to yield a maximum R value (eq 1); as optimal values for more parameters were determined, these were incorporated into the binding conditions. Periodically throughout the optimization, conditions were reexamined to determine if subsequent parameter changes had altered these values. Optimal ionic conditions of the APNH-DNA interaction were determined by incubating saturating amounts of DNA and APNH at 25 °C in 0.01 M Tris-HCl (pH 7.5)–5% Me_2SO with various concentrations of either NaCl, KCl, or LiCl. As shown in Figure 1, maximal binding occurred at 0.19 M NaCl, 0.05 M KCl, or 0.01 M LiCl. The optimal pH for binding was determined by equilibrating saturating amounts of DNA and APNH in 0.19 M NaCl plus 5% Me_2SO and either 0.01 M Tris-HCl or 0.01 M PB in the pH range of 6 to 9. Maximal binding occurred at pH 7.4–7.5 in phosphate buffer and pH 6.9–7.5 in Tris-HCl buffer (data not shown). The kinetics of the DNA-APNH association reaction were studied by incubating a reaction mixture at one-half saturation. The results of several experiments are shown in Figure 2. It is clear that the DNA-APNH interaction is very rapid ($t_{1/2} \sim 10$ s); equilibrium is approached within 2–3 min. The effect of temperature on the DNA-APNH interaction was studied by preincubating identical DNA solutions at various temperatures followed by the addition of identical amounts of APNH with continued incubation at each temperature. Figure 3 indicates that

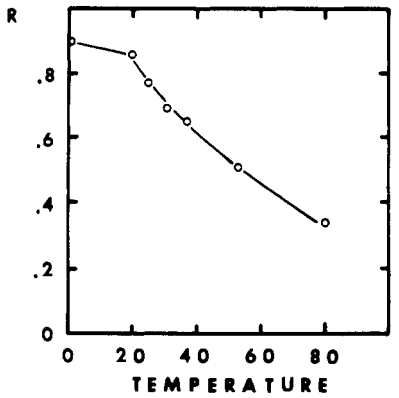

FIGURE 3: Temperature effect on DNA-APNH association. Five milliliters of ^{125}I-labeled rat DNA (0.08 μg/ml) in SB buffer plus 5% Me_2SO was incubated at each temperature for 1 h. APNH was added to 1 μg/ml, and the samples were incubated at each temperature for an additional hour and immediately filtered and processed as in Figure 1. Background retention at each temperature has been subtracted.

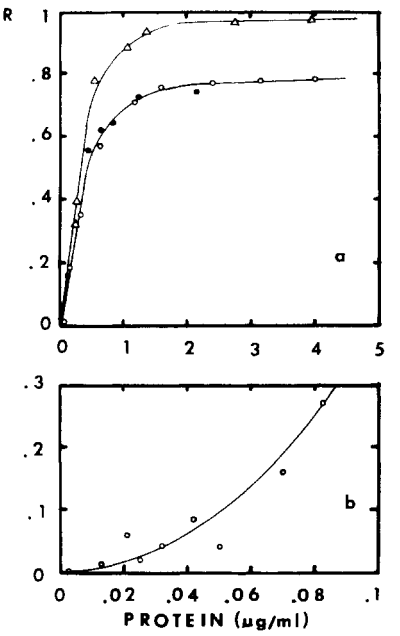

FIGURE 4: Saturation of rat liver and T7 DNA with increasing APNH concentration. (a) Increasing amounts of APNH were mixed with ^{125}I-labeled rat DNA or ^{3}H-labeled T7 DNA (0.08 μg/ml) in a final volume of 3.1 or 5.1 ml of buffer SB plus 5% Me_2SO. Following a 60-min incubation at 25 °C, 1-ml aliquots were processed to determine R as described in Figure 1. (△) represent T7 DNA; (○, ●) represent independent experiments with rat DNA. (b) Same as Figure 4a except at APNH concentrations less than 0.1 μg/ml.

APNH binds less DNA at higher temperatures. The effect of temperature is moderate in the 0–25 °C range, but the extent of the reaction is markedly decreased between 25 and 80 °C. Divalent cations or sulfhydryl reagents had little or no effect on the interaction of APNH and DNA (data not shown).

Initial protein concentrations for saturation and one-half saturation were determined by incubating fixed amounts of labeled DNA with increasing amounts of APNH in a fixed volume of SB buffer plus 5% Me_2SO. Figure 4a is representative of such saturation curves for both ^{125}I-labeled rat DNA and ^{3}H-labeled T7 DNA. Saturation occurs at a protein:DNA ratio of 13–15 (w/w); one-half saturation occurs at a $P_{1/2}$ of 0.3 and 0.33 μg/ml for rat and T7 DNA, respectively. $P_{1/2}$ is defined as the protein concentration required for 50% saturation of the DNA. Rat liver DNA (80%) and essentially all of the T7 DNA are retained by the filter at saturation. The cause for the different saturation levels of the two DNAs is not at present known, although it may be due to the in vitro labeling procedure employed. By all criteria available, there is no difference between the binding of APNH to rat or T7 DNA except for the saturation level difference. ^{3}H-Labeled T7 DNA can be purified as an intact molecule and yields substantially lower backgrounds than the in vitro labeled rat DNA. Consequently, we have employed T7 DNA in most of the experiments to be discussed. In almost all cases, however, the same experiments were performed employing ^{125}I-labeled rat liver DNA with comparable results. At very low protein concentrations (Figure 4b), the binding curve for rat liver DNA is distinctly nonlinear. A similar curve, although not as pronounced, results if T7 DNA is employed (data not shown). If the APNH concentration is fixed at 1 μg/ml and the concentration of labeled T7 DNA is varied, a curve of the form in Figure 5 results; saturation of APNH occurs at 0.08 μg/ml of DNA or a P:DNA ratio of 13 (w/w). Similar results were obtained also with rat liver DNA (data not shown). Taken together these data suggested that the observed interaction was specific and was not just a trapping phenomenon and also suggested a fixed number of DNA binding sites per unit mass of binding protein.

From the preceding data, it was possible to establish optimal conditions for the APNH–DNA interaction. These conditions were 0.19 M NaCl–0.01 M Tris-HCl–0.001 M EDTA (pH 7.5) plus 5% Me_2SO at 25 °C with a P:DNA of 12.5 (w/w). Although equilibrium was attained within 10 min, reactions were normally incubated for 1 h to ensure complete equilibration. It should be noted that, although 25 °C is not the optimal temperature, it was chosen as a standard temperature to facilitate comparison with other studies of DNA–protein interactions. Furthermore, 0.05 M KCl or 0.01 M LiCl could have just as easily been employed in the place of 0.19 M NaCl. This differential monovalent cation effect has greatly facilitated our subsequent studies on the effects of APNH on the helix–coil transition of DNA.

(*b*) *Specificity of APNH–DNA Interaction as Determined by Equilibrium Competition Experiments.* From previous DNA binding experiments (Patel and Thomas, 1973; Thomas et al., 1973) employing sucrose density gradient centrifugation and DNA affinity chromatography, partial species and single-strand specificity was implicated. With the optimal DNA binding conditions established above, a more detailed investigation of the specificity of the DNA–APNH interaction was possible. This was approached by employing equilibrium competition experiments (Riggs et al., 1970a,b; Lin and Riggs, 1972) and kinetic competition experiments (Riggs et al., 1970b).

Equilibrium competition experiments were designed to discriminate between the apparent species and single-strand specificity of the APNH–DNA interaction. Equilibrium competition experiments involved incubating a fixed amount of labeled DNA and APNH in the presence of increasing amounts of unlabeled competing DNA and monitoring the decrease in the number of DNA–protein complexes as a function of competing DNA concentration. In principle, the competing DNA which has the highest affinity for the bound proteins should compete to the greatest extent and at much lower initial DNA concentrations. Al-

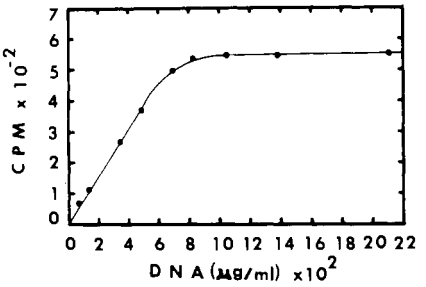

FIGURE 5: Saturation of APNH with increasing T7 DNA concentration. Increasing amounts of ^{3}H-labeled T7 DNA were mixed with APNH (1 μg/ml) in a final volume of 3.1 ml of buffer SB plus 5% Me_2SO. After incubation at 25 °C for 60 min, 1-ml aliquots were filtered in triplicate and washed with the above buffer. The amount of ^{3}H-labeled T7 DNA retained was determined by counting the dried filters. Each point is corrected for background retention of ^{3}H-labeled T7 DNA in the absence of APNH.

Table I: Competition Parameters of Various DNAs.[a]

Type and Conformation of Competing DNA	$D^{1/2}$ (μg/ml)[b]	$k' \times 10^4$ (s^{-1})[c]	Mol %[d] A-T
Poly d(A-T)			
Double strand	0.04	3.94 ± 0.31[g]	100
Single strand	0.04	ND[e]	
Rat Liver			
Double strand	0.23	1.85 ± 0.19	54.8
Single strand	0.04	5.69 ± 0.41	
E. coli (double strand)	0.40	ND	46.9
M. luteus (double strand)	2[f]	1.18 ± 0.21	27.3
Poly d(G-C)			
Double strand	14[f]	0.96 ± 0.05	0.0
Single strand	7[f]	ND	

[a] All DNAs were exhaustively digested with S1 nuclease. [b] $D^{1/2}$ is the initial competitor concentration required to reduce the amount of ^{3}H labeled T7 DNA retained to 50% of its initial value in the equilibrium competition experiments. Data extrapolated from Figure 6. [c] k' is the pseudo-first-order rate constant obtained from kinetic competition experiments. Slopes calculated from lines in Figure 7. [d] All values from Sober (1968). [e] Not determined. [f] Obtained by linear extrapolation. [g] Standard error of the slope calculated by linear regression methods.

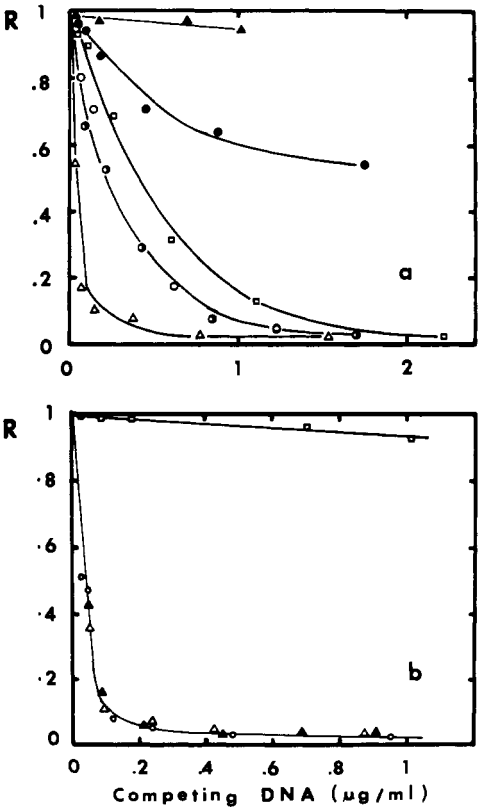

FIGURE 6: Equilibrium competition experiments with various DNAs. (a) A fixed amount of APNH (1 μg/ml) and ^{3}H-labeled T7 DNA was mixed with increasing amounts of S1 trimmed competing DNAs in a final volume of 3.1 ml of buffer SB plus 5% Me_2SO. After a 60-min incubation at 25 °C, 1-ml aliquots, in triplicate, were processed to determine R. The competing DNAs are poly[d(G-C)·d(G-C)] (▲), *M. luteus* (●), *E. coli* (□), rat liver (preparation a, ○; preparation b, ◑), and poly[d(A-T)·d(A-T)] (△). All data are normalized to R = 1 at zero competition. (b) Same conditions as Figure 6a except that increasing amounts of heat denatured rat liver DNA (○), poly[d(A-T)] (▲), and poly[d(G-C)] (□). Poly[d(A-T)·d(A-T)] (△) was used simultaneously as a control. R is normalized to one at zero competition.

though the extent of competition is often a good indication of the relative affinities of the DNAs for the protein, this is often difficult to interpret. A more meaningful and more accessible parameter to monitor is the initial competitor concentration required for 50% competition, $D^{1/2}$, where a lower $D^{1/2}$ indicates higher affinity of the bound proteins for the competing DNA (Lin and Riggs, 1972).

Initial equilibrium competition experiments employing ^{125}I-labeled rat liver DNA, double-stranded rat liver, *E. coli*, and salmon sperm DNA, and poly[d(A-T)·d(A-T)] and poly[d(G-C)·d(G-C)] as competitors indicated that there was no apparent species specificity of binding (Patel and Thomas, 1975). However, the relative competing abilities of these DNAs seemed to correspond to the A-T content of the DNA. To further investigate the effect of A-T content on the $D^{1/2}$ of the competition reaction, rat liver, *E. coli*, and *M. luteus* DNA along with poly[d(A-T)·d(A-T)] and poly[d(G-C)·d(G-C)] were employed in equilibrium competition experiments. These represent a gradient of 0–100% A-T. To ensure that small amounts of single-stranded material on the duplexes did not affect the results of this experiment, the duplexes were exhaustively digested with S1 nuclease (a single-strand specific nuclease) and the S1 trimmed duplexes were repurified on CsCl and Cs_2SO_4 gradients. Since there appeared to be no species specificity of binding, ^{3}H-labeled T7 DNA was employed in the place of ^{125}I-labeled rat DNA. This facilitated the studies by lowering the background retention of the DNA and eliminating possible artifacts due to the in vitro labeling procedure. ^{3}H-Labeled T7 DNA was mixed with increasing amounts of each competing DNA in a fixed volume and was incubated with sufficient APNH to saturate the T7 DNA alone. Figure 6a shows the results of one such experiment. It is clear that poly[d(A-T)·d(A-T)] is a better competitor than the other DNAs and, in fact, the relative competitive abilities of the DNAs correspond to the A-T content of the competing DNAs. Table I summarizes the results of both equilibrium and kinetic competition experiments as well as the mole per cent A-T and the conformation of the competing DNAs. Here we refer only to the double-strand data. From Table I, it is seen that $D^{1/2}$ for poly[d(A-T)·d(A-T)] is 350-fold lower than the extrapolated value for poly[d(G-C)·d(G-C)]. $D^{1/2}$ for *M. luteus* DNA is 50-fold higher than $D^{1/2}$ for poly[d(A-T)·d(A-T)], while $D^{1/2}$ for *E. coli* and $D^{1/2}$ for rat liver are tenfold and sixfold greater, respectively, than $D^{1/2}$ for poly[d(A-T)·d(A-T)]. Thus, APNH exhibits a marked affinity for A-T rich DNA.

However, there was also reason to suspect that APNH

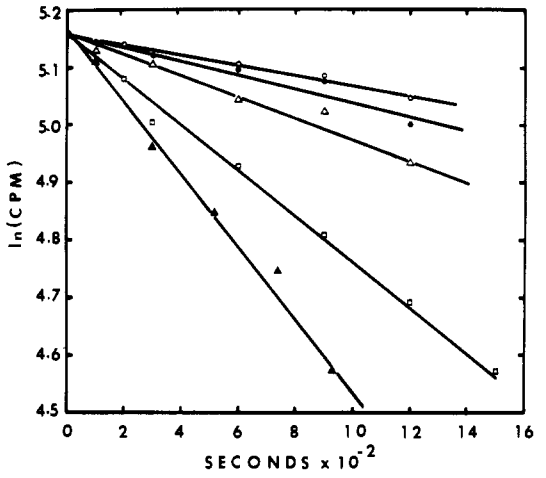

FIGURE 7: Kinetic competition experiments with various DNAs. ^{3}H-Labeled T7 DNA-APNH complexes were formed under optimal conditions (see text) and allowed to equilibrate at 25 °C for 60 min. At t = 0, a 50-fold excess of either of the following S1 trimmed duplexes were added, poly[d(G-C)·d(G-C)] (○), *M. luteus* (●), rat liver (△), single strand rat liver (▲), or poly[d(A-T)·d(A-T)] (□). Aliquots (0.35 ml) were filtered in triplicate at each time point and the total cpm retained was determined. This value was corrected for background retention and the natural logarithm of the cpm retained was plotted vs. time.

might bind to single-stranded DNA (Patel and Thomas, 1973; Thomas et al., 1973; and Thomas and Patel, 1974). Consequently, the relative affinities of these proteins for single-stranded and double-stranded DNA were investigated employing the equilibrium competition assay. Heat denatured rat liver DNA, poly[d(A-T)] and poly[d(G-C)] were used as competitors; native rat liver DNA and poly[d(A-T)·d(A-T)] were employed as controls. Under these conditions, the DNAs employed were estimated by hyperchromicity measurements to be 85–100% single stranded. The results of such experiments are shown in Figure 6b, and the $D^{1/2}$'s for the single stranded DNAs employed are summarized in Table I. What is of interest is that the $D^{1/2}$ for single-stranded poly[d(A-T)] is identical with that for poly[d(A-T)·d(A-T)]. On the other hand, $D^{1/2}$ for double-stranded rat liver DNA is six times the $D^{1/2}$ for single-stranded rat liver DNA; in fact, $D^{1/2}$ for single-stranded rat liver DNA is identical, within experimental error, with that obtained for poly[d(A-T)]. Conversely, the $D^{1/2}$ for single-strand poly[d(G-C)] is 150 times the $D^{1/2}$ for poly[d(A-T)] and single-stranded rat liver DNA. These results confirmed our initial observations that APNH indeed had a higher affinity for single-stranded DNA. However, these experiments further revealed that, although APNH prefers single-stranded DNA, this specificity is also dependent on the base composition of the DNA. Consequently, APNH appears to have the greatest specificity for single-stranded regions which contain deoxyadenosine or thymidine residues. Subsequent experiments with poly[d(A)] and poly[d(T)] show that APNH binds preferentially to poly[d(T)] (data not shown).

(*c*) *Specificity of APNH–DNA Interaction as Determined by Kinetic Competition Experiments.* As a further measure of the apparent single-stranded and A-T specificity of APNH, the dissociation kinetics of DNA–APNH complexes was investigated as a function of the conformation or A-T content of the competing DNA. The rate of dissociation of preformed DNA–protein complexes can be measured by adding a large excess of unlabeled competing DNA and following the decrease in the amount of labeled DNA retained on the nitrocellulose filter as a function of time (Riggs et al., 1970b; Jobe and Bourgeois, 1972; Wang et al., 1974). This can be represented more formally in the following manner:

$$\text{D*–P} + \text{D} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{D–P} + \text{D*} \qquad (2)$$

where the * represents radioactively labeled DNA, and k_{-2} and k_2 represent the respective rate constants for the reactions as written. The time derivative of D*–P is

$$\frac{-\mathrm{d(D^*\text{–}P)}}{\mathrm{d}t} = k_{-2}(\text{D*–P})(\text{D}) - k_2(\text{D–P})(\text{D*}) \qquad (3)$$

Initially, however, k_2(D–P)(D*) is very small and eq 3 becomes

$$\frac{-\mathrm{d(D^*\text{–}P)}}{\mathrm{d}t} = k_{-2}(\text{D*–P})(\text{D}) \qquad (4)$$

But if (D) $\gg$ (D*–P), (D) is essentially constant and k_{-2}(D) = k', the pseudo-first-order rate constant. Substituting k' into eq 4 and integrating over the time interval 0 to t and applying the boundary conditions yields

$$\ln (\text{D*–P}) = -k't + \ln (\text{D*–P})_{t=0} \qquad (5)$$

A similar equation has been derived for the *lac* operator–repressor system by Riggs et al. (1970b). Since the amount of radioactivity retained on the filter is directly proportional to (D*–P), a plot of ln (cpm retained) vs. time should yield a straight line with a slope of $-k'$ and a y intercept of ln (D*–P)$_{t=0}$. When D and D* are identical, k' is only a function of the rate of dissociation of the DNA–protein complex. But when D and D* are not identical, k' is a measure of the intrinsic dissociation rate of the complex and the relative affinity of the protein for the competing DNA. It is, however, difficult to separate these two parameters. Consequently, it was necessary to fix all DNA concentrations at the same value since these enter into the composite rate constant (k' = k_{-2}(D)). All competing DNA concentrations were fixed at 50-fold excess for the kinetic competition experiments described here. In our hands, a 50–100-fold DNA excess yielded ideal first-order kinetics over the first 30–40% of the reaction.

Experimentally, the dissociation kinetics of DNA–APNH complexes were investigated by preforming the complexes with ^{3}H-labeled T7 DNA and APNH. After equilibrium was attained, a 50-fold excess of either S1 trimmed rat liver, *M. luteus,* single-stranded rat liver DNA, S1 trimmed poly[d(A-T)·d(A-T)] or poly[d(G-C)·d(G-C)] was added to the mixture, and the amount of labeled DNA–protein complexes was monitored as a function of time. A plot of ln (cpm retained) vs. time for the various DNAs is shown in Figure 7. It should first be noted that the data are quite linear, thus justifying our assumption of pseudo-first-order rate constant, k'; these values are tabulated in Table I along with the $D^{1/2}$'s for the corresponding equilibrium competition experiments. In conjunction with lated in Table I along with the $D^{1/2}$s for the corresponding equilibrium competition experiments. In conjunction with the A-T content and conformation of the DNA, three things are evident.

(1) The rate constants increase with increasing A-T content or in going from double-stranded to single-stranded DNA of the same A-T content.

(2) The A-T effect on the rate constant is not as pro-

nounced as that observed with the equilibrium competition experiments.

(3) The rate constant for single-stranded rat liver DNA is 1.5 times greater than that for poly[d(A-T)·d(A-T)]. From these data we conclude again that APNH exhibits a greater affinity for A-T rich DNA and single-stranded DNA; however, in this instance there appears to be an even greater affinity for single-stranded DNA as opposed to double-stranded poly[d(A-T)].

(*d*) *Effect of Molecular Weight on the Specificity of the APNH-DNA Interaction.* Single-stranded and double-stranded molecular weights for the competing DNAs were determined by band sedimentation (Studier, 1965) to ascertain whether the observed binding specificities were due to differential nicking or a fortuitous molecular weight gradient of the DNAs. The results of these determinations are tabulated in Table II. The S1 trimmed competing DNAs are listed in order of descending G + C content. The double-stranded molecular weights of the competing DNAs do not follow any pattern relative to the G + C content of the DNA. In fact, the DNA preparations seem to be randomly distributed about an average of 14×10^6 daltons, but the poly[d(A-T)·d(A-T)] and poly[d(G-C)·d(G-C)] are essentially the same size. Consequently, the A-T gradient effect observed in the equilibrium and kinetic competition experiments (Figure 6a, Table I, and Figure 7) does not appear to be a result of a molecular weight gradient of the competing DNAs. The last column in Table II indicates the number of single-stranded breaks per single strand of competing DNA. There is no gradient of nicks as a function of G-C content. Perhaps of most significance is the comparison of the two rat liver DNA preparations. Preparation b contains three times the number of single-stranded breaks as preparation a; yet in Figure 6a, the two preparations fall on the same competition curve suggesting that, at this level of resolution at least, the number of nicks present in the competing DNA does not affect its $D^{1/2}$ or k'.

The S1 nuclease employed functioned as expected since the single- and double-stranded molecular weights of rat liver preparation b were 65 and 34%, respectively, of that of the same DNA preparation without S1 digestion (data not shown). At the same time there was no detectable hydrolysis of double-stranded ^{3}H-labeled T7 DNA under identical conditions. Hyperchromicity measurements suggested that 95–100% of the S1 trimmed DNAs are in duplex form. As a reference, the single- and double-stranded molecular weights of the T7 DNA employed are also included in Table II. We conclude that the apparent A-T and single-stranded specificity of APNH cannot be attributed to either a differential number of single-stranded breaks or a fortuitous gradient of molecular weights. Therefore APNH has a much higher affinity for single-stranded and A-T rich DNA.

Table II: Single and Double Strand Molecular Weights of DNAs Employed.[a]

Type DNA	$M \times 10^{66}$ (daltons) Double Strand	$M \times 10^{66}$ (daltons) Single Strand	Single Strand Breaks per Single Strand[c]
Poly d(G-C)·d(G-C)00	0.205	0.052	0.97
M. luteus	15.0	2.62	1.86
E. coli	21.6	1.99	4.4
Rat Liver			
a	12.8	2.36	1.7
b	34.0	2.73	5.2
Poly d(A-T)·d(A-T)	0.456	0.227	0
[^{3}H]T7	25.0	12.4	0

[a] All DNAs except ^{3}H-labeled T7 were exhaustively digested with S1 nuclease. [b] Calculated from alkaline and neutral $s_{20,w}{}^0$'s employing Studier's equations (Studier, 1965). [c] Calculated from the equation, $N = (M_{ds}/2M_{ss}) - 1$, where N is the average number of single strand breaks per single strand and M_{ds} and M_{ss} are the double- and single-strand molecular weights, respectively.

Discussion

The role of the NHCPs in the overall structure of the eukaryotic chromosome is presently a subject of some debate. Although the NHCP have been implicated in the specific modulation of chromosomal function (Gilmour, 1975; Stein et al. 1975), we have no a priori reason to suspect that this is the only role of NHCP in the cell nucleus. In this laboratory, we have worked under the hypothesis that proteins involved in chromatin function should have affinity for DNA. We have concentrated our efforts on characterizing the DNA binding properties of a particular subclass, APNH, of chromatin proteins. It is our belief that by correlating the DNA binding properties of APNH with other DNA binding proteins of known biological functions, we can gain some insight into its possible biological roles. We have shown here that APNH exhibits high affinity for DNA but, unlike other preparations of NHCP, it does not interact with DNA in a species-specific manner (Kleinsmith et al., 1970; Teng et al., 1971). Instead, these proteins interact with DNA on the basis of its A-T content—the higher the A-T content of the DNA, the greater the interaction. Furthermore, they also interact to the greatest extent with single-stranded DNA as opposed to double-stranded DNA of the same G-C content.

In considering the DNA binding properties of APNH, it must be noted that more than one protein species could be binding simultaneously and, as yet, we have not determined the number and types of binding sites on the DNA. We do know, however, that the two major components of APNH bind to single-stranded DNA very tightly (Thomas et al., 1973). Also, since our assay employed only purified DNA, and since chromatin consists of NHCP, histones, RNA, and DNA, we cannot be sure whether these interactions are representative of an in vivo interaction. Our use of purified DNA may be justified, however, since Clark and Felsenfeld (1971) have shown that at least 50% of the chromatin DNA is accessible to nuclease digestion and poly(lysine) precipitation.

At this stage of the investigation, we do not interpret the optimal conditions for APNH binding to DNA as being indicative of a specific mechanism or a particular type of interaction. However, it should be noted that maximal interaction does occur at physiological ionic strength and pH (0.19 M Na^+ and pH 7.5). We interpret this as an indication of the specificity and nativeness of the APNH-DNA interaction. We further interpret both the pH and ionic strength effects as being indicative of a Coulombic component in the interaction. The optimal ionic strength is comparable to that determined by Sevall et al. (1975) for a different preparation of NHCP. The temperature effect observed was not unexpected and suggests further experiments to determine more precisely the effect of temperature on the equilibrium. The absence of a marked divalent cation

effect was not altogether unexpected as many proteins including DNA polymerase I (Englund et al., 1969) and gene 32 protein of bacteriophage T4 (Alberts and Frey, 1970) do not require Mg^{2+} for DNA binding, although their actual in vivo and some in vitro functions seem to require Mg^{2+}. These optimal conditions for binding provide conditions for further experiments, especially employing the differential monovalent cation and temperature effects as keys to the further purification of APNH.

Although the saturation levels of APNH and DNA are in themselves uninformative, the protein concentration at 50% saturation is a good indication of the affinity of a protein for a given DNA ($P^{1/2}$ is inversely proportional to the association equilibrium constant, K_a). $P^{1/2}$ for APNH binding to rat liver and T7 DNA is 0.3–0.32 μg/ml, whereas, $P^{1/2}$'s for other nuclear protein fractions isolated in this laboratory are three- to sixfold higher (Patel and Thomas, 1975), suggesting that APNH binds to DNA with a much higher affinity. The fact that the same protein to DNA ratio (13) at saturation is obtained when either DNA or protein concentration is varied (see Figures 4 and 5) argues strongly for a specific interaction between APNH and DNA. Two explanations seem reasonable for this apparently high protein/DNA ratio (approximately 1 protein/2 base pairs). First, a certain fraction of the APNH may be impurities or irreversibly denatured during the isolation procedure; thus, the actual concentration of DNA binding proteins is much less and the mass of APNH required to saturate the DNA, as a result, is much higher. Second, if the observed APNH/DNA ratio is a true reflection of the interaction, it is still of considerable interest since Alberts et al., (1972) have reported that the gene 5 protein of the bacteriophages M13 and fd bind DNA with a stoichiometry of one protein per four DNA nucleotides. At present both explanations are equally plausible. The nonlinearity of the rat DNA–APNH saturation curve (Figure 4b) suggests several possibilities. If we assume that only one bound protein is sufficient for retention on the filter of a DNA-protein complex, then the apparent nonlinearity could be attributed either to cooperativity of binding (multiple DNA sites), to an oligomerization reaction where the oligomer is the only binding form, or to multiple binding proteins with different equilibrium constants.

The specificity of the DNA–APNH interaction was both surprising and interesting. Both the equilibrium competition and kinetic competition experiments suggested that APNH has a greater affinity for A-T base pairs and single-stranded DNA. It should also be noted that this single-stranded specificity is corroborated by saturation experiments with S1 nuclease trimmed rat liver DNA (Patel and Thomas, 1975). The equilibrium competition experiments suggest that APNH has an equal affinity for poly[d(A-T)·d(A-T)] and single-stranded rat DNA, but the kinetic competition experiments suggest that, in fact, APNH actually has a higher affinity for single-stranded DNA than for poly[d(A-T)·d(A-T)], since k'(ss) > k'd(A-T). Proteins with high affinity for A-T rich DNA are not uncommon. The *lac* repressor in binding to nonoperator DNA shows an A-T preference (Lin and Riggs, 1972) as does the NHCP described by Sevall et al. (1975). Furthermore gene 32 protein, isolated after bacteriophage T4 infection (Alberts and Frey, 1970), exhibits increased affinity for the coil form of DNA. Proteins, which bind preferentially to the coil form as opposed to the duplex, destabilize the helix and lower the T_m of the duplex (von Hippel and McGhee, 1972). If these proteins preferentially bind A-T rich regions, the A-T rich duplexes will be preferentially destabilized and the T_m of such regions will be lowered accordingly. From the preceding, we would predict that APNH, on interacting with double-stranded DNA, would lower the T_m of the A-T rich regions of the duplex. Preliminary thermal denaturation experiments suggest that APNH indeed lowers the T_m of poly[d(A-T)·d(A-T)] (Thomas and Patel, unpublished results). Several calculations suggest that the equilibrium constant for the APNH–single-stranded rat DNA interaction is three orders of magnitude higher than the APNH–double-stranded rat DNA interaction at 25 °C. These results, although very preliminary and dependent upon several assumptions, are a further indication of the predicted effect of APNH on the helix–coil transition.

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Photochemical Cross-Linking of Neighboring Residues in Protein–Nucleic Acid Complexes: RNase and Pyrimidine Nucleotide Inhibitors†

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ABSTRACT: Irradiation of the stable complexes formed between RNase and its competitive inhibitors cytidine 2′(3′),5′-diphosphate (pCp), and uridine 2′(3′),5′-diphosphate (pUp), resulted in covalent bond formation between the pyrimidine nucleotides and the enzyme. The photochemical reactions were initiated by ultraviolet light of $\lambda > 300$ nm, employing acetone as a photosensitizer. This method was found to yield less undesired by-products, particularly photolyzed amino acids and aggregates resulting from protein-to-protein cross-linking, than the direct method of irradiation with light of $\lambda > 260$ nm. Tryptic digestion of the modified protein, and chromatographic analysis of the peptides thus obtained, revealed a single and specific peptide which became covalently linked to both nucleotide inhibitors. The amino acid composition of this peptide is consistent with the sequence Asn-67-Arg-85 of RNase. Part of this peptide (residues 78 through 83) is close to the enzyme's binding site for the pyrimidine moiety of the nucleotides. Denatured RNase failed to cross-link to the inhibitors, and the extent of pUp cross-linking could be reduced by the addition of another competitive inhibitor (3′-UMP). Finally, the presence of excess inhibitor in the irradiation mixture did not lead to nonspecific cross-linking. This indicates that specificity is also achieved due to the fact that unbound excited inhibitor molecules do not react with the protein but prefer to follow different and faster reaction paths.

The active study of protein–nucleic acid interactions in recent years has emphasized the critical role these interactions play in life processes. The control and regulation of gene expression in cellular metabolism involve, for example, protein–DNA interactions (von Hippel and McGhee, 1972), while the protein synthesis machinery involves a variety of protein–RNA interactions (Kurland, 1972). In order to account for the high affinities observed in protein–nucleic acid complexes, simultaneous interactions of a number of functional groups on the two partners must be involved. These groups must be positioned in a specific conformational relationship which permits a favorable interaction to take place. The molecular mechanism upon which these highly developed specific interactions are based is not understood in detail for any of these systems.

Photochemistry provides a promising approach to the study of such interactions since proteins and nucleic acids cross-link covalently when irradiated with ultraviolet light. This has been shown in several systems including DNA and proteins in bacteria (Smith, 1975), bovine serum albumin and DNA (Smith, 1964; Braun and Merrick, 1975), DNA polymerase and DNA (Markovitz, 1972), RNA polymerase and Br-substituted DNA (Weintraub, 1973), the *lac* repressor and the *lac* operon-containing Br-substituted DNA (Lin and Riggs, 1974), and a variety of complexes of amino acyl tRNA synthetases and their cognate tRNAs (Schoemaker and Schimmel, 1974; Budzik et al., 1975; Schoemaker et al., 1975). The proposed approach to the problem of protein–nucleic acid interactions utilizes photochemistry in an attempt to "freeze" existing contact points in protein–nucleic acid complexes, and thereby allowing the identification and chemical characterization of the interacting residues. The major advantage of this approach is that photo-

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