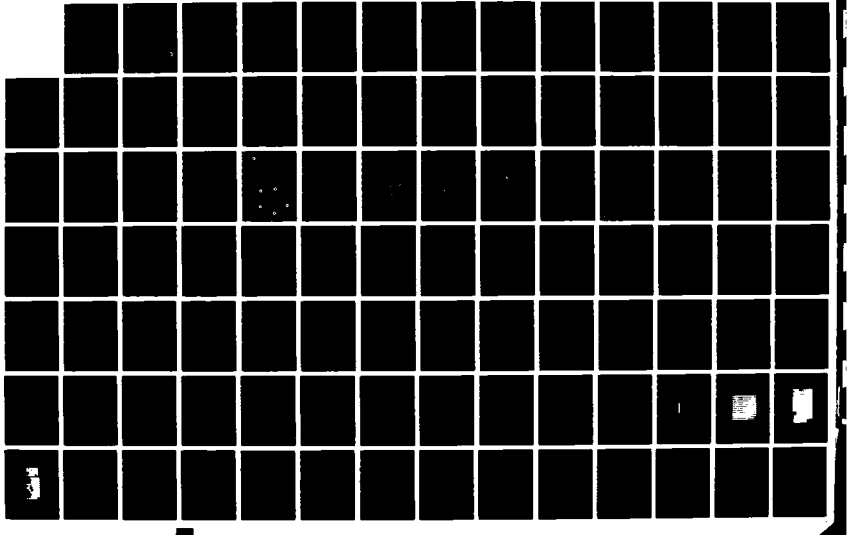
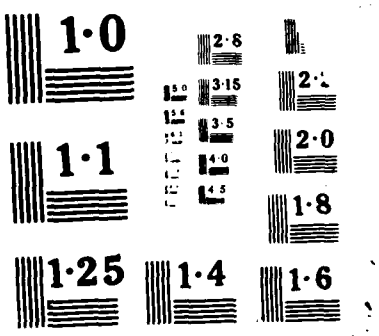
AD-A191 557 MOLECULAR TOXICOLOGY OF CHROMATIN(U) CALIFORNIA UNIV SAN FRANCISCO CARDIOVASCULAR RESEARCH INST E KUN 31 DEC 87 AFOSR-TR-88-0009 AFOSR-86-0064 1/2

UNCLASSIFIED F/G 6/11 NL




1·0
1·1
1·25
1·4
1·6
1·8
2·0
2·8
3·15
3·5
4·0
4·5

AFOSR-TR- 88-0009

AFOSR - 86 - 0064

PROGRESS REPORT

Molecular Toxicology of Chromatin

Period of January 1, 1987 - December 31, 1987

Submitted by: Professor Ernest Kun
Principal Investigator
University of California, San Francisco
Department of Pharmacology and the
Cardiovascular Research Institute
San Francisco, California 94143-0130

Tel. (415) 476-4543

December 17, 1987

SECURITY CLASSIFICATION OF THIS PAGE

ADA191554

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S): AFOSR-TR- 88-0009
6a. NAME OF PERFORMING ORGANIZATION: Cardiovascular Res. Inst Univ. of Calif.,SF; 6b. OFFICE SYMBOL (If applicable):	7a. NAME OF MONITORING ORGANIZATION: AFOSR Bolling Air Force Base, D.C. 20332
6c. ADDRESS (City, State, and ZIP Code): 1327-M, CVRI, UCSF San Francisco, CA 94143-0130	7b. ADDRESS (City, State, and ZIP Code): Bolling Air Force Base D.C. 20332
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: AFOSR; 8b. OFFICE SYMBOL (If applicable): NL	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: AFOSR-86-0064
8c. ADDRESS (City, State, and ZIP Code): Bolling Air Force Base D.C. 20332	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 61102F; PROJECT NO. 2312; TASK NO A5; WORK UNIT ACCESSION NO

11. TITLE (Include Security Classification): Molecular Toxicology of Chromatin

12. PERSONAL AUTHOR(S): Dr. Ernest Kun

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
ANNUAL	FROM 1/1/87 TO 12/31/87	December 17, 1987	100

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

I. Benzamide-dna Interactions: Deductions from Binding, Enzyme-Kinetics and from X-Ray Structural Analysis of A 9-Ethyladenine-Benzamide Adduct.

II. Molecular Interactions Between DNA, Poly(ADP-ribose) Polymerase and Histones.

III. Analysis of the Molecular Contacts Between Poly(ADP-ribose) Polymerase and DNA.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL: T. JAN CERVENY, Major, USAF	22b. TELEPHONE (Include Area Code): (202) 767-5021; 22c. OFFICE SYMBOL: NL

DD Form 1473, JUN 86 Previous editions are obsolete SECURITY CLASSIFICATION OF THIS PAGE

18 DEC

Table of Contents

I

Part I

BENZAMIDE-DNA INTERACTIONS; DEDUCTIONS FROM BINDING, ENZYME-KINETICS AND FROM X-RAY STRUCTURAL ANALYSIS OF A 9-ETHYLADENINE-BENZAMIDE ADDUCT

ABSTRACT.

The interaction of benzamide with the isolated components of calf thymus poly(ADP-ribose) polymerase and with liver nuclei has been investigated. A benzamide-agarose affinity gel matrix was prepared by coupling o-aminobenzoic acid with Affigel 10, followed by amidation. The benzamide-agarose matrix bound the DNA that is coenzymic with poly(ADP-ribose) polymerase; the matrix, however, did not bind the purified poly(ADP-ribose) polymerase protein. A highly radioactive derivative of benzamide, the [^{125}I]-labelled adduct of o-aminobenzamide and the Bolton-Hunter reagent, was prepared and its binding to liver nuclear DNA, calf thymus DNA and specific coenzymic DNA of poly(ADP-ribose) polymerase was compared. The binding of labelled benzamide to coenzymic DNA was several fold higher than its binding to unfractionated calf thymus DNA. A DNA-related enzyme inhibitory site of benzamide was demonstrated in a reconstructed poly(ADP-ribose) polymerase system, made up from purified enzyme protein and varying concentrations of a synthetic octadeoxynucleotide that serves as coenzyme.

As a model for benzamide binding to DNA, a crystalline complex of 9-ethyladenine and benzamide was prepared and its X-ray crystallographic structure determined; this indicated a specific hydrogen bond between an amide hydrogen atom and N-3 of adenine. The benzamide also formed a hydrogen bond to another benzamide molecule. The aromatic ring of benzamide does not intercalate between ethyladenine molecules, but lies nearly perpendicular to the planes of stacking ethyladenine molecules in a manner reminiscent of the binding of ethidium bromide to polynucleotides.

Thus we have identified DNA as a site of binding of benzamide; this binding is critically dependent on the nature of the DNA and is high for coenzymic DNA that is isolated with the purified enzyme as a tightly associated species. A possible model for such binding has been suggested from the structural analysis of a benzamide-ethyladenine complex.

INTRODUCTION

Transformation of synchronized human fibroblasts, induced by ultimate carcinogens, has been found to be prevented by benzamide when both carcinogens and benzamide are present at non-toxic concentrations in the early S phase (1,2). Similar observations were made with C3H10T1/2 hamster embryo cells in culture (3). Although benzamide is known to be a competitive inhibitor of poly(ADP-ribose) polymerase (E.C. 2.4.99) with respect to NAD^+ (which also contains an amide group) (4), its antitransforming biological effect does not correlate with enzyme inhibition at the NAD^+ binding site (2). On the other hand, toxicity of benzamides on CHO-KI cells has been reported to be related to their K_i with respect to the NAD^+ binding site of the polymerase (5).

A possible cellular site of action of benzamide which may be important in the regulation of cell transformation was provided by preliminary experiments with autoradiography. We observed that [^{14}C]-labelled benzamide was localized at nuclear membrane sites (1), suggesting an apparent binding of benzamide to nuclear membrane macromolecules. The present work is concerned with the identification of DNA as a benzamide-binding nuclear macromolecule. The binding of benzamide to DNA appears to be stronger than the association of benzamide with the poly(ADP-ribose) polymerase enzyme protein (presumably at the NAD^+ binding site) (4). The evidence for this is that the binding of benzamide to the enzyme could not be demonstrated under conditions when benzamide was associated with DNA. We also show that, of the DNA forms bound by benzamide, the coenzymic DNA of poly(ADP-ribose) polymerase is a preferred species for such binding. Benzamide exhibits also a second, non-competitive enzyme inhibitory action that is not related to NAD^+. This was determined from experiments with purified enzyme and a specific octadeoxynucleotide as a coenzyme

substituting for coenzymic DNA. These results indicate that the poly(ADP-ribose) polymerase system has at least two types of benzamide binding sites.

The molecular mechanism of benzamide-DNA binding is not known at present. However, an X-ray diffraction analysis of crystals of a complex of 9-ethyl-adenine with benzamide revealed a unique mode of hydrogen bonding that may provide a model for benzamide-nucleotide interactions.

EXPERIMENTAL PROCEDURES

1. Calf thymus poly(ADP-ribose) polymerase and enzyme-associated active DNA were isolated according to the method of Yoshihara, et al. (6). The procedure and its results are illustrated in Table 1. The enzyme at Stage V is 95% homogenous, as determined by gel electrophoresis. The enzyme protein was first eluted from hydroxylapatite with a linear phosphate gradient (30 → 300 mM), then the enzyme-associated DNA (coenzymic DNA) eluted with 2 M KCl containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM Na azide, 1 mM reduced glutathione and 0.5 mM dithiothreitol. Fractions containing the DNA were dialyzed against 10 mM Tris-HCl, pH 7.4, 1 mM EDTA buffer, then freeze-dried. The yield was 3.65 mg DNA, with an estimated average size of 340 bp of double-stranded DNA after S_1 nuclease digestion of single-stranded DNA. Highly purified enzyme protein, exhibiting only traces of proteolytic peptide products, was also obtained by substituting red-agarose affinity chromatography for DNA cellulose followed by protein separation by high performance liquid chromatography (MONO-S, Pharmacia).*

---

*Buki, K., Kirsten, E. and Kun, E., unpublished results.

2. Rat liver nuclei were isolated according to Liew and Chan (7) as modified by Hakam, et al. (8). The protein content (9) of the nuclear suspensions used routinely was 29 mg/ml. Nuclear DNA was determined by fluorometry (10). The poly(ADP-ribose) polymerase activity of nuclei was within 20% of 1.85 nmol ADP-ribose bound per mg protein per min. assayed in a test system of a volume of 50-100 µl composed of 150 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1.5 mM dithiothreitol, (reaction buffer) and 100 to 200 µM NAD labelled with [^{3}H] or [^{14}C] or [^{32}P] (11). The enzyme activity was determined at 20°C, terminated with 3 ml of 10% trichloroacetic acid (4°C) and the thoroughly acid-washed precipitable radioactive material assayed either directly on fiber filters (11), or after separation of poly(ADP-ribose), by high performance liquid chromatography (8). When the purified enzyme was tested, the assay system also contained 10 µg/ml specific coenzymic DNA or the octadeoxynucleotide added at varying concentrations.

3. Synthesis of o-aminobenzamide-agarose affinity matrix and assay for DNA binding. o-aminobenzoic acid (2.56 g) was dissolved in 50 ml of 0.5 M $NaHCO_3$ and the pH adjusted to 10.7 with 2 N NaOH. Affigel 10 (Bio-Rad) was suspended and washed in the $NaHCO_3$-NaOH buffer then 20 ml of the gel suspension (settled by gentle suction filtration) was added to the o-aminobenzoic acid solution, the mixture slowly agitated for 3 hours at room temperature and left overnight at 4°C. The gel was washed successively with 200 ml portions of 1M Na_2HPO_4, H_2O, 1M KH_2PO_4, H_2O and finally with 1M $(NH_4)_2SO_4$. Twenty ml of the wet gel was suspended in 40 ml of 1M $(NH_4)_2SO_4$ and EDAC [1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide] was added to a final concentration of 0.1 M (19). The pH was adjusted to 4.7 and thereafter maintained between 3.3 and 5.5 during

gentle agitation at room temperature for 4 hours, and the suspension was left overnight at 4°C. The gel was again washed with 200 ml portions of 1M Na_2HPO_4, H_2O, 1M KH_2PO_4 and H_2O. The quantity of agarose bound o-aminobenzamide was determined as follows. The o-aminobenzamide gel was hydrolyzed in 6 M HCl at 100°C for 48 hours; thereafter, the sample was freeze-dried, dissolved in H_2O and again freeze-dried. This process was repeated 3 times, and finally the material was taken up in 1 ml H_2O and the pH adjusted to 7.5-8.0. An aliquot was analyzed by high performance liquid chromatography (Altex, Ultrasphere-ODS, 4.6 mm x 25 cm) and resolved in a linear gradient of methanol (0-25%) in 50 mM phosphate, pH 4.25, time = 40 min. One-ml fractions were collected and analyzed fluorometrically (374 nm excitation, 420 nm emission) in 25% glycerol and compared to o-aminobenzoate standards, prepared identically to the samples to be analyzed. The amount of o-aminobenzamide bound per ml gel was calculated to be 7.3 µmoles per ml.

Benzamide-agarose suspensions (0.2 or 0.4 ml) were incubated with agitation with 2.3 µg coenzymic DNA in a total volume of 0.65 ml reaction mixture, containing Tris-Mg^{2+}-dithiothreitol reaction buffer (see above) for 30 min. at 25°C. At the end of this period, the affinity gel was spun down at 2000 x g for 10 min. and 200 µl of the supernatant tested for coenzymic DNA content in an enzymatic system containing 32.5 µg polymerase (Stage IV) protein/ml, 108 µM NAD [^{14}C]-labelled in the adenine moiety (2.5 µCi per ml) at 25°C in

a reaction volume of 400 µl. Aliquots of 70 µl of the supernatant were assayed for the quantity of auto-poly-ADP-ribosylated enzyme protein as shown in Figure 2, which was proportional to the quantity of free coenzymic DNA in solution within the limits of the enzymatic test. The rate of auto-poly-ADP-ribosylation of the enzyme was linear with time and was also a linear function of the concentration of free coenzymic DNA in this system below saturation of the enzyme with DNA.

4. DNA-cellulose was prepared according to Alberts (12) and the quantity of cellulose-bound DNA, determined spectrophotometrically (following hydrolysis for 4 hours at 90°C in 10 mM phosphate + 1 mM EDTA at pH 7.4), was 0.7 mg DNA per ml resin.

5. Iodination of poly(ADP-ribose) polymerase protein. Ten to 20 µg of IODO-GEN (Pierce, 1,3,4,6-tetrachloro-3α, 6α-diphenlyglyco-uril) was dissolved in 200 µl $CHCl_3$ and the solvent evaporated, leaving a film on the surface of the tube. Two hundred µg purified enzyme protein, dissolved in 133 µl 100 mM Tris-HCl (pH 7.4) 150 mM NaCl, was added and the mixture incubated for 10 min. at 4°C with 1 mCi [^{125}I]-NaI, the latter added in a volume of 10 µl. The enzyme protein was separated from the unreacted [^{125}I] by gel filtration on a Sephadex G-75 column (0.7 x 6 cm) equilibrated with the Tris-NaCl buffer (pH 7.4), containing 2 mM dithiothreitol. Enzymatic activity of the iodinated enzyme was determined by chromatographic analysis of the polymer following NaOH and proteinase K hydrolysis of the polymer-protein adducts (cf. 8). After iodination more than 95% activity was recovered.

6. Preparation of the adduct of the Bolton-Hunter (BH) reagent [3-(p-hydroxyphenyl)-propionyl-N-hydroxy succinimide ester] with o-aminobenzamide, and its iodination.

(a) Synthesis. A solution of 1 g of BH reagent (38 mmol) and 2.27 g (20 mmol) o-aminobenzamide in 150 ml of absolute ethanol was allowed to stand overnight at room temperature. The product was isolated on preparative TLC silica gel plates (Analtech) with a developing solvent composed of $CHCl_3$, MeOH, CH_3COOH (90:10:2). The separated product was scraped off the plate and extracted with ethanol for 2 hours at room temperature. The solvent was evaporated from the pooled eluates. Final purification was achieved by rechromatography in the above-described system which removes traces of unreacted o-aminobenzamide. The product gave a single spot on TLC using the above developing solvent. RF for o-aminobenzamide = 0.61; 0.45 for BH adduct of o-aminobenzamide; the BH reagent decomposes to two products RF: 0.31, 0.87.

(b) Iodination. Fifty µg of IODO-GEN was dissolved in 100 µl $CHCl_3$, and the solvent evaporated in order to coat the inner surface of a tube with the reagent. Twenty-five µg (88 nmol) BH-o-aminobenzamide was dissolved in 25 µl M NaAc (pH 5.6) and added to the IODO-GEN-coated tubes followed by 1 mCi [^{125}I]-NaI. The reaction was allowed to proceed for 10 min. at room temperature and the products were separated by TLC in the system described above. The radioactive product was located by auto-radiography (RF:0.85) and eluted with ETOH; assuming the same specific activity of [^{125}I] in the product as in [^{125}I]-NaI, its quantity was 0.195 nmol (43% yield).

7. Preparation of crystalline complex of benzamide and 9-ethyladenine.

In a 25 ml beaker, 36.34 mg (0.30 mmol) of benzamide (m.p. 128-130°C) and 48.96 mg (0.30 mmol) of 9-ethyladenine (m.p. 190-192°C) were dissolved in 6 ml of deuterochloroform. The beaker was covered with aluminum foil and the solution was allowed to slowly evaporate to dryness over 2 days at room

temperature. Most of the resultant crystals were either those of pure benzamide or 9-ethyladenine (checked by melting points), but included in the deposition were a few small, but visually discernable clusters of prisms with a single-crystal melting point in the range of 150-165°C. One of these prisms was selected for X-ray analysis.

8. Preparation of the deoxyribonucleotide octamer.

Complementary octameric single strands were synthesized by the solid phase method (17), then combined and annealed to obtain the duplex shown in Figure 1. We have shown (22) that coenzymic DNA can be replaced by octamers of specific composition, representing catalytically active domains of coenzymic DNA.

Figure 1

Benzamide (Ring U [^{14}C]) and benzoic acid (7.2 mCi/mmol) were purchased from Pathfinder Labs (St. Louis, MO.). All other reagents were of analytical grade. [^{14}C]-labelled NAD was obtained from Amersham Corporation; [^{32}P]-labelled NAD from New England Nuclear Corporation. Benzamide was purchased from Aldrich Co. and 9-ethyladenine and deuterochloroform from Sigma.

9. X-Ray Crystallography.

The 2:1 complex of 9-ethyladenine with benzamide, $2(C_7H_9N_5):C_7H_7NO$, M_r = 447.50, is triclinic, space group $P\bar{1}$, a = 9.482(3), b = 14.937(3), c = 8.553(3)Å, α = 110.59(2), β = 96.70(2), γ = 91.71(2)°, V = 1123.1(5)Å^3, Z = 2, D_x = 1.323 g cm^{-3}, λ(CuKα) = 0.15418 nm, μ = 0.646 mm^{-1}, F(000) = 472e, t = 22°C. Three-dimensional X-ray diffraction data were collected on a Nicolet computer-controlled 4-circle diffractometer using a crystal 0.20 x 0.20 x 0.40 mm in

dimensions with the $\theta - 2\theta$ scan technique in the 2θ range 0 - 138° ($(\sin\theta/\lambda)_{max}$ = 6.0557 nm^{-1}) with a minimum scan rate of 29.3° min^{-1}. In this way, 4208 unique reflections were scanned of which 3219 had $\underline{I} \geq 2.0\ \sigma(\underline{I})$. These were considered as "observed" and were used in further calculations. Structure amplitudes were obtained from the raw data by correcting for geometric factors and placement on an absolute scale. There was no crystal decay as a function of time as indicated by the monitoring of the intensities of four standard reflections during the data collection. Values of $\sigma(\underline{F})$ were obtained from the relation $\sigma(\underline{F}) = (\underline{F}/2)[\sigma^2(\underline{I})/\underline{I}^2 + \delta^2]^{1/2}$, where $\underline{I}$ is the integrated intensity observed, $\sigma(\underline{I})$ is derived from counting statistics and δ is the instrumental uncertainty obtained from the standard reflections and was 0.025 for this data set. The structure was solved using the direct methods computer program MULTAN80 (13) which gave the positions of all non-hydrogen atoms. These were refined, first with isotropic and then anisotropic temperature factors. All hydrogen atom positions were found from a difference Fourier map and were included with isotropic temperature factors in the final refinement using a least-squares procedure (14,15). The quantity minimized during the refinement was $\Sigma w||\underline{F}_{obs}|-|\underline{F}_{calc}||^2$ with the weight, $w = 1/\sigma^2(\underline{F})$. The final residuals are R_{obs} = 0.065, wR_{obs} = 0.068.* The final difference Fourier map had no peak higher than 0.26 $e/\text{Å}^3$ and

---

* $\underline{R} = \Sigma||\underline{F}_o|-|\underline{F}_c||/\Sigma|\underline{F}_o|$, where $\underline{F}_o$ is an observed structure factor and $\underline{F}_c$ is a structure factor calculated from a postulated structure. $\underline{R}$ is a measure of the extent to which the measured X-ray diffraction pattern of a structure agrees with that calculated from the parameters of the structure determined by analysis of the diffraction data.

$\underline{wR} = (\Sigma w(||\underline{F}_o|-|\underline{F}_c||)^2/\Sigma w(|\underline{F}_o|)^2)^{1/2}$ is another measure of this agreement incorporating the weight, w, of each observation.

the maximum parameter shift to e.s.d. quotient was 0.45. Other computer programs used are from the program library of the Molecular Structure Laboratory of the Institute for Cancer Research. The atomic scattering factors used are from a compilation of published values (16).

RESULTS

The extent of binding of benzamide to components of the purified poly (ADP-ribose) polymerase protein (Table I) was determined with the aid of a benzamide affinity matrix (see Experimental Procedures). When 0.5 ml of benzamide agarose gel suspended in the Reaction Buffer (see Experimental Procedures) was incubated with either 1.0 mg of poly(ADP-ribose) polymerase of purification Step I or with 0.1 mg enzyme protein of purification Step IV for 30 min. at 4°C in a total volume ranging from 0.75 to 1 ml, 94% to 103% of the enzymatically active protein was recovered in the gel supernatant after centrifugal separation of the gel. This supernatant contained non-absorbed enzyme protein. These results were indistinguishable from the non-specific adsorption of traces of the enzymatic protein to agarose, containing no benzamide affinity label. Therefore, it was concluded that the enzyme protein does not bind to the benzamide-agarose affinity matrix.

Table I

On the other hand, the binding of the specific coenzymic DNA of poly (ADP-ribose) polymerase to the benzamide-agarose was readily demonstrable. The binding of DNA to the affinity column was determined by an enzymatic test which quantitatively assays the unbound coenzymic DNA in a reconstructed *in vitro* enzymatic system, containing purified polymerase protein as a catalyst. As shown in Figure 2, the top line represents results of the control experiment, where DNA was incubated with 0.4 ml of Affigel 10 containing no benzamide

Figure 2

affinity label but otherwise treated in a manner identical to the test containing the o-aminobenzamide Affigel 10 (see Experimental Procedures), whereas the second and third curves indicate diminishing DNA content in the gel supernatant, demonstrating the binding of DNA to increasing quantities of the benzamide gel matrix. The experiment shown in the fourth curve contains no added DNA in the incubation system - only the gel supernatant - and represents blank values. It was demonstrated in separate tests that incubation of the benzamide affinity gel with the buffer does not release an enzyme inhibitor, o-aminobenzamide, and 100% enzymatic activity could be recovered when gel supernatants were incubated with the standard poly(ADP-ribose) polymerase system, (see inset to Figure 2). The DNA that was bound to the affinity column could be quantitatively recovered from the gel by overnight incubation with M NaCl at 4°C. Furthermore, the coenzymic DNA present in commercial calf thymus DNA could be bound to the affinity gel and after recovery with M NaCl, the eluted DNA exhibited the same coenzymic function in the poly(ADP-ribose) polymerase test as crude thymus DNA which is about 1/50th of that of coenzymic DNA. The enzymatic test for the binding of coenzymatically active DNA to the benzamide-agarose matrix demonstrates a certain degree of specificity of coenzymic DNA as compared to crude thymus DNA. The enzymatic assay, which responds to the quantity of coenzymic DNA used in our tests, could not have detected an amount of non-specific DNA, i.e., unfractionated calf thymus DNA, at concentrations used in the benzamide gel binding experiments of coenzymic DNA because the thymus DNA contains only 1-2% of coenzymatically active species.

We tested the possibility of interference by benzamide with the DNA-association of the purified enzyme protein. The test was carried out with [^{125}I]-labelled enzyme and DNA-cellulose affinity matrix (12). First, the association of [^{125}I]-labelled enzymatically 95% active poly(ADP-ribose)

polymerase protein to DNA-cellulose was determined. These concentrations (0, 25. 50, and 78 nM)of enzyme protein (107 cpm [^{125}I]/fmol of enzyme) were incubated with a constant amount of DNA-cellulose in 270 µl total volume of reaction mixture containing 7 µg DNA/ml resin suspension, 100 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM dithiothreitol and 100 µg/ml bovine serum albumin for 20 min. at 25°C. For displacement (i.e., determination of specific binding), control tubes contained in addition to the above components 250 µg/ml calf thymus DNA. At the end of incubation the resin was spun down for 4 min. at 2500 x g and the bound and free enzyme were determined by [^{125}I] counts. From Scatchard plots (not shown) K_D was calculated to be 3.7 nM.

The binding of 3.6, 14.4 and 33.2 nM [^{125}I]-labelled poly(ADP-ribose) polymerase protein to DNA-cellulose was determined following incubation of DNA-cellulose with 0 to 1 mM benzamide for 20 hrs. Benzamide added to the DNA cellulose suspension did not interfere with the binding of the polymerase protein to this matrix.

Incubation of [^{14}C]-labelled benzamide with isolated liver nuclei was assumed to facilitate detection of benzamide-DNA binding because of a hydrophobic environment prevailing in nuclei. The DNA concentration dependence and time course of binding of benzamide to DNA, present in isolated liver nuclei, are illustrated in Figure 3. The time of incubation was 2.5 hours at 25°C in order to minimize DNA breakdown. Increasing quantities of nuclei, proportional to DNA bound more benzamide (Figure 3). The rate of binding was linear with time up to 8 hrs. (inset of Figure 3), a time limit chosen because significant decay of nuclear DNA was likely beyond this period. In contrast [^{14}C]-labelled benzoic acid of the same specific

Figure 3

activity as benzamide exhibited no binding to nuclear DNA.

The highly radioactive [^{125}I]-labelled Bolton-Hunter reagent derivative of o-aminobenzamide (see Experimental Procedures) was tested as a ligand for DNA present in liver nuclei,and its association with coenzymic DNA and with commercial calf thymus DNA were compared. When increasing quantities of liver nuclei, equivalent to 66, 99 and 132 µg DNA were incubated for 8 hrs. with 0.96 mM non-radioactive iodinated Bolton-Hunter reagent adduct of o-amino-benzamide, charged with tracer quantity of [^{125}I]-containing adduct (603 MBq/µg equivalent to 3.8 x 10^6 cpm) in 100 µl Tris-Mg^{2+}-dithiothreitol buffer (see above), 0.33, 0.68 and 1.1 nmol ligand was proportionally bound to DNA as a function of the quantity of DNA in liver nuclei. In these experiments 2 mM unlabelled non-radioactive iodinated ligand was used for quenching. It was apparent that the [^{125}I]-containing ligand, which has a specific radioactivity 10 times higher than the [^{14}C]-labelled benzamide, was suitable for DNA binding studies since both [^{14}C]-benzamide and the [^{125}I]-labelled ligand gave similar results. It was of importance to ascertain that DNA in liver nuclei is the only macromolecular species that binds benzamide. To test this we exposed liver nuclei briefly to DNA-ase I and determined the rate of DNA degradation by direct chemical analysis (10). Simultaneously we also assayed the binding of the [^{125}I]-labelled ligand to untreated nuclei and to nuclei which were exposed to 5 µg DNA-ase ./ml. Results of three separate experiments, which gave nearly identical values, are shown in Table II. Reducing DNA content of nuclei to about 1/5th completely abolished the binding of the [^{125}I]-labelled benzamide derivative to liver nuclei. No detectable proteolysis occurs under the same conditions.

Table II

If the association of benzamide to DNA has relevance to poly ADP-ribosylation-related control mechanisms in cellular systems, it would be expected that the coenzymic DNA of poly(ADP-ribose) polymerase should exhibit some degree of specificity of benzamide binding. We have tested this possibility by comparing the association of the [^{125}I]-labelled ligand with calf thymus DNA and with purified coenzymic DNA, and found at least a four-to five-fold increase of benzamide binding to "coenzymic" DNA, as compared to crude thymus DNA (results not shown). The possible relevance of this observation to the structure of coenzymic DNA is unknown and is subject to further studies.

Figure 4

Kinetic evidence (Figure 4) supports the conclusion drawn from preceding results that benzamide also binds to a component of the poly(ADP-ribose) polymerase system at a site that is not identical with the catalytic NAD^+-binding site. At a constant concentration of NAD^+ (25 nM) and purified enzyme protein (1 nM) the concentration of coenzymic octamer (Figure 1) was varied from 0 to 0.4 μM and the rate of enzymatic activity in this system depended on the concentration of the octamer, as a coenzyme (Figure 4). Benzamide non-competitively inhibited the coenzymic action of the octamer with a K_i = 2 μM.

The atomic coordinates of the benzamide-9-ethyladenine complex, determined by X-ray diffraction analysis (see Experimental Procedures) are listed in Table III. The crystal contains two molecules of 9-ethyladenine and one molecule of benzamide in the asymmetric unit. Interatomic distances and some angles are diagrammed in Figure 5. This crystal structure, illustrated in

Figures 6,7a, 7b

Figure 6, is characterized by pairs of 9-ethyladenine molecules hydrogen-bonded in sheets that are connected through hydrogen bonds to pairs of benzamide molecules that are hydrogen-bonded across space group symmetry centers. The amide hydrogen atoms not involved in benzamide dimer formation connect the sheets of

adenine bases in this way. Thus, one hydrogen atom of each amino group of two benzamide molecules is involved in hydrogen bonding to the amide carbonyl oxygens to form a dimer. The other amino hydrogens form hydrogen bonds to N(3) of 9-ethyladenine, linking adjacent sheets. The hydrogen bonding in sheets is illustrated in Figure 7(a); this shows the stacking of purine rings above or below each other in planes parallel to the plane of this Figure, with the benzamide ring systems lying nearly perpendicular to these planes. This is further shown in Figure 7(b) which is a view perpendicular to the view in Figure 7(a). Figure 7(b) shows that the 9-ethyladenine groups are approximately planar (apart from the out-of-plane-CH_3 of the ethyl group). It also shows the manner by which two benzamide molecules, hydrogen bonded to each other, can also form an association between stacking 9-ethyladenine molecules by hydrogen bonding involving the second hydrogen atom on the nitrogen atom of the amide group of benzamide.

It was of interest to compare the benzamide-9-ethyladenine structure with that of ethidium bromide-dinucleoside monophosphate (18). In the hydrogen-bonded benzamide complex, studied here, the benzene ring is approximately perpendicular to the stacked adenine plane. In a similar manner the 9-phenyl group of ethidium bromide is also approximately perpendicular to the plane of stacked bases. This is demonstrated in Figure 8. Thus, the phenanthridinium moiety of ethidium bromide intercalates between the nuclei and bases while the 9-phenyl group does not. Thus the aromatic ring of benzamide appears to behave in such complexation more like the 9-phenyl group of ethidium bromide than its phenanthridinium group. No intercalation of benzamide occurs in this crystalline complex. The limited solubility of dinucleotide monophosphates in chloroform,in contrast to ethyladenine, thus far prohibited crystallization of possible benzamide adducts. Further work with synthetic oligo-deoxyribonucleotides is in progress.

igure 8

DISCUSSION

The direct binding assay of coenzymic DNA to *o*-aminobenzamide matrix sharply distinguishes between the polymerase protein and DNA and identifies benzamide-DNA association. This behavior seems specific for the benzamide that

is linked to the matrix at the ortho-position. An affinity resin containing 3-aminobenzamide as ligand effectively binds the enzyme protein (19), but no information exists with respect to DNA binding to this matrix. Apparently the 3-aminobenzamide resin is recognized by the enzyme at the NAD^+-binding sites, whereas the ortho-substituted resin used in our experiments preferentially serves as DNA oriented ligand. The molecular reasons for the differences in the behavior of the two resins are unknown. It can be expected from the hydrogen-bonding model that H_2O under certain conditions would interfere with benzamide-DNA association by competing for hydrogen bonds. That benzamide does not inhibit the binding of the enzyme to DNA-cellulose may be explained by prolonged exposure to H_2O in this test system. However, in nuclei, where DNA in chromatin structures most probably contains hydrophobic domains, the binding of benzamide or its BH-derivative is readily demonstrable (Figure 3). Unfortunately none of these binding models are suitable for the calculation of binding constants of benzamide to DNA. Kinetics of inhibition by benzamide, that is observed at constant concentration of NAD^+ but at varying concentrations of a coenzymic octadeoxynucleotide, identifies a novel site of action of benzamide. Non-competitive kinetics may be compatible with the binding of benzamide to the enzyme-octamer complex, presumably at a hydrophobic purine domain of the octadeoxynucleotide. The sequence of the octamer has been based on the known hormone-receptor binding DNA domains (cf. 20) and we assume that the structure of catalytically active regions of coenzymic DNA may be related to the synthetic octadeoxynucleotide. The hydrogen-bonded structure of the 9-ethyladenine-benzamide adduct provides a model for the binding of benzamide to certain DNA structures. It appears

from the structure of the model complex that benzamide does not intercalate between the bases, but is a hydrogen bond donor to N(3) of the purine ring. The aromatic ring in benzamide lies nearly perpendicular to the bases. The pairing of benzamide with N2, N3 of guanine (in the minor groove) or adenine N6, N7 (in the major groove) are possible.

It is of interest that the N(3) position of adenine in DNA exhibits special reactivity as, for example, with the alkylating 9-anthryloxirane (21). A hydrogen-bonded interaction of benzamide and other antitransformers at the N(3) sites of adenine in DNA, with specific consequences on DNA conformation, is possible. For example, benzamide could complex to N(3) of adenine and the oxygen of an adjacent ribose.

ACKNOWLEDGMENTS

This research was supported by USPH Grant HL-27317, the Air Force Office of Scientific Research Grants AFO-85-0377, and AFO .-86-0064, USPHS Grants CA-10925, CA-06927, RR-05539 and CA-22780 from the National Institutes of Health, BC-242 from the American Cancer Society, and by an appropriation from the Commonwealth of Pennsylvania. Ernest Kun is a recipient of the Research Career Award of the USPHS.

References

1. Kun, E., Kirsten, E., Milo, G.E., Kurian, P. and Kumari, H.L. (1983) Proc. Natl. Acad. Sci. USA 80, 7219-7223.

2. Milo, G.E., Kurian, P., Kirsten, E. and Kun, E. (1985) FEBS Letts. 179, 332-336.

3. Borek, C., Morgan, W.F., Ong, A. and Cleaver, J.E. (1984) Proc. Natl. Acad. Sci. USA 81, 243-247.

4. Purnell, M.R. and Whish, W.J.D. (1980) Biochem. J. 185, 775-777.

5. Purnell, M.R., Kidwell, W.R., Minshall, L. and Whish, W.J.D. (1985) in "ADP-Ribosylation of Proteins" pp. 98-105 (Althaus, F.R., Hilz, H., and Shall, S., eds.) Springer-Verlag, Berlin.

6. Yoshihara, K., Hashida, T., Tanaka, Y., Oghushi, H., Kamiya, T. (1978) J. Biol. Chem. 253, 6459-6466.

7. Liew, C.C. and Chan, P. (1976) Proc. Natl. Acad. Sci. USA 76, 3458-3462.

8. Hakam, A., McLick, J. and Kun, E. (1984) J. Chromatogr. 296, 369-377.

9. Bradford, W. (1976) Anal. Biochem. 72, 248-254.

10. Labarca, C. and Paigen, K. (1980) Anal. Biochem. 102, 344-352.

11. Kirsten, E., Jackowski, G., McLick, J., Hakam, A., Decker, K. and Kun, E. (1985) Exptl. Cell Res. 161, 41-52.

12. Alberts, B. and Herrick, G. (1971) Methods in Enzymology, Vol. XXI (Grossman, L. and Moldave, K., eds.) pp. 198-217, Academic Press, New York.

13. Main, P., Fiske, S.J., Hull, S.E., Lessinger, L., Germain, G., Declerq, J-P., and Woolfson, M.M. (1980) "MULTAN80. A System of Computer Programs for the Automatic Solution of Crystal Structures from X-Ray Diffraction Data," Physics Department, Universities of York, England and Louvain, Belgium.

14. Carrell, H.L. (1975) "ICRFMLS. Modification of UCLALS4," Institute for Cancer Research, Philadelphia, PA.

15. Gantzel, P.K., Sparks, R.A., Long, R.E. and Trueblood, K.N. (1969) "UCLALS4. Full - Matrix Least-Squares Program in FORTRAN IV."

16. International Tables for X-Ray Crystallography (1974), Vol. 4, pp. 72-102, Kynoch Press, Birmingham, England.

17. Matteucci, M.D. and Caruthers, M.H. (1981) J. Amer. Chem. Soc. 103, 3185-3191.

References
Continued

18. Jain, S.C. and Sobell, H.M. (1984) J. Biomol. Structure and Dynamics 1, 1179-1194.

19. Butscher, H.J., Auer, B., Locker, H., Schweiger, M. and Hirsch-Kauffmann, M. (1986) Anal. Biochem. 152, 285-290.

20. Kun, E., Minaga, T., Kirsten, E., Hakam, A., Jackowski, G., Tseng, A. and Brooks, M. (1986) in "Biochemical Action of Hormones" Vol. XIII (Litwack, J., ed.) pp. 33-55, Academic Press, New York.

21. Nien-Chu, C. Yang and Chia-Wun Chang (1985) Proc. Natl. Acad. Sci. USA 82, 5250-5254.

22. Hakam, A., McLick, J., Buki, K., and Kun, E. (1987) FEBS Lett. (in press)

LEGENDS TO FIGURES

FIGURE 2. Binding of specific coenzymic DNA to benzamide-agarose matrix. The binding of DNA to benzamide agarose was assayed enzymatically as described in Experimental Procedures. Ordinate: quantity of enzymatic product, that is the auto-poly-ADP-ribosylated enzyme protein; abscissa: time of enzymatic reaction at 25°C; —●—●— : eluate of agarose, containing no ligand (control); —o—o—: eluate of 0.2 ml benzamide agarose incubate; —□—□— : eluate of 0.4 ml of benzamide agarose incubate; —■—■— : eluate of benzamide agarose in the absence of added DNA (control 2). Inset indicates that no benzamide was released from benzamide-agarose matrix during incubation. In this experiment benzamide-agarose or agarose were incubated without added DNA (0 to 10 min. = abscissa) in the same system as described in Experimental Procedures. Aliquots of the supernatant were tested in the poly(ADP-ribose) polymerase assay in order to determine possible "leakage" of enzyme inhibitor from the benzamide gel. Ordinate: % enzymatic activity in the absence or presence of gel eluates. The enzyme assay contained 10 µg/ml specific coenzymic DNA.

FIGURE 3. The binding of [^{14}C]-benzamide to DNA of rat liver nuclei. Increasing amounts of liver nuclei containing 0.1 to 0.5 mg DNA (abscissa) were incubated with 3 mM [^{14}C]-labelled benzamide at pH 8.0 in 500 µl of the reaction buffer (see Experimental Procedures) for 2.5 hrs. at 25°C in the presence or absence of 20 mM unlabelled benzamide as a quenching agent. One ml of cold incubation buffer, containing 1 mg serum albumin per ml was added and nuclei rapidly sedimented (30 sec.) in a Beckman microfuge. The pellet was washed, then dried, the tip of the Eppendorf centrifuge tube cut off and radioactive material determined by scintillation counting following solubilization

in the scintillator for 12 hours. The time curve of benzamide binding to nuclei equivalent to 0.2 mg DNA is shown in the inset.

FIGURE 4. Non-competitive inhibition of poly(ADP-ribose) polymerase by benzamide (BA) using 8 base-paired synthetic deoxynucleotide as variable component. Vo is expressed as fmol of ADP-ribose formed (from 25 mM [^{32}P]-NAD) per 2 min. per 0.1 ml reaction mixture. The enzyme concentration was 10 µg/ml and the octadeoxynucleotide was varied from zero to 2 µg/ml. For assay condition see Experimental Procedures. Δ—Δ denotes 10 µM BA; ▲—▲ 4 µM BA; o—o 2 µM BA; ●—● no inhibitor. $(dN{\cdot}dN)_8$ = octadeoxynucleotide (see Figure 1), expressed in reciprocal mols (abscissa).

FIGURE 5. Bond lengths and interbond angles for 9-ethyladenine:benzamide complex. The upper value of each pair in 9-ethyladenine refers to the primed molecule. The average estimated standard deviation (e.s.d.) in bond lengths is 0.02 Å for those involving hydrogen and 0.003 Å for all others. The average e.s.d. for interbond angles is 1.0° for those involving hydrogen and 0.2° for all others.

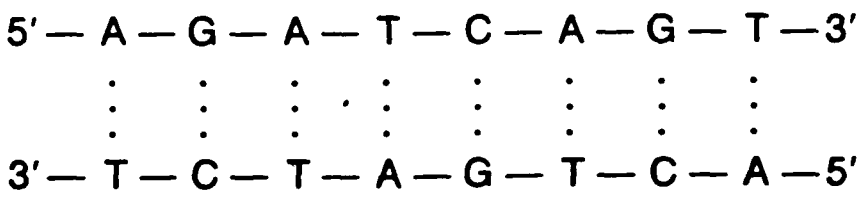
FIGURE 6. Idealized view of the crystal structure, showing the hydrogen-bonding pattern in the unit cell.

FIGURE 7. (a) View onto the best least-squares plane through the 9-ethyladenine pair (except methyl groups) showing the overlap of 9-ethyladenine in successive sheets. The atoms of some 9-ethyladenine hydrogen-bonded sheets are filled in, in order to accentuate the sheet separation. (b) View parallel to the plane in (a) showing the separation of 9-ethyladenine sheets, with distances given in Angstrom units.

FIGURE 8. View of the benzamide structure(designated number 1) docked so that the phenyl groups overlap onto the crystal structure of an ethidium bromide-dinucleoside phosphate (designated number 2). Note that while the bases of the two structures are not directly superposed by this calculation, the relationship between the phenyl group and the planes in which the bases lie is the same for both.

FIGURE 1

Structure of the deoxynucleotide octamer duplex.



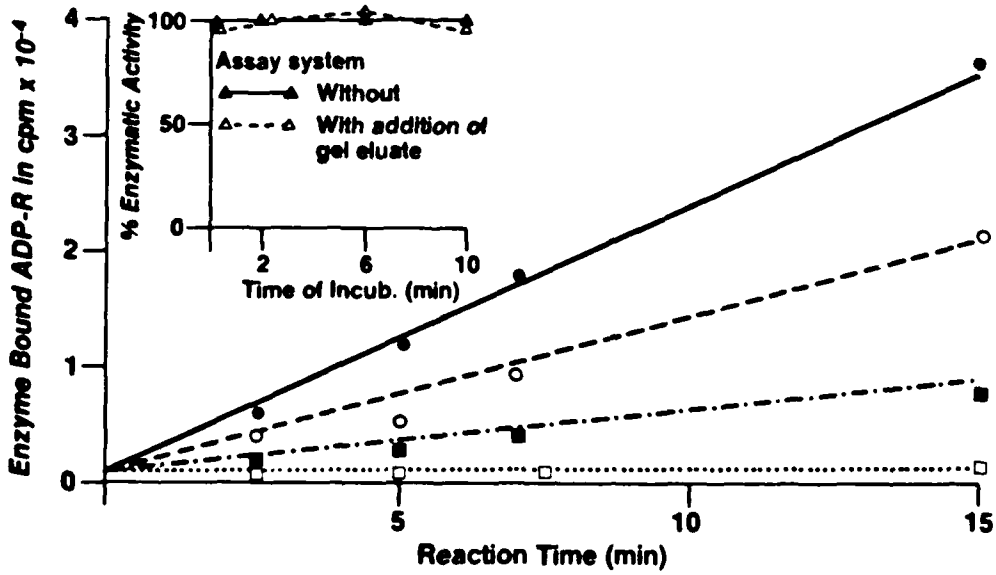

Enzyme Bound ADP-R in cpm x 10⁻⁴
4
3
2
1
0
5
10
15
Reaction Time (min)
% Enzymatic Activity
100
50
0
2
6
10
Time of Incub. (min)
Assay system
Without
With addition of gel eluate

Fig 2

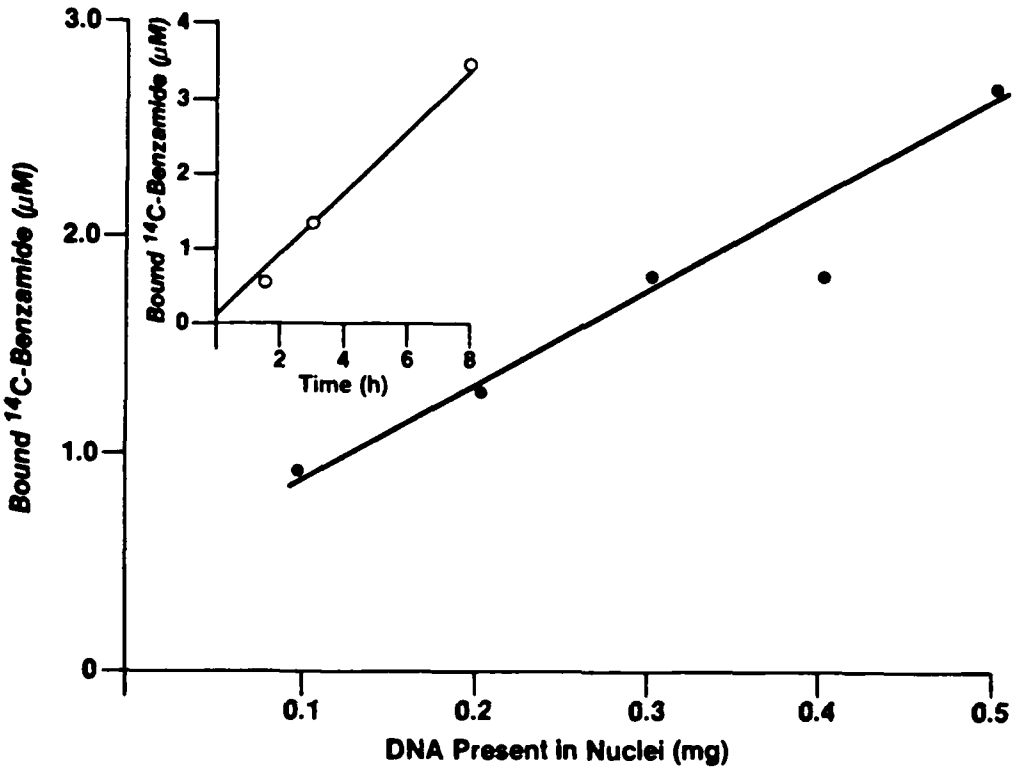
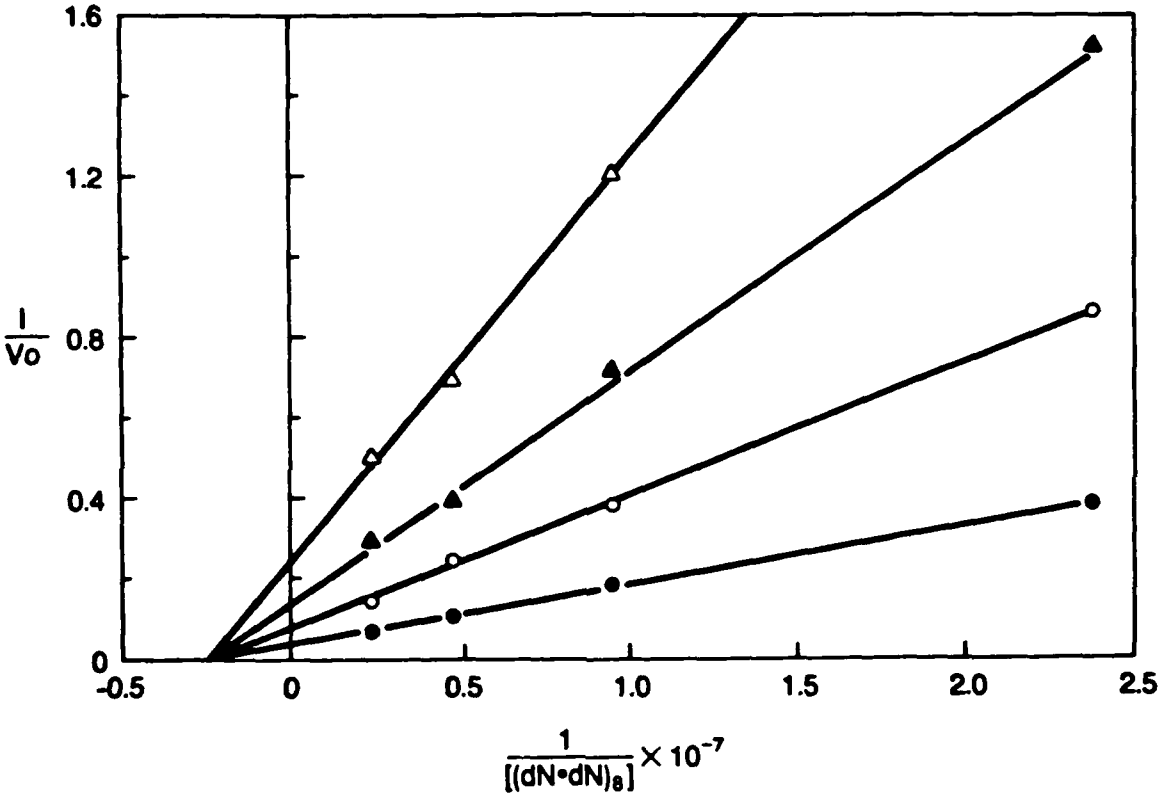
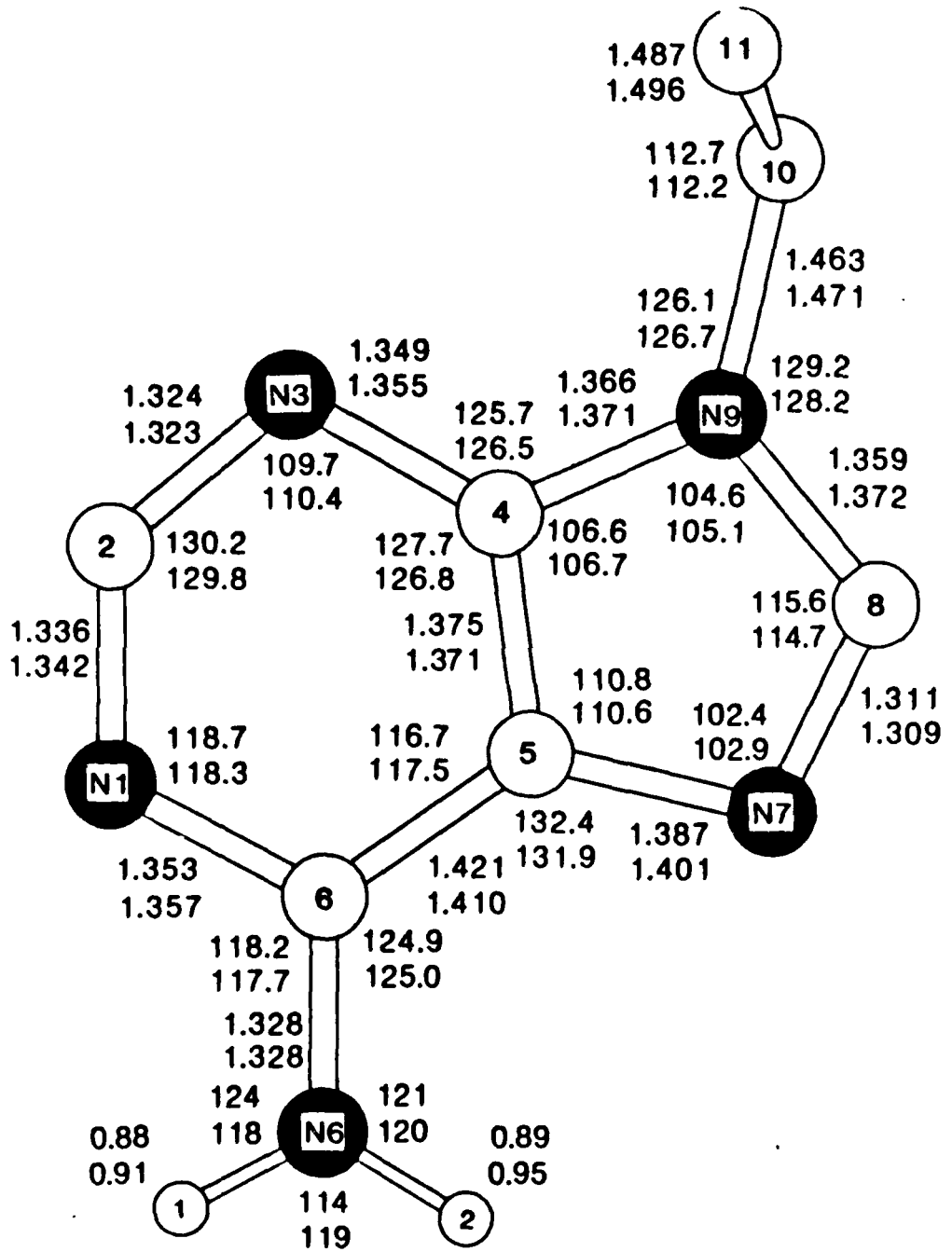
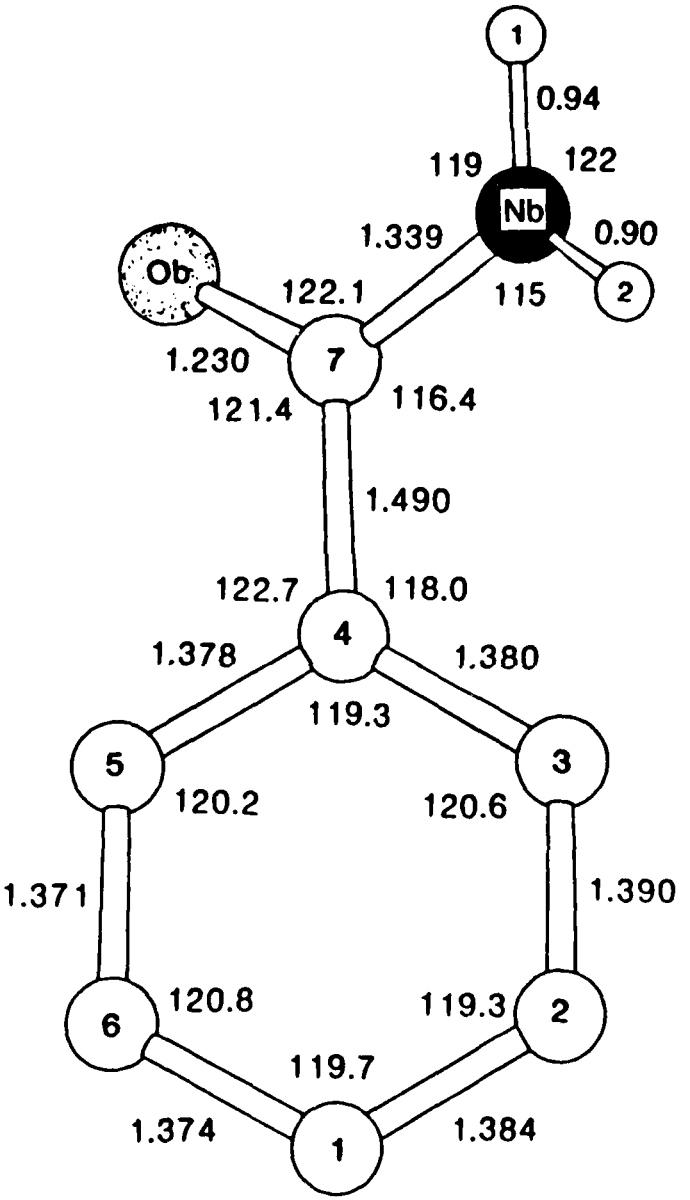

Bound ^{14}C-Benzamide (μM)
3.0
2.0
1.0
0
0.1
0.2
0.3
0.4
0.5
DNA Present in Nuclei (mg)
Bound ^{14}C-Benzamide (μM)
4
3
2
1
0
2
4
6
8
Time (h)

Fig. 3

Fig 4.




1.487
1.496
11
112.7
112.2
10
1.463
1.471
126.1
126.7
1.349
1.355
1.324
1.323
N3
1.366
1.371
125.7
126.5
N9
129.2
128.2
1.359
1.372
109.7
110.4
104.6
105.1
2
130.2
129.8
4
106.6
106.7
127.7
126.8
115.6
114.7
8
1.375
1.371
1.336
1.342
110.8
110.6
102.4
102.9
1.311
1.309
118.7
118.3
116.7
117.5
N1
5
N7
132.4
131.9
1.387
1.401
1.353
1.357
6
1.421
1.410
118.2
117.7
124.9
125.0
1.328
1.328
124
118
121
120
0.88
0.91
N6
0.89
0.95
1
2
114
119


1
0.94
119
122
Nb
1.339
0.90
Ob
122.1
115
2
1.230
7
121.4
116.4
1.490
122.7
118.0
4
1.378
1.380
119.3
5
3
120.2
120.6
1.371
1.390
6
120.8
119.3
2
119.7
1.374
1
1.384

F.25

Fig 6

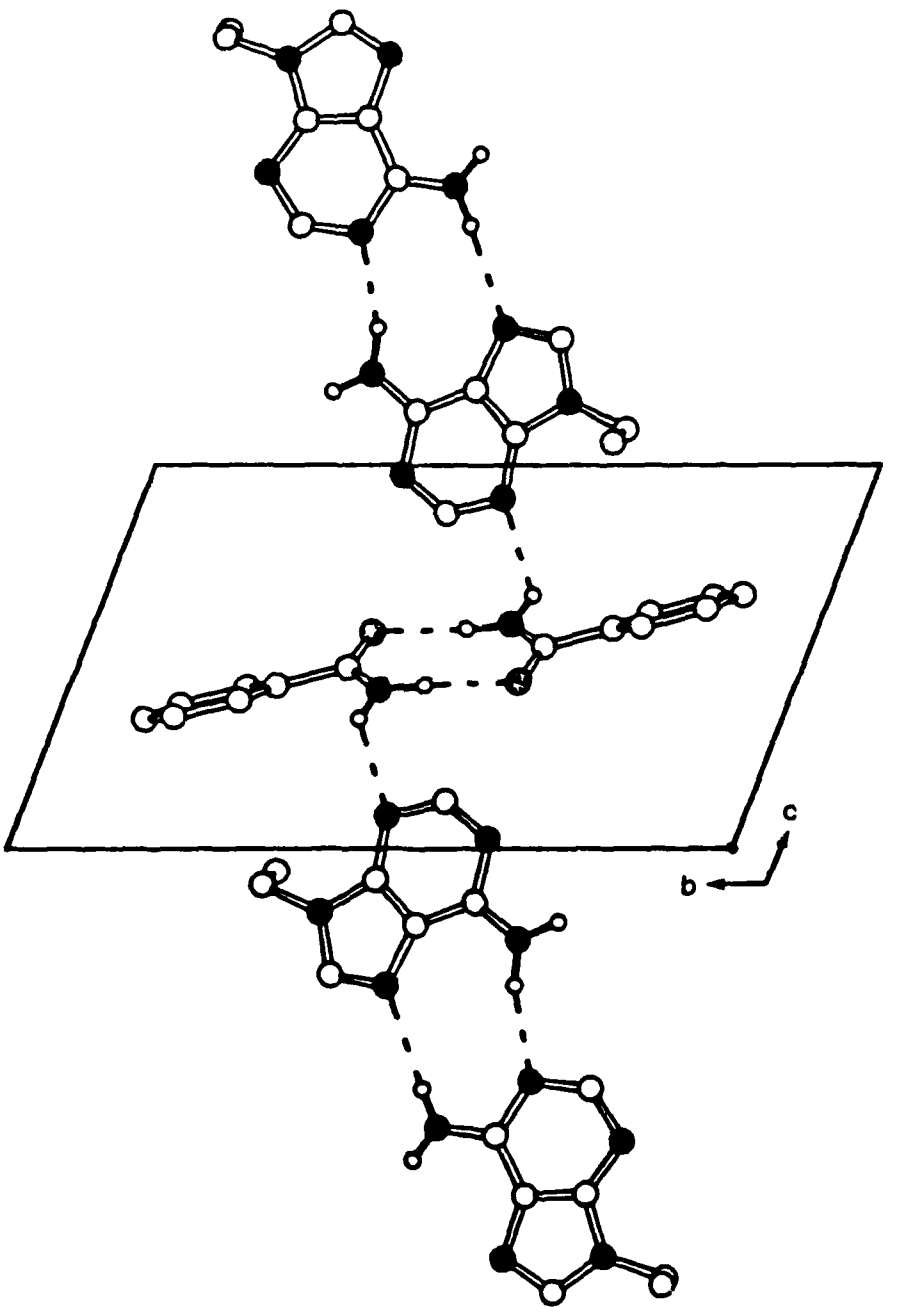


Fig 7a

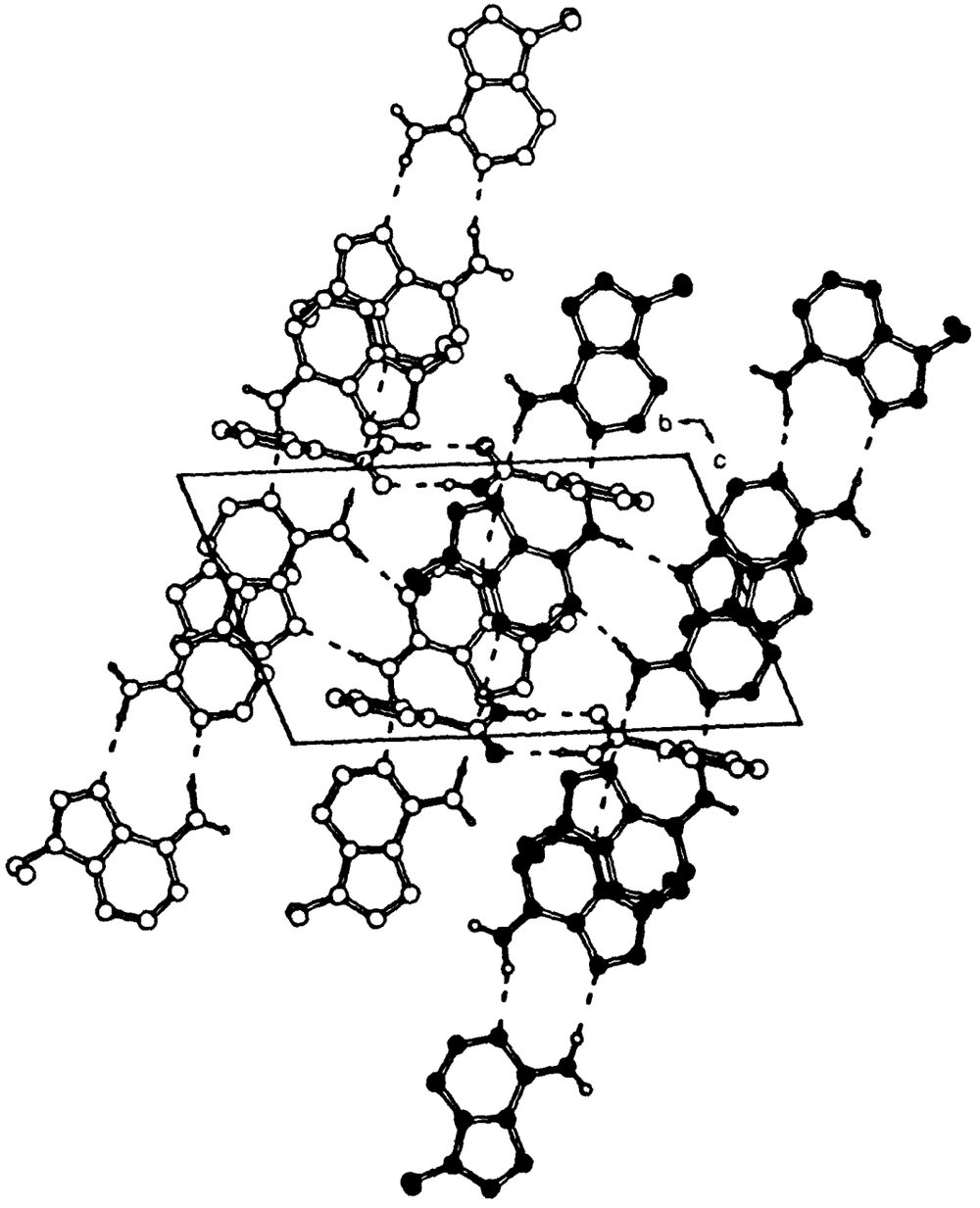
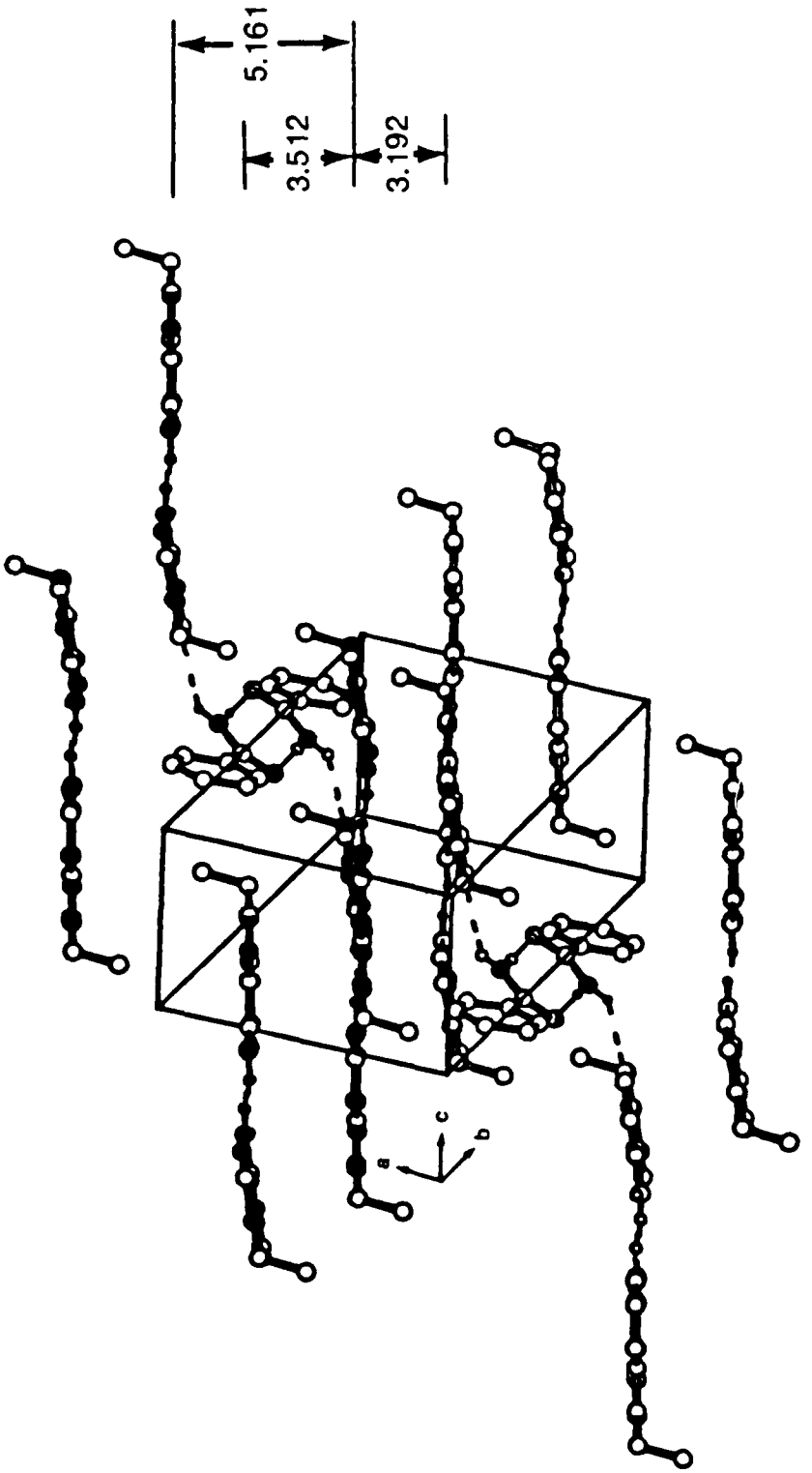


Fig 7b



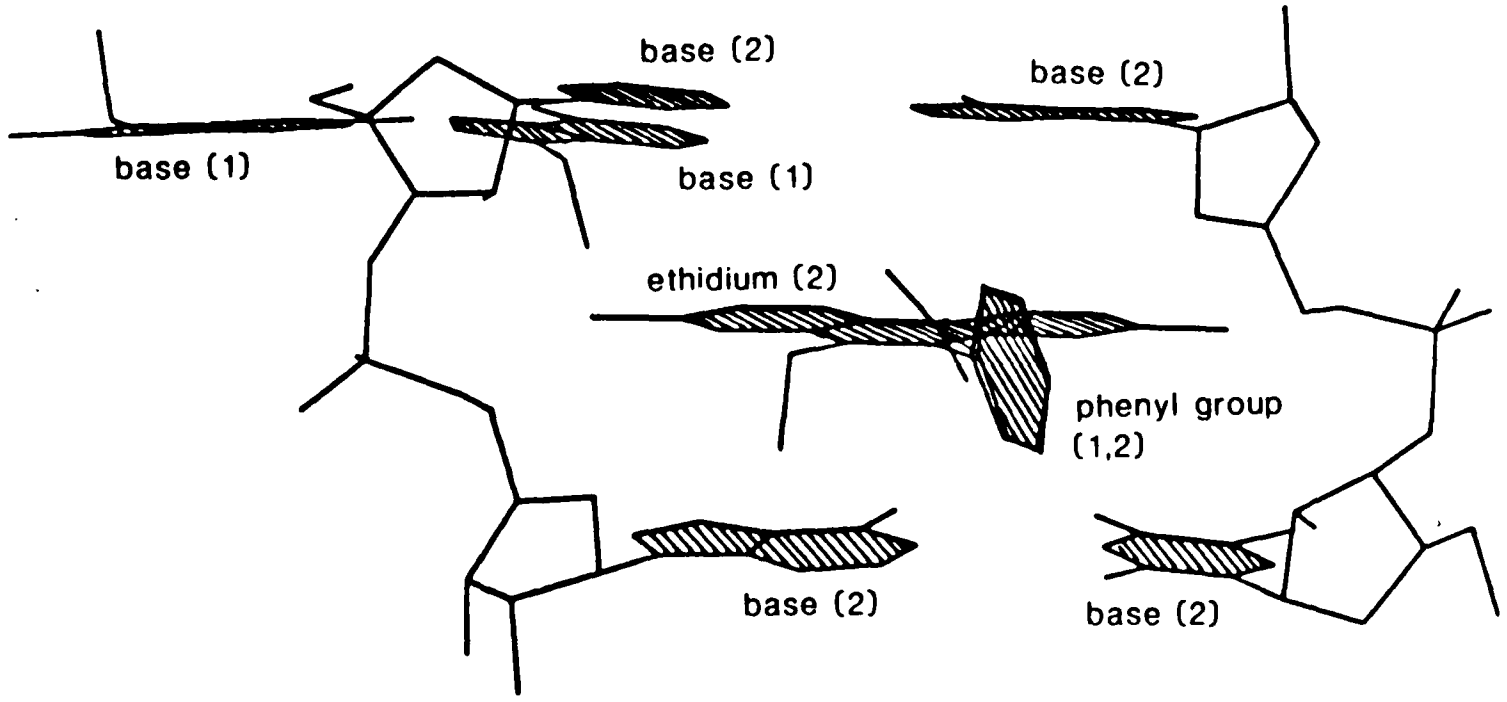

base (2)
base (2)
base (1)
base (1)
ethidium (2)
phenyl group
(1,2)
base (2)
base (2)

TABLE I

Purification scheme of poly(ADP-ribose) polymerase from calf thymus (according to Ref. 6).

Purification Stages	Preparation	Volume ml	Protein mg	Specific* Activity	Total* Activity	Purification	Yield
I	crude extract	800	32540.0	1.3	42300	1.0	100%
II	$(NH_4)_2SO_4$	300	9150.0	4.2	38430	3.2	91%
III	DNA-cellulose	350	129.5	224.0	29008	172.0	68.5%
IV	Hydroxyl apatite	4	8.5	1120.0	9520	861.0	22.5%
V	Sephacryl-S-200	3	4.5	1500.0	6750	1153.0	15.9%

*n mol ADP-R/mg protein x min.

Table II

Correlation between DNA content of liver nuclei, prior and after digestion with DNA-ase I, with the binding of the [^{125}I]-labelled adduct of the Bolton Hunter reagent and o-aminobenzamide.

	Time of incubation with DNA-ase I (min)	DNA content (µg DNA/mg protein)	[^{125}I]-ligand binding (pmol/mg protein)	% decrease in binding
1.	0	345	981	0
2.	10	242	392	60
3.	30	76	0	100

These results are an average of 3 tests, deviating not more than ± 15% of the average values listed.

TABLE III

9-Ethyladenine - Benzamide 2:1 Complex

Final Atomic Coordinates(esd) and Average B(esd)

Atom	x	y	z	Bav
N1	0.5826(2)	0.3433(1)	0.5263(2)	3.90(8)
C2	0.6601(3)	0.4244(2)	0.6248(3)	4.1(1)
N3	0.7096(2)	0.4966(1)	0.5860(2)	3.74(8)
C4	0.6733(2)	0.4796(2)	0.4195(3)	3.55(9)
C5	0.5951(3)	0.4007(2)	0.3032(3)	3.33(9)
C6	0.5486(3)	0.3283(2)	0.3600(3)	3.58(9)
N6	0.4733(2)	0.2473(1)	0.2636(2)	4.36(9)
N7	0.5750(2)	0.4096(1)	0.1445(2)	3.98(8)
C8	0.6430(3)	0.4924(2)	0.1723(3)	4.2(1)
N9	0.7059(2)	0.5387(1)	0.3353(2)	3.84(8)
C10	0.7908(3)	0.6315(2)	0.4067(3)	4.7(1)
C11	0.9463(3)	0.6201(2)	0.4404(4)	5.8(1)
N1'	0.4225(3)	0.1561(1)	-0.1057(2)	4.76(9)
C2'	0.3651(4)	0.0697(2)	-0.1236(3)	5.9(1)
N3'	0.3121(3)	-0.0031(1)	-0.2616(3)	5.4(1)
C4'	0.3216(3)	0.0207(2)	-0.3984(3)	4.0(1)
C5'	0.3788(3)	0.1053(2)	-0.4030(3)	3.67(9)
C6'	0.4319(3)	0.1775(2)	-0.2455(3)	3.73(9)
N6'	0.4887(3)	0.2637(1)	-0.2261(2)	4.59(9)
N7'	0.3724(3)	0.1020(1)	-0.5678(3)	4.62(9)
C8'	0.3120(3)	0.0159(2)	-0.6545(3)	4.7(1)
N9'	0.2777(2)	-0.0371(1)	-0.5619(3)	4.23(9)
C10'	0.2061(4)	-0.1334(2)	-0.6185(4)	5.5(1)
C11'	0.0483(4)	-0.1327(2)	-0.6354(4)	6.5(2)
C1b	0.9508(3)	0.1204(2)	1.1618(4)	5.9(1)
C2b	0.8297(3)	0.1629(2)	1.1259(4)	5.1(1)
C3b	0.8431(3)	0.2431(2)	1.0804(3)	4.4(1)
C4b	0.9757(3)	0.2814(2)	1.0744(3)	3.39(9)
C5b	1.0953(3)	0.2389(2)	1.1126(4)	5.2(1)
C6b	1.0824(3)	0.1588(2)	1.1546(4)	6.4(1)
C7b	0.9829(3)	0.3683(2)	1.0274(3)	3.8(1)
Ob	0.8849(2)	0.3845(1)	0.9359(2)	5.24(8)
Nb	1.1035(3)	0.4254(1)	1.0848(3)	5.16(9)

Bav = 1/3 [trace orthogonalized Bij matrix]

TABLE III (continued)

9-Ethyladenine - Benzamide 2:1 Complex

Atom	x	y	z	Biso
HC2	0.682(2)	0.432(2)	0.748(3)	5.0(6)
H1N6	0.459(3)	0.230(2)	0.144(3)	6.2(7)
H2N6	0.459(2)	0.202(2)	0.308(3)	5.1(6)
HC8	0.654(3)	0.523(2)	0.085(3)	5.2(6)
H1C10	0.757(2)	0.672(2)	0.511(3)	5.0(6)
H2C10	0.770(3)	0.662(2)	0.329(3)	6.4(7)
H1C11	1.002(3)	0.681(2)	0.487(3)	5.3(6)
H2C11	0.959(4)	0.582(2)	0.510(5)	12.(1)
H3C11	0.993(4)	0.588(2)	0.336(5)	11.(1)
HC2'	0.365(3)	0.061(2)	-0.012(3)	5.8(7)
H1N6'	0.523(3)	0.306(2)	-0.124(3)	6.7(7)
H2N6'	0.508(3)	0.282(2)	-0.310(4)	7.4(8)
HC8'	0.293(3)	-0.010(2)	-0.779(3)	6.1(7)
H1C10'	0.236(3)	-0.171(2)	-0.542(3)	6.8(7)
H2C10'	0.238(3)	-0.167(2)	-0.716(3)	5.9(7)
H1C11'	-0.006(3)	-0.193(2)	-0.672(4)	7.9(8)
H2C11'	0.006(3)	-0.094(2)	-0.528(4)	8.3(9)
H3C11'	0.015(3)	-0.092(2)	-0.706(4)	8.4(9)
HC1b	0.935(3)	0.061(2)	1.192(4)	8.4(9)
HC2b	0.733(3)	0.136(2)	1.130(4)	7.4(8)
HC3b	0.758(3)	0.273(2)	1.054(3)	6.4(7)
HC5b	1.192(3)	0.272(2)	1.107(3)	6.1(7)
HC6b	1.169(3)	0.131(2)	1.180(4)	8.5(9)
H1Nb	1.108(2)	0.486(2)	1.073(3)	4.5(6)
H2Nb	1.164(3)	0.412(2)	1.161(4)	8.7(9)

TABLE IV

Hydrogen bonding in the 2:1 complex of 9-ethyladenine with benzamide

D-H...A	D...A (Å)	D-H (Å)	H...A (Å)	D-H...A (°)
N(6)-H(1N6)...N(1')	2.940(3)	0.95(2)	2.01(3)	164(1)
N(6')-H(1N6')...N(7)	3.145(3)	0.89(3)	2.26(3)	171(2)
N(6)-H(2N6)...N($7'^{i}$)	3.178(2)	0.91(2)	2.29(2)	167(2)
N(6')-H(2N6')...N(1^{ii})	2.973(2)	0.88(2)	2.10(2)	169(2)
Nb-H(1Nb)...Ob^{iii})	2.906(2)	0.94(1)	1.97(1)	176(2)
Nb-H(2Nb)...N(3^{iii})	2.971(3)	0.90(3)	2.28(3)	133(2)

Symmetry code: superscript: none x,y,z; (i) x,y,1+z; (ii) x,y,-1+z;

(iii) 2-x,1-y,2-z.

Nb,Ob = N,O of benzamide.

11

Molecular Interactions Between DNA, Poly(ADP-Ribose) Polymerase and Histones

Abstract.

Molecular interactions between purified poly(ADP-ribose) polymerase, whole thymus histones, histone H1, rat fibroblast genomic DNA, and closed circular and linearized SV40 DNA were determined by the nitrocellulose filter binding technique. Binding of the polymerase protein, or histones, to DNA was greatly augmented when both the enzyme protein and histones were present simultaneously. The polymerase protein also associated with histones in the absence of DNA. The cooperative or promoted binding of histones and the enzyme to relaxed covalently closed circular SV40 DNA was greater than the binding to the linearized form. Binding of the polymerase to SV40 DNA fragments, in the presence of increasing concentrations of NaCl, indicated a preferential binding to two restriction fragments as compared to the others. Polymerase binding to covalently closed relaxed SV40 DNA resulted in the induction of superhelicity. The simultaneous influence of the polymerase and histones on DNA topology were more-than-additive. Topological constraints on DNA induced by poly(ADP-ribose) polymerase were abolished by auto ADP-ribosylation of the enzyme. Benzamide, by inhibiting poly ADP-ribosylation, reestablished the effect of the polymerase protein on DNA topology. Polymerase binding to *in vitro*-assembled core particle-like nucleosomes was also demonstrated.

Chromatin structure plays an important role in the control of gene expression in eukaryotic cells (cf. 1, 2, 3). A variety of interactions between double-stranded DNA and various nuclear proteins may be involved in gene regulation (cf. 4, 5, 6). Protein-DNA associations can influence at least two types of related phenomena. First, the availability of certain DNA sequences for transcription may depend on proteins binding to DNA. Examples include the nucleosome octamer (7,8) and histone H1-DNA interactions (9) which have been proposed to repress or curtail transcription. The second type of DNA-protein associations may result in conformational alterations of DNA itself, as documented in prokaryotes for the *CAP-* lac promoter (10, 11), the *cro*-operator (12, 13), and EcoRI endonuclease-recognition sequence interactions (14), and in eukaryotes for nucleosome-DNA (15), RNA polymerase III transcription factor-tRNA ^{leu} gene (16), and a heat shock transcription factor-promoter DNA binding (17).

Our interest in this subject arose as a consequence of studies concerned with the biological function of the poly(ADP-ribose) polymerase enzyme molecule (E. C. 2.4.2.30), a DNA-binding nuclear protein (cf. 18, 19, 20) which has been implicated in the control of a variety of cellular functions such as differentiation, toxic damage to DNA (18, 19), aging (21), hormonal effects (22), and tumorigenic growth (23, 24). The catalytic activity of poly(ADP-ribose) polymerase enzyme has an absolute requirement for DNA (18, 19). Besides the polymerase protein itself, histones can also serve as ADP-ribose acceptors (18). The present paper deals with the molecular association of poly(ADP-ribose) polymerase with histones and DNA.

and with coinciding topological changes in DNA. An analysis of macromolecular binding processes is a prerequisite to an interpretation of enzymatic mechanisms of poly ADP-ribosylation (25) in terms of cell physiology.

MATERIALS AND METHODS

Proteins and DNA:

Poly(ADP-ribose) polymerase was isolated from calf thymus by a novel procedure (26) and also by a published method (27). The protein was at least 95 percent homogeneous, containing only traces of small peptide degradation products of the enzyme protein, as determined by NaDod SO_4-PAGE (28) and immunoblotting, and had a specific activity of 1000 to 1200 nmol ADP-ribose mg^{-1} min^{-1} as tested in a standard assay (25). Calf thymus whole histones were purchased from Boehringer-Mannheim (Indianapolis, IN). Histone H1, chicken core histones, and HMGs (1 + 2) were a gift from Dr. David Cole (University of California, Berkeley, CA). *E. coli* **recA** protein was from USB Corp. (Cleveland, OH). [I^{125}] labeling of proteins was performed with iodo-beads as recommended by the manufacturer (Pierce Chemical Co., Rockford, IL). The molecular masses of labeled proteins (10^3-10^5 cpm/μg) was verified by NaDod SO_4-PAGE (28) and autoradiography. Isolation of DNA of high molecular mass was carried out by a standard method (29) from confluent cultures of 14C cells (10^8 cells), a cell line derived from rat-1 fibroblasts (24). SV40 DNA was purchased from BRL (Gaithersburg, MD). The high molecular weight 14C DNA was partially digested with MboI (New England Biolabs, Beverly, MA)

at 0.1 units/μg DNA for 30 minutes at 37^{0}C to give DNA fragments ranging between 0.5 kb and 15 kb as determined by agarose gel electrophoresis (29). SV40 DNA (Form I) was linearized with EcoRI (New England Biolabs) and labeled at the 5'-ends with γ-[^{32}P] ATP and T4 polynucleotide kinase (Pharmacia, Piscataway, NJ) (30). Aliquots of this end-labeled DNA were ligated with T4 DNA ligase (New England Biolabs) at low DNA concentrations (50 μg/ml) (29) to minimize the formation of multimers and linear concatemers (Fig. 3B inset).

Nitrocellulose filter binding assay:

The association of proteins with DNA in solution took place within 1 minute, therefore the filter binding technique as applied here determines end points. The macromolecular complexes formed in solution were stable for at least two hours and a 2 to 5 fold dilution did not dissociate them. It was determined in preliminary experiments that the largest quantities of proteins or DNA employed in these assays, either alone or in combination, did not saturate the membrane filter, a criterion strictly followed in all binding tests. Based on these considerations, the following technique was employed: varying quantities (see Results) of [^{32}P] DNA (in 1-5 μl) were added to 0.2 ml of the ice cold binding buffer (31) (25 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 M NaCl) and mildly agitated at 25^{0}C for 1 minute. Binding of proteins to DNA was assayed by adding aliquots of the proteins (5-10 μl) to the DNA solution, followed by incubation for 10 minutes at 25^{0}C. Samples were transferred onto ice, followed by filtration through nitrocellulose filters (BA 85, Schleicher & Schuell,

0.47 μm pore size, 27 mm diameter), presoaked in the binding buffer for 30-60 minutes. Each filter was washed 4 times under suction with 2.5 ml of ice cold binding buffer containing 5% DMSO, which considerably reduced the background counts. The amount of radioactive material bound to the filters was determined by scintillation spectrometry following drying of filters. When the association of [^{125}I] labeled proteins with unlabeled DNA or proteins was determined, the filters were washed 10x with 2.5 ml of the binding buffer to minimize nonspecific associations. All tests were carried out in duplicates. Typically, < 5% of input [^{32}P] DNA (cpm) was bound to filters in the absence of protein and ≡ 25% of input protein (cpm) was retained in the absence of DNA.

Nucleosome reconstitution *in vitro*:

A 209 bp EcoRI-PstI fragment of SV40 DNA (position 1783-1992 (32) of the SV40 sequence), [^{32}P] labeled at the 3' end of the EcoRI site with Klenow enzyme (Boehringer-Mannheim) and α-[^{32}P]-dATP, was purified from 5% polyacrylamide gels. A single nucleosome was assembled onto this fragment following the salt-step procedure of Stien (33). Briefly, to monitor the formation of core particle-like nucleosomes varying amounts (see legend to Fig 6A) of core histones were mixed with the labeled fragment in 100 μl of 10 mM Tris-HCl pH 8.0, 0.2 mM EDTA, 0.8 M NaCl, at room temperature. The salt concentration was gradually lowered in steps at 2 h intervals from 0.8 M to 0.2 M (from 0.8 M to 0.6 M, to 0.4 M, to 0.2 M). This was accomplished by the addition of appropriate aliquots of 10 mM Tris-HCl pH 8.0 + 0.2 mM EDTA (TE) to the initial mixture. The samples

(≅ 250 µl each) were dialyzed overnight against the TE buffer at 4^0C. If carried out at histone : DNA mass ratios ≅ 1.0 this procedure was found to yield nucleosomes (see Results).

Sedimentation analysis:

Nucleosomes, DNA, poly(ADP-ribose) polymerase, and polymerase-nucleosome complexes were analyzed on 5-20% sucrose gradients (≅ 4.0 ml) containing 10 mM Tris-HCl pH 8.0 + 10 mM $MgCl_2$ + 0.1 mM EDTA + 1 mM PMSF + 0.1 M NaCl. Centrifugation was performed in a Beckman L5-75B preparative ultracentrifuge using an SW60 rotor at 45,000 RPM for 14 h (4^0C). Fractions containing 6 drops (≅ 150 µl) were collected by puncturing the bottom of the gradient tubes (4.5 ml capacity, cellulose nitrate). The fractions were assayed for [^{32}P] (beta) and [^{125}I] (gamma) radioactivity by direct (without scintillation fluid) spectrometry. Sedimentation values were obtained using the following markers (34) calf intestinal phosphatase (6.3 **S**); *E. coli* tRNA (5 **S**); *E. coli* rRNA (16.7 **S**); calf liver catalase (11.2 **S**).

RESULTS

Promotion of binding of poly(ADP-ribose) polymerase to DNA by histones:

Initially, the effects of whole thymus histones were determined because we could not predict if any of the histone subfractions might exhibit selectivity in terms of their interaction with DNA and the polymerase enzyme. The poly(ADP-ribose) polymerase is not known to

display, in terms of DNA binding, any DNA sequence specificity, although enzymatic studies reveal that DNA molecules of differing sequence appear to stimulate enzymatic activity to various degrees (35, unpublished results). Therefore, rather than experimenting with specific DNA sequences as substrates for DNA binding, we first employed genomic DNA as binding ligand to provide broad-based DNA sequences.

Figure 1A (lower curves) shows that addition of increasing amounts of poly(ADP-ribose) polymerase (▲) or histones (●) to a constant concentration of [^{32}P] labeled 14C DNA causes increased retention of [^{32}P] DNA on nitrocellulose filters. Similarly, if increasing amounts of [^{32}P] labeled DNA is added to a constant amount of protein, a proportional increase in retention of [^{32}P] label on filters occurs (Fig. 1B). This indicates quantitative binding of both the polymerase and histones to restricted 14C DNA. When the concentration of either histones or the polymerase protein is kept constant, and the concentration of the second protein (either histones or polymerase) progressively increased, a large augmentation of DNA binding of histones or the polymerase occurs, indicating that a relatively low concentration of either one of the two proteins significantly promotes the binding of the second protein (Fig. 1A, upper curves). The experimental points in the upper curves of Figure 1A have been obtained by subtracting the percent radioactive material contributed by the protein present in constant amounts from the total amount of radioactivity retained on the filter as a result of the binding of both proteins to DNA. This method of presentation permits a direct

comparison, yielding the lower and upper curves in Figure 1. Since the nitrocellulose binding technique does not permit the exact determination of the DNA-protein complexes, nor the identification of the nature of the interactions between individual macromolecular components of the reaction mixture (36, 37, 38), instead of cooperativity, it may be more correct to propose a mutual promotion of DNA binding between polymerase and histones. As observed by others (39), binding of histones to DNA at mass ratios of histone : DNA > 1.0 seems to decrease the extent of retention of [^{32}P] label on the filters (Fig. 1A, ●). Such an effect is not observed with either the polymerase alone (Fig. 1A, ▲) or when larger amounts of the polymerase is added with a small amount of histones (Fig. 1A, ○).

The specificity of histones in promoting the binding of poly(ADP-ribose) polymerase to DNA:

The specificity of histones in the promotion of the binding of the polymerase to DNA was tested by replacing histones with a nonspecific protein like BSA or by the DNA-binding high mobility group (HMG 1+2) proteins (40) or by the **recA** protein (41, 42) of *E. coli*. Figure 1C shows that the polymerase-enhanced filter binding of [^{32}P] DNA is not observed when BSA is employed in conjunction with the polymerase protein. BSA by itself does not bind to the DNA but it binds directly to the polymerase protein as deduced from experiments employing [^{125}I] labeled BSA (not shown). This direct binding to the polymerase apparently involves the blocking of DNA binding sites on the polymerase, hence the inhibition of the binding of the polymerase to DNA in the presence of BSA. Since BSA is not a physiologically

occurring nuclear protein we did not pursue this problem further. HMG (1 + 2) proteins, when present between 0.05-0.15 μg per test, resulted in the retention of 11-36% of input DNA. Varying concentrations of poly(ADP-ribose) polymerase (0.04 to 0.15 μg per test) added simultaneously with a small amount (0.04 μg) of HMGs affected DNA retention only slightly (10 to 15% retention). This is in contrast to a 75 to 85% increase in DNA retention observed upon addition of histones (Fig. 1). Similar results were obtained with varying quantities of **recA** protein (data not shown). Polyarginine (M_r 60,000) or polylysine (M_r 50,000) at a mass ratio of 0.5 (polypeptide : 14C DNA) did not simulate the effect of histones. At higher mass ratios (> 0.5), these polypeptides inhibited the binding of the enzyme to DNA. Polyarginine and polylysine do not appreciably bind to DNA (43). These results are consistent with an apparent specificity of histones in promoting the binding of poly(ADP-ribose) polymerase to DNA.

Significance of the order of addition of macromolecular components: As illustrated in Figure 1D, the histone-promoted binding of poly(ADP-ribose) polymerase to DNA depends on the order of addition of macromolecular components. Sequential addition of DNA + histones + enzyme, or DNA + enzyme + histones, resulted in promoted binding. On the other hand, the sequence: enzyme + histone + DNA, or histones + enzymes + DNA, failed to exhibit promotional binding kinetics.

Association of histones and the enzyme protein:

Since the apparent promotion of binding of the enzyme to DNA required histones as a third macromolecular component, it was of interest to explore a direct association between the enzyme and histones in the absence of DNA. Figure 2A illustrates the association of varying concentrations of histones with a fixed concentration of poly(ADP-ribose) polymerase (O), and the binding of increasing concentrations of polymerase to a fixed concentration of histones (Δ). At histones : polymerase mass ratios of < 1.0 the retention of increasing amounts of [^{125}I] histones is observed, but at histones : polymerase mass ratios approaching 2.0, a lesser amount of the labeled polymerase is retained on the filters (Fig. 2A, O). On the other hand, when increasing amounts of [^{125}I] polymerase is added to unlabeled histones, larger amounts of [^{125}I] polymerase is retained on the filters, even at mass ratios > 1.0. The addition of unlabeled 14C DNA to the reaction mixture results in enhanced retention of the [^{125}I] polymerase on the filters (Fig. 2B).

To rule out the possibility that the increased retention of labeled DNA could be due to nonspecific clogging of the filters by the proteins (polymerase + histones; Fig. 1A), since both proteins can, by themselves, be retained on the filters (Fig. 2A, B), the following experiment was performed. Polymerase and histones, separately or together, were added to the binding buffer (as in Fig. 2A). This solution was first added to the filters without washing. A solution of [^{32}P] DNA was then added to these filters and the filters were washed (see Materials and Methods). It was observed that < 10% of the input

[^{32}P] DNA was retained on the filters, a value which was approximately equal to the background level of [^{32}P] DNA retained in the absence of the proteins. This shows a) that the association of DNA with proteins occurs only in solution, and b) that the proteins do not saturate the filters so as to prevent the DNA from passing through the filters.

Substitution of whole histones by H1:

Figure 2C shows the binding of the polymerase to H1 in the absence of DNA. In the presence of DNA, H1 and polymerase yielded results which were essentially indistinguishable from those obtained when whole histones were employed (compare Fig. 2D with Fig. 1A).

The influence of DNA topology:

The binding of the polymerase protein and histones was determined with two forms of SV40 DNA. First, SV40 DNA (Form I) was linearized with EcoRI and labeled at the 5' ends. The second type of DNA ligand (Fig. 3 inset) consisted of religated linear SV40 DNA. Under comparable conditions, more polymerase enzyme protein was bound to circular (O) than to linear (●) DNA (Fig. 3). A fixed concentration of whole thymus histones (0.04 μg) promoted the association of the polymerase protein to SV40 DNA, in a manner similar to the enzyme binding to genomic DNA (compare Fig. 1A and 3). The apparent differences between the shape of the isotherms observed with genomic (Fig. 1A) and SV40 DNAs (Fig. 3) may reflect sequence and size differences in the DNAs which could influence protein binding. Since the DNA-background counts of the two forms of DNA were

nearly identical, artefacts due to a preferential binding of circular over linear DNA to nitrocellulose membranes are unlikely. It has been reported that poly(ADP-ribose) polymerase also binds to supercoiled ColE1 (44) and pBR322 DNAs (personal communication, Dr. Gilbert de Murcia, Univ. of Strasbourg, France).

Binding of the polymerase to SV40 DNA restriction fragments as a function of the ionic strength:

It is generally known that protein-DNA interactions are sensitive to the ionic strength of the binding medium. Therefore we tested the effect of increasing concentrations of NaCl on the binding of the polymerase to various restriction fragments of SV40 DNA. SV40 DNA was digested with MboI and the fragments were [^{32}P] labeled at their 5' ends. Filter binding assays were performed at a constant polymerase : DNA mass ratio but with increasing concentrations of NaCl in the binding and washing buffers. Filter-bound DNA was eluted and the DNA fragments separated on a polyacrylamide gel. Figure 4 shows that all the restriction fragments were retained on the filters, indicating binding of the polymerase to these fragments even at a relatively high NaCl concentration (0.6 M; lane 12). Maximal binding to all the fragments occurred at ≅ 0.02 M NaCl (lane 3). The strength, or the amount, of polymerase bound to the fragments decreased as the salt concentration was raised (Fig. 4). Only a 50-60% reduction in the strength (or amount) of polymerase binding to the DNA fragments occurred even as the NaCl concentration was raised from 0.02 M to 0.4 M. Variation in the amount of DNA retained on the filters was not an effect of the salt concentration on the filters since control

experiments demonstrated that very little of the input radioactivity was retained in the absence of the protein (lanes 6 and 11, Fig. 4), irrespective of the concentration of NaCl. Densitometry revealed that the polymerase exhibited 3-4 fold preferential binding to the 610 bp fragment (position 4100-4710 bp on the SV40 sequence), followed by the 237 bp fragment (2534-2771 bp), at all NaCl concentrations tested. It is possible that the preferential binding of the polymerase to the 610 bp and 237 bp fragments is a reflection of the sequence or conformational specificity (or both). The sequence (32) of the 610 bp and the 237 bp fragments reveals several contiguous 6-16 bp A + T stretches and the fragments have an overall A + T richness of 50-66%. A + T rich regions are known to bind proteins (45), occur in regulatory contexts (46), and have been implicated in unusual DNA conformations (47, 48). Competition binding and DNase I footprinting experiments with specific DNA fragments are underway, to study the sequence specificity, if any, of the polymerase to DNA.

Torsional constraints induced by the polymerase on relaxed DNA:
It is known that the binding of histones, especially of H3 and H4, to relaxed covalently closed circular DNA molecules induces superhelicity (39, 49, 50), and it has been proposed that DNA wraps around the polymerase protein (51). Therefore, we determined topological changes in DNA following the binding of the enzyme protein and histones alone and in combination, to relaxed covalently closed circular SV40 DNA. SV40 DNA (FormI, supercoiled duplex) was converted to relaxed covalently closed circular DNA (FormIr) by

treatment with topoI. The DNA was then deproteinized. Proteins to be tested (polymerase and histones) were mixed with FormIr DNA and the reaction mixture was incubated at 25^{0}C for 1.5 h. TopoI was added to relax any DNA twists that were not due to protein binding, and the DNA was deproteinized and run on an agarose gel (73). The addition of increasing amounts of enzyme protein, to a constant concentration of DNA, resulted in increased superhelical twisting of relaxed SV40 DNA (lanes 3-6, Fig. 5A). The number of superhelical turns induced by the polymerase appeared to plateau as the enzyme : DNA mass ratio approached 1.0, and raising the mass ratio further produced no significant increase (compare lanes 5 and 6, Fig. 5A). Histones, when present at a histone : DNA mass ratio of 0.5, induced the same number of superhelical turns as poly(ADP-ribose) polymerase at an enzyme : DNA mass ratio of 1.2 (compare lanes 6 and 7, Fig. 5A), suggesting a greater effectivity of histones as compared to the polymerase protein. The sequential addition of first histones, followed by the enzyme protein, to DNA exceeded the additive effect of histones or the enzyme protein alone (compare lanes 6 and 7 with lanes 8 and 9, Fig. 5A). This more-than-additive effect is reminiscent of the mutually promoting influence of histones and the polymerase on their binding to DNA (Fig. 1-3). For example, at an enzyme : DNA mass ratio of 0.8, the number of superhelical turns induced was about 10, whereas at an [enzyme + histone] : DNA mass ratio of 0.6, the number of superhelical turns induced increased to 15 (compare lanes 5 and 9, Fig. 5A). From these data it was calculated (52, 53) that, in the presence of topoisomerase I (nicking and closing ezyme) and one mol of

polymerase protein (mol. mass 120,000) per mol SV40 DNA (mol. mass 3.2×10^6, 5243 bp), a change in the linking number of 0.5 occurred. Since the linking number is always an integer, it is estimated that a change of one unit in linking number corresponds to the binding of two mols of enzyme in the presence of the nicking and closing activity of topoisomerase I. Resolution of the topoisomers on ethidium bromide-containing agarose gels indicated that the superhelical turns had a negative sign (52).

The above results were obtained with an enzyme protein that was not exposed to NAD^+. Incubation of the enzyme with NAD^+ in the presence of DNA results in the automodification of the enzyme by covalently bound ADP-ribose oligomers (18, 19) and a concomitant decrease in the binding of the enzyme to DNA (31, unpublished results). Incubation of the polymerase protein with histones and relaxed SV40 DNA in the presence of NAD^+,which results in poly ADP-ribosylation of both proteins, prevented DNA supercoiling in the test system (Fig 5B, lane 4). Benzamide, a specific inhibitor of poly (ADP-ribose) polymerase (54) abolished the inhibitory effect of poly ADP-ribosylation on DNA supercoiling in the above system and reestablished the topological change in DNA (lane 5, Fig5B). In the reaction containing no histones, only the enzyme protein, SV40 DNA and NAD^+ (lane9, Fig 5B) benzamide also prevented the inhibitory effect on DNA supercoiling (lane 10, Fig5B). On the other hand, histone-induced supercoiling of SV40 DNA (lane7, Fig5B) was not affected by either NAD^+ (lane 14, Fig5B) or benzamide (lane15, Fig5B) or both (not shown). The activity of topo I was not influenced by NAD^+ and benzamide (lane11, Fig 5B). These results show that the

enzymatic activity of the polymerase protein regulates DNA topology in system containing histones, poly(ADP-ribose) polymerase, NAD^+, and topo I. The effect of NAD^+ and benzamide were also tested employing the filter binding assay and the results indicated that there was a substantial decrease in the retention of [^{32}P] labeled SV40 DNA under the conditions of auto-ADP-ribosylation of the enzyme. Benzamide restored the retention of DNA onto the filters when included in the binding mixture (data not shown).

Binding of the polymerase to *in vitro*-assembled core particle-like nucleosomes:

In the preceding experiments, the binding of the polymerase to DNA in the presence of whole histones or H1 was determined. Under these conditions histones are presumed to form dimers or tetramers (55, 56) but in core particles and chromatin, histones exist as octamers (3). Chromatin isolated from nuclei may contain tightly bound poly(ADP-ribose) polymerase (18). Therefore it was considered important to determine if the polymerase protein would bind to nucleosome-like particles *in vitro*. Recent experiments have convincingly demonstrated that nucleosomes can be assembled *in vitro* onto restriction fragments of SV40 DNA (33, 57, 58, 59).

A core particle-like structure was assembled *in vitro* onto a 209 bp EcoRI-PstI fragment of SV40 DNA and these particles were employed as ligands for polymerase binding. Two recent studies have demonstrated that this fragment lies within a region of SV40 DNA where a unique array of stable nucleosomes can be reconstituted

(33, 59); the EcoRI-PstI fragment is just sufficient in length to accomodate only one octamer of histones. The formation of an octamer of histones onto this fragment was verified by two methods.
1. Gel electrophoresis of histone-DNA complexes at histone : DNA mass ratios approaching unity revealed a distinctly retarded (as compared to the naked DNA) band (Fig. 6A). This band migrates at approximately 390 bp, as observed for core particles (60).
2. The sedimentation coefficient (≅ 11 **S**) of these particles, which sedimented near the middle (Fig. 6C) of the sucrose gradients, closely agrees with the reported value for native core particles (10.5-11 **S**; 61, 62). These criteria show that the reconstituted core particle-like nucleosome resembled native core particles.

In the absence of histones, the binding of increasing amounts of poly(ADP-ribose) polymerase to the [^{32}P] labeled 209 bp SV40 DNA fragment, results in a concomitant reduction in the electrophoretic mobility of the [^{32}P] DNA fragment (Fig. 6B). At low polymerase : DNA mass ratios (< 1.4) three different retarded (as compared to free DNA) bands appear (Fig. 6B). At mass ratios (polymerase : DNA) of 1.4 and 1.8 only one retarded band and the complete absence of any free DNA is observed, indicating the binding of saturating amounts of the polymerase to the DNA. It is estimated that 2 or 3 polymerase molecules could be bound per DNA molecule at saturation, assuming full binding activity for all the macromolecules and the following molecular masses: polymerase = 120,000; 209 bp DNA = 138,000. The binding of [^{125}I] polymerase to the 209 bp [^{32}P] labeled fragment was also assayed by sucrose density gradient centrifugation. When the

mass ratio of [^{125}I] polymerase : 209 bp [^{32}P] DNA fragment was 0.1-1.8, and conditions comparable to the electrophoretic assay were employed for binding, the positions of the peaks containing the [^{125}I] label and the [^{32}P] label in the density gradient corresponded to the number of bands in the gel assay (not shown). At a polymerase : 209 bp fragment mass ratio of 1.8 only one peak containing the [^{125}I] label (representing the polymerase) coinciding with a peak of [^{32}P] label (representing the DNA fragment) was observed. This [^{125}I] peak had an approximate sedimentation coefficient of 12.5 **S** (not shown).

The addition of [^{125}I] labeled polymerase to the [^{32}P] labeled core particle-like nucleosomes results in a pronounced change in the sedimentation behavior of most of the [^{32}P] label from 11 **S** (core particle-like nucleosome), to a faster sedimenting species at 14.3 **S** (Fig. 6D). This peak of faster sedimenting [^{32}P] label also coincides with a peak of [^{125}I] label, indicating binding of the polymerase to the core particle-like nucleosomes. Some of the [^{32}P] label appears as polymerase-free nucleosome-like particles. Approximately 50% of the input [^{125}I] (polymerase) is bound to the [^{32}P] core particle-like nucleosomes, as indicated by the distribution of the [^{125}I] label (Fig. 6D). The [^{125}I] polymerase sediments at 4.8 **S** in agreement with a previous estimate (31). Assuming the mol. mass of a histone octamer to be 109,000, the approximate stoichiometry of [^{32}P] nucleosomes to [^{125}I] polymerase is 1:7, indicating considerable affinity of the polymerase to the *in vitro*-assembled nucleosomes.

Discussion:

The majority of experimental work concerned with the cellular function of poly ADP-ribosylation of nuclear proteins has been focused on enzymatic rates that are correlated with biological responses (18, 19). A notable exception is the proposed correlation between chromatin structure and poly ADP-ribosylation (63). We demonstrate here that the binding of the polymerase protein to DNA is specifically enhanced by histones (Fig. 1, 2, 3). Potential artefacts due to saturation of nitrocellulose membranes with macromolecules was ruled out by performing binding well below saturation. When histones were substituted by HMGs (1 +2), the **recA** protein of *E. coli*, or polyamino acids, no promotion of the binding of the enzyme to DNA occurred. When histones were substituted by BSA there was an inhibition of polymerase binding to DNA as discussed in Results (Fig. 1C).

It is possible that the torsional constraints imposed on DNA by the binding of histones can create a specific DNA conformation which is required for maximal polymerase binding and/or enzymatic activation (or both). Torsional constraints can be induced on both circular and linear molecules. In the case of poly(ADP-ribose) polymerase, experimental evidence (Fig. 3) indicates that this constraint may be larger with circular DNA, as compared to linear DNA hence larger promotional binding to circular DNA. It has been suggested (64) that torsional constraints can be induced on linear DNA molecules, presumably between two protein binding sites. Thus, the promoted binding of histones + enzyme protein to genomic DNA (Fig. 1 and 2) could also be explained on this basis. As shown in Figure 1D, the order

of addition of macromolecules is critical to obtain promotional association with DNA. The results show that polymerase-DNA or histone-DNA complexes must be formed for subsequent promotional binding with the second protein component (either histones or polymerase). This behavior is compatible with the following scheme. Promotional binding to DNA is present in (1) and (2) but not in (3). As described in Results, binding of one protein (polymerase or histones) to DNA induces a topological change in the DNA which apparently preceeds the promotional binding of the second protein according to this scheme.

1. $E + DNA \Rightarrow (E^* DNA) + H \Rightarrow E^*DNA^* H$
2. $H + DNA \Rightarrow (H^* DNA) + E \Rightarrow H^*DNA^* E$
3. $E + H \Rightarrow (E\text{-}H) + DNA \Rightarrow (E\text{-}H) \cdots DNA$

* indicates promotional (more than additive) binding properties

(E-H) = enzyme-histone complex (no promotional binding).

Although we have not directly demonstrated *in vivo* if any of the phenomena described in this paper are important for cell physiology, the promotion of polymerase binding to DNA by histones and the accompanying changes in DNA topology could be of biological significance. Since histones are much more abundant than the enzyme in the cell, any changes in DNA topology due to the binding of the enzyme might be confined to relatively few DNA regions. The requirement for superhelicity in transcription in eukaryotes has been demonstrated in some instances (65, 66, 67, 68, 69) even though this subject remains controversial (70, 71, 72). Polymerase binding to DNA or nucleosomes could conceivably have a localized effect on the topology of DNA and thus may indirectly influence gene expression.

Figure Legends

Figure 1A: Promotion of the binding of poly(ADP-ribose) polymerase to restricted [^{32}P] labeled 14C DNA by histones.
(▲) varying concentration of enzyme; (●) varying concentration of histones; (O) varying concentration of enzyme with a constant (0.04 μg) concentration of histones; (Δ) varying concentrations of histones with a constant (0.04 μg) concentration of the enzyme. Each assay contained 0.1 μg of DNA (10^5 Cerenkov cpm). The order of addition of the macromolecular constituents was DNA + enzyme + histones (Δ), or DNA + histones + enzyme (O). A partial digest of high molecular weight 14C DNA with MboI, labeled at the 5' ends with [^{32}P] ATP by T4 polynucleotide kinase (see Materials and Methods), was used in these and other assays
(Fig. 1 and 2).

Figure 1B: Binding of increasing amounts of [^{32}P] labeled restricted 14C DNA to a constant amount of enzyme or histones.
(▲) enzyme; (●) histone. The concentration of the enzyme or histones (0.1 μg) was constant and DNA varied (abscissa).

Figure 1C: Specificity of histones in the promotion of binding of poly(ADP-ribose) polymerase to DNA as compared to BSA. Experiments were similar to those illustrated in Figure 1A. (▲) varying concentrations of enzyme; (○) varying concentrations of BSA; (●) varying concentrations of BSA with a constant concentration (0.04 μg) of enzyme; (Δ) varying concentrations of enzyme with a constant (0.04 μg) concentration of BSA.

Figure 1D: Influence of the order of addition of macromolecules on the mutual promotional effect of histones on the binding of polymerase to DNA.

The order of additions were: (●) DNA + histones + enzyme; (□) DNA + enzyme + histones; (Δ) histones + DNA + enzyme; (▲) histones + enzyme + DNA; (○) enzyme + histones + DNA. Each assay contained 0.1 μg of histones. After addition of the first two components, the tubes were incubated for 2 minutes at 25°C and the third component was then added and incubation continued for 10 minutes before filtration.

Figure 2A: The binding of the enzyme to histones in the absence of DNA.

(O) constant concentration of [^{125}I] labeled enzyme with increasing concentrations of unlabeled histones; (Δ) constant concentration of [^{125}I] labeled histones with increasing concentrations of unlabeled enzyme. The mass ratios of enzyme/histones or histones/enzyme are indicated on the abscissa (for details see Materials and Methods).

Figure 2B: The effect of the addition of unlabeled DNA at a histone/enzyme mass ratio of 2.0.

Increasing concentrations of unlabeled restricted 14C DNA (phosphorylated at the 5' ends) were added to the [^{125}I] labeled enzyme followed, after an incubation of 1 minute, by the addition of histones to reach a mass ratio (H/E) of 2.0.

Figure 2C: The binding of the enzyme to histone H1.

The binding assays were done as described in the legend of Figure 2A. (O) constant concentration [^{125}I] labeled enzyme with varying concentration of [^{125}I] labeled H1; (Δ) constant concentration of [^{125}I] labeled H1 with varying concentrations of unlabeled enzyme. The mass ratios of enzyme/H1 or H1/enzyme are indicated on the abscissa.

Figure 2D: Promotion of the binding of the enzyme to [^{32}P] labeled restricted 14C DNA by H1.

Experiments were done as described in the legend of Figure 1A except that histone H1 is substituted for a mixture of histones. (●) H1 only; (▲) enzyme only; (○) varying concentrations of H1 with a constant concentration (0.04 μg) of enzyme; (Δ) varying concentrations of enzyme with a constant concentration (0.04 μg) of H1.

Figure 3: Promotion of the binding of poly(ADP-ribose) polymerase to [^{32}P] labeled linear and circular SV40 DNA by histones.

The experiments and the presentation of data are similar to those described in the legend of Figure 1A. (●) *linear* DNA with enzyme alone; (▲) *linear* DNA with increasing concentrations of enzyme and a constant amount of (0.04 μg) of histones; (○) *circular* DNA with increasing concentrations of enzyme; (Δ) *circular* DNA with increasing concentrations of the enzyme and a constant amount (0.04 μg) of histones. Inset: Ethidium bromide stained 1.0% agarose gel showing the positions of supercoiled (**S**, lane 1), linear (**L**, lane 2), and religated linear, i.e. relaxed (**R**, lane 3), SV40 DNA. Lanes 4 and 5 are the same as lanes 2 and 3 except that they are presented as an autoradiogram of the dried gel. Each lane contains approximately 20-30 ng of SV40 DNA.

Figure 4: Binding of poly(ADP-ribose) polymerase to restriction fragments of SV40 DNA at different NaCl contrations. SV40 DNA Form I was digested with MboI and the fragments [^{32}P] labeled at their 5' ends. A constant amount (150 ng) of polymerase was added to the [^{32}P] DNA fragments (50 ng = 20,000-30,000 cpm) and the binding assay performed in: 25 mM Tris-HCl (pH 8.0) + 10 mM $MgCl_2$ + 0.5 mM DTT + different concentrations of NaCl. Conditions for binding and nitrocellulose filtration are described in Materials and Methods. The DNA fragments were eluted off the filters by incubation of the filters in 0.5 ml of 25 mM HEPES (pH 7.5) + 10 mM NaCl + 0.2% SDS for 1 h at 37^{0}C. The eluates were phenol : chloroform extracted, EtOH precipitated, and run on a 4% nondenaturing polyacrylamide gel. The gel was fixed in 5% TCA, dried, and autoradiographed overnight. The autoradiogram was scanned in a Joyce-Lobel chromoscan 3 densitometer. All tests were done in duplicates but only one sample for each NaCl concentration is shown. For unknown reasons the 396 bp and 384 bp fragments were labeled to a lesser extent than the other fragments. The faint band below the 945 bp fragment is presumed to be a partial digestion product; the 60 bp fragment migrated off the gel. Figure 4 is a composite of different lanes from the same gel.

Lane 1: 0 M NaCl; lane 2: 0.01 M NaCl; lane 3: 0.02 M NaCl; lane 4: 0.05 M NaCl; lane 5: 0.1 M NaCl; lane 6: 0.1 M NaCl + no enzyme; lane 7: 0.15 M NaCl; lane 8: 0.2 M NaCl; lane 9: 0.3 M NaCl; lane 10: 0.4 M NaCl; lane 11: 0.6 M NaCl + no enzyme; lane 12: 0.6 M NaCl; lane 13: input DNA.

Figure 5: Torsional constraints induced by poly(ADP-ribose) polymerase protein and histones on SV40 DNA Form Ir (relaxed covalently closed circular duplex).

Torsional constraints were determined by the method of Germond, et al. (73) as follows: SV40 DNA Form I (supercoiled duplex) was converted to Form Ir by topoisomerase I (topo I), 10 units/μg DNA, in the binding buffer by incubation at 37°C for 30 minutes, followed by extraction with phenol/chloroform and precipitation of DNA with EtOH. This DNA was taken up in 50 μl binding buffer and proteins to be tested were added and incubated for 1.5 h at 25°C. Topo I (2 units/μg DNA) was then added and incubation continued for 5 minutes at 37°C. The reaction was stopped with 1% SDS, followed by phenol/chloroform extraction and precipitation of DNA with EtOH. The DNA was subjected to electrophoretic separation on a 1.0% agarose gel run in TBE and stained with ethidium bromide (1 μg/ml).

Fluorescent bands show topoisomers with differing linking numbers. Each lane in Figure 5A and 5B contained approximately 1.0 μg of SV40 DNA. The order of addition of the components in the reaction mix are as stated in each lane.

Figure 5A: Lane 1: SV40 DNA Form I, superhelical DNA (I), and nicked circular DNA (II); lane 2: SV40 DNA Form Ir; lanes 3-6: Form Ir + polymerase at E/DNA mass ratios of 0.2, 0.6, 0.8, and 1.2, respectively; lane 7: Form Ir + histones at H/DNA mass ratio of 0.5; lane 8: Form Ir (1 μg) + histones (0.2 μg) + polymerase (0.2 μg); lane 9: Form Ir (1 μg) + histones (0.3 μg) + polymerase (0.3 μg).

Figure 5B: Lane 1: SV40 DNA Form I, superhelical DNA (I) and nickedcircular DNA (II); lane 2: SV40 DNA Form Ir; lane 3: Form Ir (1 μg) + histones (0.2 μg) + polymerase (0.2 μg); lane 4: Form Ir (1 μg) + NAD^+ (0.05 mM) + histones (0.2 μg) + polymerase (0.2 μg); lane 5: Form Ir (1 μg) + NAD^+ (0.05 mM) + BA (0.05 mM) + histones (0.2 μg) + polymerase (0.2 μg); lane 6: Form Ir + polymerase [E/DNA = 0.6]; lane 7: Form Ir + histones [H/DNA = 0.5]; lane 8: Form Ir + BA (0.05 mM) + polymerase [E/DNA = 0.6]; lane 9: Form Ir + NAD^+ (0.05 mM) + polymerase [E/DNA = 0.6]; lane 10: Form Ir + NAD^+ (0.05 mM) + BA (0.05 mM) + pclymerase [E/DNA = 0.6]; lane11: Form I + NAD^+ (0.05 mM) + BA

(0.05 mM) + topo I; lane12: SV40 DNA (1 μg): Form I (superhelical DNA) and Form II (nicked circular DNA); lane13: Form Ir (relaxed covalently closed circular DNA); lane14: Form Ir (1 μg) + histones (0.5 μg) + NAD^+ (0.05 mM); lane15: Form Ir (1 μg) + histones (0.5 μg) + BA (0.05 mM). Lanes 1-11 and lanes 12-15 are from different gels.

Figure 6A & 6B:

Polyacrylamide gel electrophoresis of histone-DNA and poly(ADP-ribose) polymerase-DNA complexes.

A) histones + [^{32}P] labeled 209 bp EcoRI-PstI fragment of SV40 DNA were mixed in various proportions and aliquots were applied onto the gel (see Materials and Methods). The formation of core particle-like nucleosomes was monitered by gel electrophoresis. Mass ratios of Histones : DNA are given. N =core particle-like nucleosomes; F = naked DNA. B) Polymerase + [^{32}P] 209 bp fragment were mixed in varying proportions in binding buffer (final volume 20 μl), incubated at 25^{0}C for 15 min and the total reaction mixture was loaded onto gels. The polymerase : DNA mass ratios are indicated. F = naked DNA; B = fragment fully saturated with polymerase. Both 6A and 6B show autoradiograms of 4% nondenaturing polyacrylamide gels fixed in 5% TCA, dried, and exposed to film overnight.

The gels were run in TE (10 mM Tris-HCl pH 8.0 + 1 mM EDTA) at 4^0C for 16 h at 0.5 V/cm^2. Each lane in both 6A and 6B contains approximately 20 ng (≅ 15,000 Cerenkov cpm) of [^{32}P] DNA.

Figure 6C & 6D

Sucrose density gradient sedimentation of polymerase-core particle-like nucleosome complexes.

Sucrose gradients were prepared and centrifuged as described in Materials and Methods. C) The peak radioactivity in the various fractions was identified to be as follows: 4.8 **S** = [^{125}I] polymerase (200 ng); 5.1 **S** = naked [^{32}P] DNA, 209 bp fragment (≅ 40 ng); 11 **S** = core particle-like nucleosomes (≅ 50 ng of [^{32}P] DNA) reconstituted at histones : [^{32}P] DNA mass ratio of 1.2. All these peaks of radioactivity were obtained from different gradient tubes. For convenient presentation of the data, only the fractions containing the peak radioactivity are shown, although the other fractions were assayed for radioactivity. D) [^{32}P] core particle-like nucleosomes (≅ 50 ng of DNA) were mixed with [^{125}I]polymerase (≅ 300 ng) in binding buffer at 25^0C for 15-20 min and layered on top of the gradients. 14.3 **S** = core particle-like nucleosomes + polymerase complex. O---O, [^{32}P] DNA; ●---●, [^{125}I] polymerase.

References

1. Wientraub, H. (1985) *Cell* 42: 705-711.
2. Eissenberg, J. C., Cartwright, I. L., Thomas, G. H. and Elgin, S. C. R. (1985) *Annu. Rev. Genet.* 19: 485-536.
3. McGhee, J. D. and Felsenfeld, G. (1980) *Annu. Rev. Biochem.* 49: 1115-1156.
4. Pabo, C. O. and Sauer, R. T. (1984) *Annu. Rev. Biochem.* 55: 293-321.
5. Echols, H. (1986) *Science* 233: 1050-1056.
6. McKnight, S. and Tjian, R. (1986) *Cell* 46: 795-805.
7. Knezetic, J. A. and Luse, D. S. (1986) *Cell* 45: 95-104.
8. Lorch, Y., LaPointe, J. W. and Kornberg, R. D. (1987) *Cell* 49: 203-210.
9. Weintraub, H. (1984) *Cell* 38: 17-27.
10. Wu, H. and Crothers, D. M. (1984) *Nature* (London) 308: 509-513.
11. Gronenborn, A. M., Nermut, M. V., Eason, P. and Clore, G. M. (1984) *J. Mol. Biol.* 179: 751-757.
12. Ohlendorf, D. H., Anderson, W. F., Fisher, R. G., Takeda, Y. and Mathews, B. W. (1982) *Nature* (London) 298: 718-723.
13. Brennan, R. G., Takeda, Y., Kim, J., Anderson, W. F. and Mathews, B. W. (1986) *J. Mol. Biol.* 188: 115-118.
14. Frederick, C. A., Grable, J., Melia, M., Samudzi, C., Jen-Jacobsen, L., Wang, B.-C., Green, P., Boyer, H. W. and Rosenberg, J. M. (1984) *Nature* (London) 309: 327-331.

15. Richmond, T. J., Finch, S. T., Rushton, B., Rhodes, D. and Klug, A. (1984) *Nature* 311: 532-537.

16. Stillman, D. J., Better, M., and Geiduschek, E. P. (1985) *J. Mol. Biol.* 185: 451-455.

17. Shuey, D. J. and Parker, C. S. (1986) *Nature* 323: 459-461.

18. Ueda, K. and Hayaishi, O. (1985) *Annu. Rev. Biochem.* 54: 73-100.

19. Gaal, J. C. and Pearson, C. K. (1985) *Biochem. J.* 230: 1-18.

20. Yoshihara, K. and Kamiya, T. (1982) in *ADP-ribosylation reactions*, eds. Hayaishi, O. and Ueda, K. (Academic Press, N. Y.) pp. 157-171.

21. Jackowski, G. and Kun, E. (1981) *J. Biol. Chem.* 256: 3667-3670.

22. Kun, E., Minaga, T., Kirsten, E., Hakam, A., Jackowski, G., Tseng, Jr., A. and Brooks, M. (1986) in *Biochemical Action of Hormones*, eds. Litwack, J. (Academic Press, N. Y.) Vol. 13, pp. 33-55.

23. Kun, E. Kirsten, E., Milo, G. E., Kurian, P. and Kumari, H. L. (1983) *Proc. Natl. Acad. Sci. USA* 80: 7219-7223.

24. Tseng, Jr., A., Lee, W. M. F., Kirsten, E., Hakam, A., McLick, J., Buki, K. and Kun, E. (1987) *Proc. Natl Acad. Sci. USA* 84: 1107-1111.

25. Bauer, P., Hakam, A. and Kun, E. (1986) *Fed. Eur. Biochem. Soc. Letts.* 195: 331-338.

26. Buki, K. Kirsten, E. and Kun, E. (1987) *Anal. Biochem.* (in press).

27. Yoshihara, K., Hashida, T., Tanaka, Y., Ohgushi, H., Yoshihara, H. and Kamiya, T. (1978) *J. Biol. Chem.* 263: 6459-6466.

28. Laemmli, U. K. (1970) *Nature* (London) 227: 680-685.

29. Maniatis, T., Fritch, E. and Sambrook, J. (1982) *Molecular Cloning - A Laboratory Manual* (Cold Spring Harbor Laboratory, N. Y.).

30. Maxam, A. M. and Gilbert, W. (1980) in *Methods in Enzymology*, eds. Grossman, L. and Moldave, K. (Academic Press, N. Y.) Vol. 65, pp. 499-560.

31. Ohgushi, H., Yoshihara, K. and Kamiya, T. (1980) *J. Biol. Chem.* 255: 6205-6211.

32. Buchman, A. R., Burnett, L. and Berg, P. (1980) in *DNA Tumor Viruses*, ed. Tooze, J. (Cold Spring Harbor Laboratory, N. Y.) pp. 799-829.

33. Stein, A. (1987) *J. Biol. Chem.* 262: 3872-3879.

34. Price, C. A. (1982) in *Centrifugation in Density Gradients*(Academic Press, N. Y.), pp. 323-324.

35. Hakam, A., McLick, J. Buki, K. and Kun, E. (1987) *Fed. Eur. Biochem. Soc. Letts.* 212: 73-78.

36. Bailey, J. M. (1979) *Anal. Biochem.* 93: 204-206.

37. McGhee, J. D.,and von Hippel, P. H. (1974) *J. Mol. Biol.* 86: 469-489.

38. Woodbury, C. P. and von Hippel, P. H. (1983) *Biochemistry* 22: 4730-4737.

39. Stein, A., Whitlock, Jr., J. P. and Bina, M. (1979) *Proc. Natl. Acad. Sci. USA* 76: 5000-5004.

40. Johns, E. W. (1982) (ed.) *The HMG Chromosomal Proteins* (Academic Press, London).

41. Radding, C. M. (1982) *Annu. Rev. Genet.* 16: 405-437.

42. McEntee, K., Weinstock, G. M. and Lehman, I. R. (1981) *J. Biol. Chem.* 256: 8835-8844.

43. Vogel, T. and Singer, M. F. (1976) *J. Biol. Chem.* 251: 2334-2338.

44. Zahradka, P. and Ebisuzaki, K. (1984) *Eur. J. Biochem* 142: 503-509.

45. Soloman, M. J., Strauss, R. and Varshavsky (1986) *Proc. Natl. Acad. Sci. USA* 83: 1276-1280.

46. McClellan, J. A., Palecek, E. and Liley, D. M. J. (1986) *Nucl. Acids Res.* 14: 9291-9309.

47. McClean, M. J., Blano, J. A., Kilpatrick, M. W. and Wells, R. D. (1986) *Proc. Natl. Acad. Sci. USA* 83: 5884-5888.

48. Koo, H.-S., Wu, H.-M. and Cothers, D. M. (1986) *Nature* 320: 501-506.

49. Camerini-Otero, R. D. and Felsenfeld, G. (1977) *Nucl. Acids Res.* 4: 1159-1181.

50. Jorcano, J. L. and Ruiz-Carrillo, A. (1979) *Biochemistry* 18: 768-774.

51. de Murcia, G., Jongstra-Bilen, J., Ittel, M. E., Mandel, P. and Delain, E. (1983) *EMBO J.* 2: 543-548.

52. Keller, W. (1975) *Proc. Natl. Acad. Sci. USA* 72: 4876-4880.

53. Boyles, S. S. and Pettijohn, D. E. (1986) *J. Mol. Biol.* 187: 47-60.

54. Purnell, M. R. and Whish, W. J. D. (1980) *Biochem. J.* 185: 775-777.

55. Kornberg, R. D. and Thomas, J. O. (1974) *Science* 184: 865-868.

56. D'Anna, Jr., J. A. and Isenberg , I. (1974) *Biochemistry* 13: 2098-2104.

57. Clarke, M. F., Fitzgerald, P. C., Brubaker, J. M. and Simpson, R. T. (1985) *J. Biol. Chem.* 260: 12394-12397.

58. Nobile, C., Nickol, J. and Martin, R. G. (1986) *Mol. Cell. Biol.* 6: 2916-2922.

59. Poljak, L. G. and Gralla, J. D. (1987) *Biochemistry* 26: 295-303.

60. McMurray, C. T. and Van Holde, K. E. (1986) *Proc. Natl. Acad. Sci. USA* 83: 8472-8476.

61. Stein, A. (1979) *J. Mol. Biol.* 130: 103-134.

62. Simpson, R. T. (1978) *Biochemistry* 25: 5224-5231.

63. Poirier, G. G., DeMurcia, G., Jonstra-Bilen, J., Niedergang, C. and Mandel, P. (1982) *Proc. Natl. Acad. Sci. USA* 79: 3423-3427.

64. Wang, J. C. (1985) in *Nucleases*, eds. Linn, S. and Roberts, R. J. (Cold Spring Harbor Laboratory, N. Y.), pp 41-57.

65. Shen, C.-K. J. and Hu, W. S. (1986) *Proc. Natl. Acad. Sci. USA* 83: 1641-1645.

66. Harland, R. M., Weintraub, H. and McKnight, S. (1983) *Nature* (London) 302: 38-43.

67. Luchnik, A. N., Bakayev, V. V., Zbarsky, I. B. and Georgiev, G. P. *EMBO J.* 1: 1353-1358.

68. Ryoji, M. and Worcel, A. (1984) *Cell* 37: 21-32.

69. Weintraub, H., Cheng, P. F. and Conrad, K. (1986) *Cell* 46: 115-122.

70. Wolfe, A. P., Andrews, M. T., Crawford, E., Losa, R. and Brown, D. D. (1987) *Cell* 49: 301-302.

71. Worcel, A. (1987) *Cell* 49: 302-303.

72. Petryniak, B. and Lutter, L. C. (1987) *Cell* 48: 289-295.

73. Germond, J. E., Hirt, B., Oudet, P., Gross-Bellard, M. and Chambon, P. (1975) *Proc. Natl. Acad. Sci. USA* 72: 1843-1847.

Abbreviations:

H = whole thymus histones;

BA = benzamide;

BSA = bovine serum albumin;

E = enyme, poly(ADP-ribose) polymerase;

PMSF = phenylmethanesulfonyl fluoride;

topo I = topoisomerase I

FIG 1

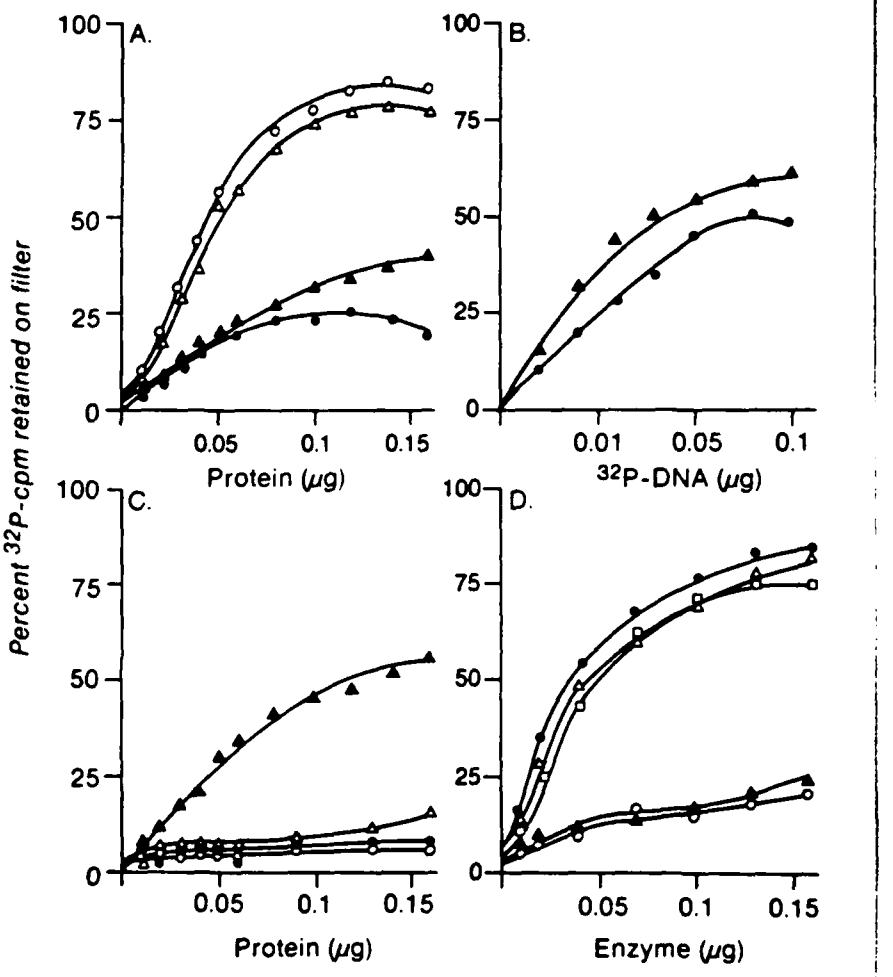

A.
B.
C.
D.
100
75
50
25
0
0.05
0.1
0.15
0.01
Protein (μg)
32P-DNA (μg)
Enzyme (μg)
Percent 32P-cpm retained on filter

FIG 2

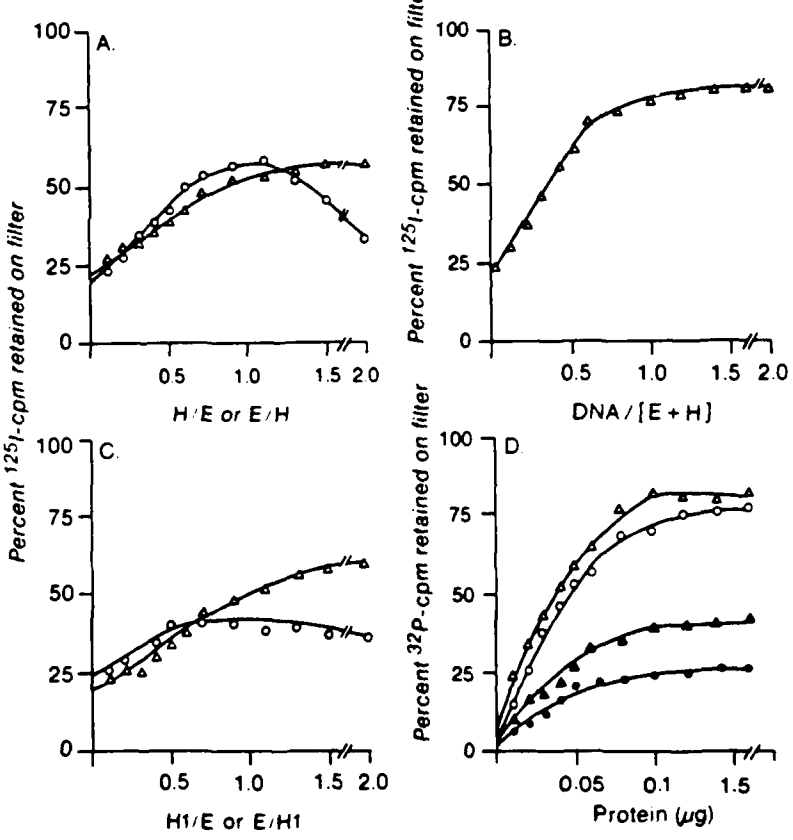

A.
B.
C.
D.
Percent ^{125}I-cpm retained on filter
Percent ^{32}P-cpm retained on filter
100
75
50
25
0
0.5
1.0
1.5
2.0
0.05
0.1
H/E or E/H
DNA/[E+H]
H1/E or E/H1
Protein (μg)

Figure 3

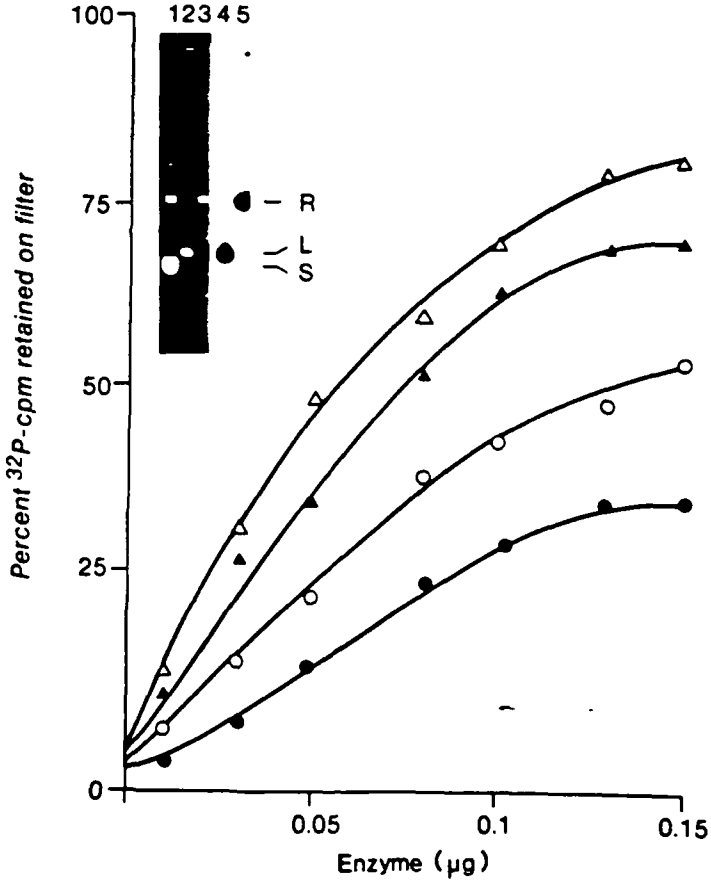

100
75
50
25
0
1 2 3 4 5
R
L
S
Percent ^{32}P-cpm retained on filter
0.05
0.1
0.15
Enzyme (μg)

Figure 4

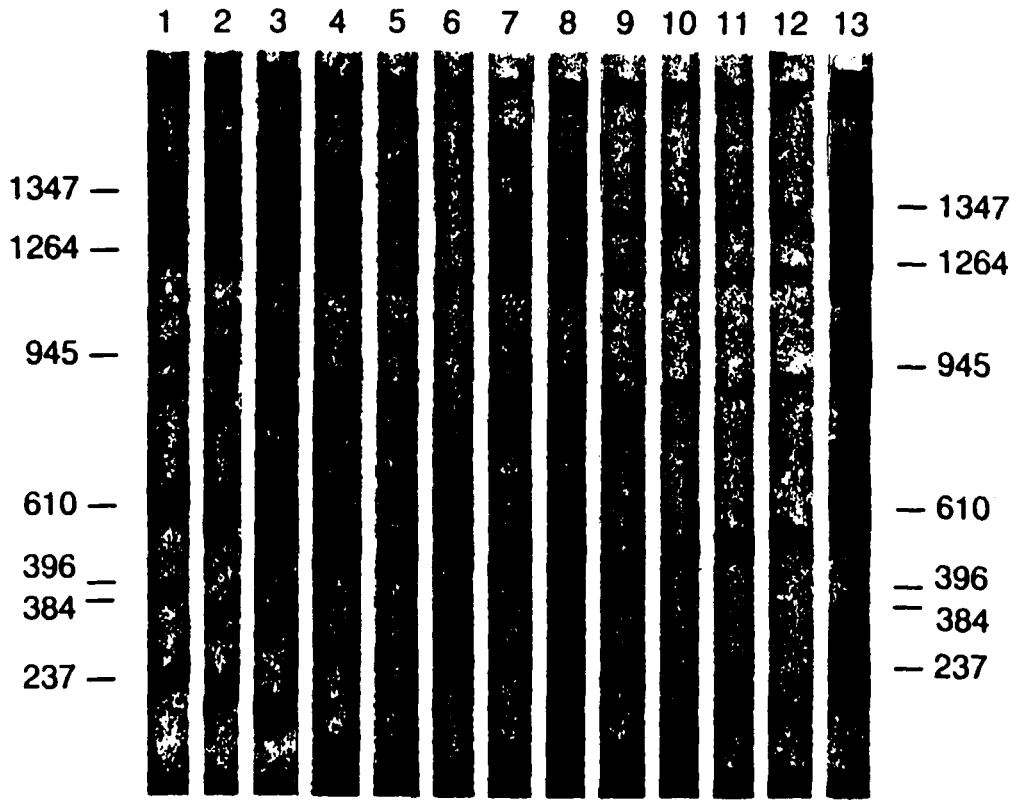

1 2 3 4 5 6 7 8 9 10 11 12 13
1347
1264
945
610
396
384
237
1347
1264
945
610
396
384
237

Figure 5A

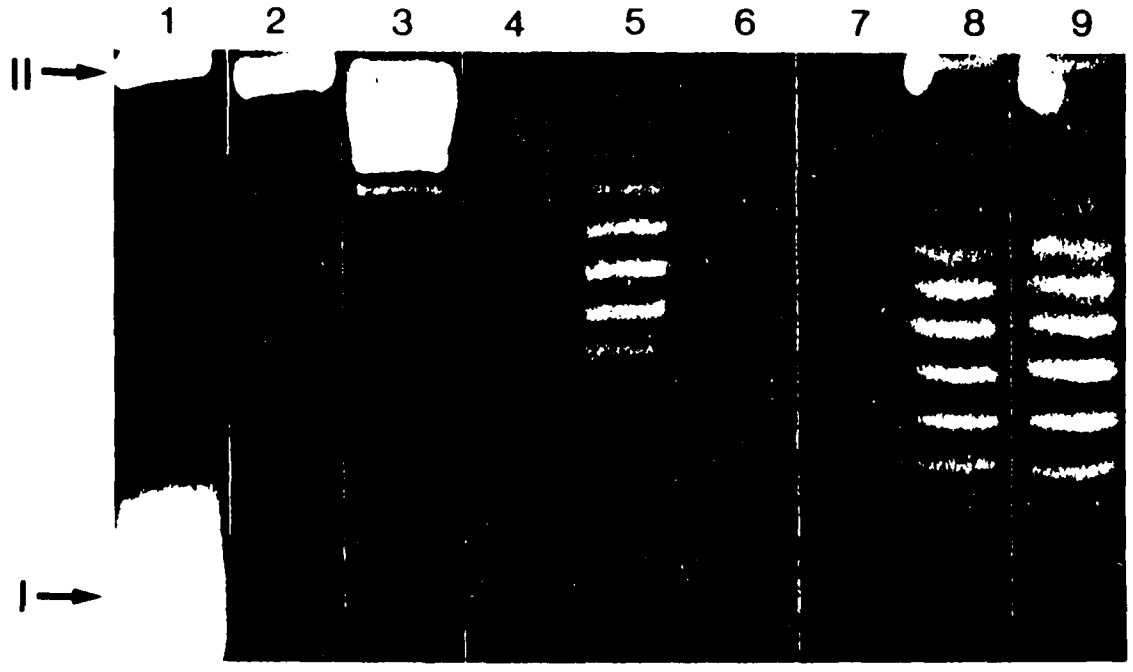
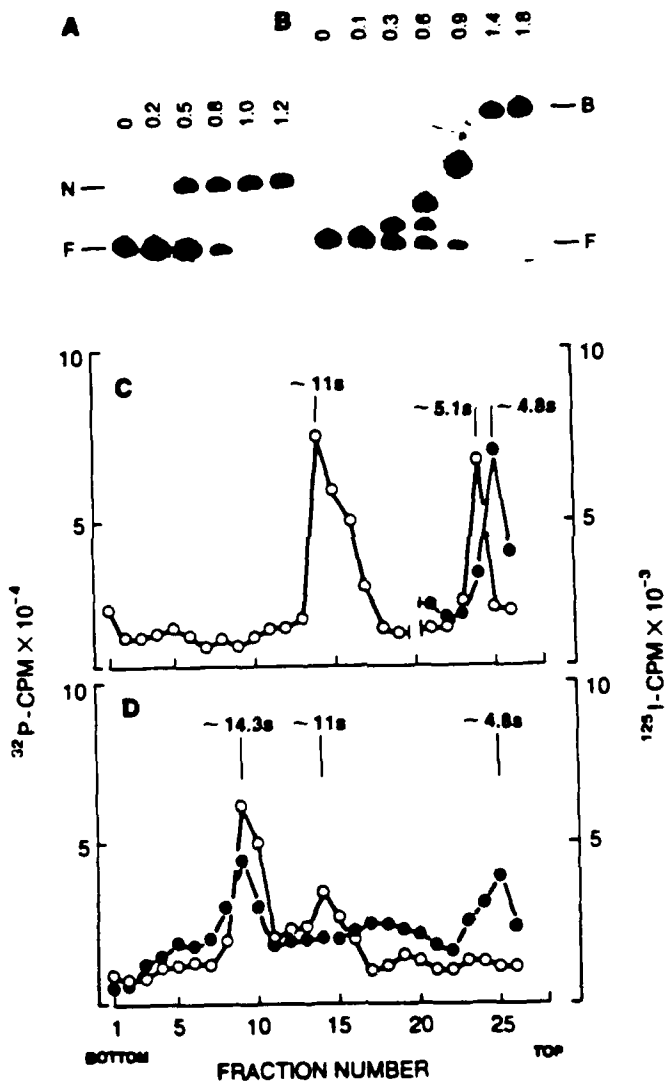


FIG 5B

Figure 6


A
B
0
0.2
0.5
0.8
1.0
1.2
0
0.1
0.3
0.6
0.9
1.4
1.8
N
F
B
F
C
~11s
~5.1s
~4.8s
D
~14.3s
~11s
~4.8s
10
5
^{32}P-CPM X 10^{-4}
^{125}I-CPM X 10^{-3}
1
5
10
15
20
25
BOTTOM
TOP
FRACTION NUMBER

III

Part III

Analysis of the Molecular Contacts Between Poly (ADP-ribose) Polymerase and DNA*

III-1

Summary

The interaction between poly (ADP-ribose) polymerase and various uniquely end labeled restriction fragments from SV40 and *pBR*322 DNAs was studied employing nuclease protection experiments. DNaseI footprinting indicated that approximately 66-85 bp of DNA was protected by poly (ADP-ribose) polymerase from DNaseI attack, and that a segment of DNA probably lies outside on the surface of the polymerase protein in the polymerase-DNA complex. Of the four different restriction fragments assayed for polymerase binding, only the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment showed clear poly (ADP-ribose) polymerase footprints, indicating the specific binding of the polymerase to this DNA fragment. Since this 209 bp *Eco*RI-*Pst*I SV40 DNA fragment is overall A+T rich and is intrinsically bent, we conclude that the specific binding of the polymerase to the internal length of DNA is dictated perhaps by A+T richness and DNA bending. The polymerase also binds to the 5' and 3' termini of other DNAs as assayed by digestion of the polymerase-DNA complexes with λ exo and exo III. Binding of the polymerase to the DNA termini is nonspecific since the polymerase binds the 5' and 3' ends of all the restriction fragments and polynucleotides tested. The polymerase does not generate DNaseI footprints on these DNAs with the exception of the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment. Poly (dI-dC) : poly (dI-dC) can compete for polymerase binding to the 209 bp *Eco*RI-*Pst*I SV40 DNA in DNaseI and exo III footprinting assays, presumably because the polymerase binds to the ends of poly (dI-dC) : poly (dI-dC). Poly (dA-dT) : poly (dA-dT) binds the polymerase 20%

more effectively than poly (dG-dC) : poly (dG-dC) as tested by the nitrocellulose filter-binding assay. Methylation protection experiments and the binding of the polymerase to glucosylated and nonglucosylated T4 DNA suggest that the polymerase makes major groove contacts. Proteolysis of the polymerase with plasmin and western blotting revealed that a 36 Kd polypeptide fragment contained the DNA-binding domain of the polymerase. The DNA-binding 36 Kd polypeptide footprinted on the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment exactly like the whole polymerase.

1. Introduction

Poly (ADP-ribose) polymerase (E.C. 2.4.2.30) is a chromatin-bound nuclear protein (Ueda and Hayaishi, 1985; Gaal and Pearson, 1985). In the presence of DNA and NAD^+ the enzyme catalyzes mono ADP-ribosylation (Bauer et al., 1986) and poly ADP-ribosylation (Ueda and Hayaishi, 1985) of itself as well as other acceptor proteins (Ueda and Hayaishi, 1985). Poly (ADP-ribose) polymerase has been implicated in the control of a variety of cellular functions such as differentiation, damage to DNA (Ueda and Hayaishi,1985; Gaal and Pearson, 1985), aging (Jackowski and Kun, 1981), hormonal effects (Kun et al., 1986), and tumorigenic growth (Kun et al., 1983; Tseng et al., 1987).

The exact role of DNA in the catalytic activity of poly (ADP-ribose) polymerase is unknown but indications are that DNA might play a role in (ADP-ribose) chain elongation (Bauer et al., 1986). During the purification of the poly (ADP-ribose) polymerase protein, a polymerase-associated DNA species (co-enzymic DNA), which greatly enhances the catalytic activity of the polymerase as compared to whole thymus DNA, is copurified (Hashida et al., 1979). DNAs differing in size and sequence have been tested for their effectivity to stimulate the enzymatic activity of poly (ADP-ribose) polymerase *in vitro* (Hakam et al.,1987; Berger and Petzold.,1985; Benjamin and Gill, 1980a, & Benjamin and Gill, 1980b). For example, a synthetic duplex octadeoxynucleotide derived from the SV40

promoter-enhancer region was found to stimulate the catalytic activity of the polymerase as effectively as co-enzymic DNA and better than calf thymus DNA (Hakam et al.,1987).

The mechanism by which the polymerase binds to DNA has not been studied in detail. Most of the information available on the nature of the interaction between the polymerase and DNA has been deduced from experiments involving the measurement of the catalytic activity of the polymerase, i.e.,in the presence of NAD^+. The polymerase has reduced affinity to DNA in the presence of NAD^+ (Ohghushi et al., 1980; our unpublished results). Therefore, in the present report all experiments were carried out in the absence of NAD^+, emphasizing the DNA-binding property of the polymerase. Elucidation of the mechanism of binding of the polymerase to DNA is considered important for an understanding of the role of the polymerase in cell physiology.

We have previously shown that the polymerase interacts cooperatively with histones to bind to DNA, and the binding of the polymerase to closed circular SV40 DNA induces superhelicity (Sastry and Kun, 1987). In the following report we present a detailed investigation of the interaction between the polymerase and specified DNA restriction fragments and with synthetic polynucleotides. Histones were not included in these experiments due to obvious difficulties that would arise in the interpretation of the nuclease footprints in terms of distinguishing the patterns of protection due to the histones and those due to the polymerase (or both).

2. Materials and Methods

(a) Poly (ADP-ribose) polymerase, nucleases and DNA

Poly (ADP-ribose) polymerase (MW 120,000) was purified from calf thymus as described elsewhere (Buki et. al., 1987). The polymerase preparation was greater than 95% homogeneous and the characteristics of a similar preparation have been described previously (Buki et al., 1987). DNase I and micrococcal nuclease were obtained from Cooper Biomedical (Malvern, PA) . Exonuclease III and lambda exonuclease were purchased from Bethesda Research Labs (Gaithersburg, MD). Restriction endonucleases were from New England Biolabs (Beverley, MA). Distamycin A hydrochloride was purchased from Sigma Chemical CO. (St. Louis, MO). T4 polynucleotide kinase, Klenow fragment, T4 and *E. coli* DNA polymerases, calf terminal transferase, and bacterial alkaline phosphatase were obtained from Boerhinger Mannheim (Indianapolis ,IN). γ-[^{32}P]-ATP, α -[^{32}P]-dATP, and α-[^{32}P]-ddATP were from either Amersham Corp. (Arlington Heights, IL) or New England Nuclear (Boston, MA). SV40 DNA was purchased from Bethesda Research Labs. *pBR*322 was prepared locally or obtained from Promega Biotech (Madison,WI). *pSVO*7 (containing the *Eco*R II 'G' fragment of SV40 DNA) was a gift of Dr. Robert Tjian (Univ. of California at Berkeley, Berkeley, CA). Phage T4 DNA was a gift of Dr. Bruce Alberts (Univ. of California at San Fransisco, San Fransisco, CA). Poly (dI-dC) : poly (dI-dC) (average length = 500 bp), poly (dA-dT) : poly (dA-dT) (average length = 300 bp), and poly (dG-dC) : poly

(dG-dC) (average length = 300 bp) were purchased from Pharmacia (Piscataway, NJ).

(b) Nuclease footprinting

1. Preparation of DNA fragments: SV40 DNA was digested with *Eco*RI and the 5' ends labeled with γ-[^{32}P]-ATP and T4 Polynucleotide kinase, or the 3' ends labeled with α-[^{32}P]-dATP and Klenow fragment. The DNA was then digested with *Pst*I and the 209 bp fragment was purified from 5% non-denaturing polyacrylamide gels (Maniatis et al., 1982). The 3' end of the *Pst*I site of the 209 bp *Eco*RI-*Pst*I fragment of SV40 was labeled with α-[^{32}P]-ddATP and terminal nucleotydyl transferase (Roychaudhury and Wu, 1980). The 5' end of the *Pst*I site (with a 3' overhang and recessed 5' end) could not be efficiently ^{32}P labeled by conventional protocols. Similarly, uniquely end-labeled fragments were generated by digestion of SV40 DNA with *Acc*I-*Eco*RI and the digestion of pBR322 with *Bam*HI-*Sal*I.

2. DNaseI Digestion: The DNA fragments to be footprinted (Galas and Schmitz, 1978) were mixed with purified poly (ADP-ribose) polymerase in the presence or absence of poly (dI-dC) : poly (dI-dC) (as competitor) in a final volume of 50 µl of binding buffer (25 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 M NaCl) and incubated at 25^0 C for 15 min. A freshly prepared solution of DNaseI (0.5 U/50 µl reaction) was added and the mixture was incubated at 37^0 C for 30-60 sec. The digestion was stopped by the addition of EDTA

(10 mM final concentration) and SDS (0.1% final concentration). The samples were heated in a 90°C water bath for 3 min, precipitated with 0.3 M Na Acetate and EtOH, rinsed once with 70% EtOH, and resuspended in formamide dyes and subjected to electrophoresis on denaturing polyacrylamide gels (Maxam and Gilbert, 1980).

3. Micrococcal Nuclease Digestion: Micrococcal nuclease digestion reactions were similar to DNaseI digestion reactions except that after the initial binding reaction at 25°C for 15 min, $CaCl_2$ (to a final concentration of 2.5 mM) was added to the DNA-protein complexes followed by the addition of micrococcal nuclease (1.5 U/50 µl reaction). The reaction mixture was further incubated at 37°C for 7-10 min. The reactions were terminated as described for DNaseI.

4. Exonuclease III and λ exonuclease digestions: Exo III reactions were carried out in binding buffer as described for DNaseI reactions. Five units of exo III were added to the initial binding mixture (50 µl) and the nuclease digestion was carried out for 30 min at 37°C. In the case of λ exo digestions (10 U/50 µl reaction at 37°C for 20 min) the initial polymerase binding reaction and the subsequent λ exo digestion were carried out in 67 mM glycine-KOH pH 8.1, 2.5 mM $MgCl_2$, and 2-mercaptoethanol. Exo III and λ exo digestions were terminated as described for DNaseI reactions.

5. Gel electrophoresis: The nuclease-digested DNA samples were resuspended in 7 µl of formamide dyes (80% vol/vol formamide, 0.02% wt/vol xylene cyanol, and 0.02% wt/vol bromophenol blue) and heated in a 90°C water bath for 3 min before loading them on a 40 X 35 cm 8% acrylamide - 7 M urea sequencing gel (Maxam and Gilbert,

1980). The gels were run at 1500V until the bromophenol blue dye was about 8-10 cm from the bottom of the gel. Maxam-Gilbert chemical cleavage reactions were run alongside of the nuclease-digested DNA samples to serve as markers (Maxam and Gilbert, 1980). After the electrophoresis was completed the gels were either dried or directly exposed to X-ray film (Kodak XOMAT AR-5) with or without an intensifying screen for usually 12-72 hrs. The positions of the nuclease cleavage sites could be accurately identified when the fragments were 50-70 bases long. For longer DNA fragments, mapping of the nuclease cleavage sites could have an error of up to ± 3 bases.

(c) Western Blotting

Digestion of poly (ADP- ribose) polymerase protein with plasmin (a trypsin-like serine protease which cuts predominantly at lysines and arginines; Robbins & Summaria, 1970), polyacrylamide gel electrophoresis have been described elsewhere (Buki and Kun, 1987).

1. Electroblotting and immunostaining: The proteins from the polyacrylamide gel were electroblotted on to nitrocellulose membranes in 25mM 3-(cyclohexylamino)-2-hydroxy-1-propane sulfonate buffer (pH 9.5) containing 20% methanol (Szewczyk and Kozloff, 1985) at 100V for 1.5 h using a BioRad trans-blot apparatus as described by the manufacturer. For immunostaining the nitrocellulose membrane was washed 2x in a solution of 0.05% Tween20 + 0.01% thimersol in phosphate buffered saline (PBS)

followed by incubation in a solution of rabbit anti-poly (ADP-ribose) polymerase antibody (described in Buki and Kun, 1987) which was diluted 5000-fold in PBS. The membrane was incubated for 1h at 22^0C with the polyclonal antibody. The membrane was then washed 3X with PBS and exposed to a solution containing peroxydase-labeled anti-rabbit goat serum (1000-fold dilution in PBS) at 22^0C for 1h. The membrane was further washed 5X with PBS and placed in a freshly prepared solution of 3,3'- diaminobenzidine (1 mg/ml in 30 mM TRIS-Cl, pH 7.3 + 150 mM NaCl) and 1 µl/ml of 30% H_2O_2 was added and membrane was shaken rapidly. After ~ 5 min brown-colored bands could be seen. The staining reaction was stopped by the addition of 6 N HCl to bring the pH of the solution to ~ 1.0. Finally, the membranes were rinsed with water and photographed under visible light.

2. Detection of DNA-binding polypeptides: After electroblotting, the nitrocellulose membrane with the polypeptides bound to it, was washed for 2 h at room temperature (22^0C) in binding buffer (see 'DNaseI digestions', above). The membrane was then bathed overnight at room temperature in a solution of binding buffer containing [^{32}P]-labeled 209 bp *Eco*RI-*Pst*I fragment(≅10^6 CPM) of SV40, (see "Preparation of DNA fragments"). The membrane was then washed with several changes of binding buffer for 2 h at room temperature, air-dried at room temperature and exposed overnight to X-ray film with an intensifying screen.

3. Results

The molecular contacts between poly (ADP-ribose) polymerase and specified uniquely end labeled DNA restriction fragments were determined by nuclease protection experiments. The restriction fragments employed in this study had the following characteristics.

1. A 209 bp *Eco*RI-*Pst*I fragment of SV40 DNA from position 1783 to 1992 of the SV40 DNA sequence (Buchman et al., 1980). This fragment lies within the coding region of VP1 (a viral coat protein), has an overall A+T content of 60.2%, and has several contiguous runs of A+T residues (3-10 bp each) interspersed with not more than 4 consecutive G+C bp runs (see Fig. 16 for the DNA sequence).
2. A 154 bp *Acc*I-*Eco*RI fragment of SV40 (positions 1629-1783) that is 54.5% A+T and contains 3-9 bp A+T stretches (but fewer A+T stretches in total number than the *Eco*RI- *Pst*I fragment) punctuated by 9 blocks of G+C (3-5 bp each).
3. A 311 bp *Eco*RII'G' fragment (position 5092-160) containing the SV40 early transcriptional regulatory elements. This fragment is 48.8% A+T and contains not more than 17 bp of A+T at any place but only a total of 4 such blocks . It has a total of 11 G+C runs (3-7 bp each).
4. A 276 bp *Bam*HI-*Sal*I (position 375 - 651) fragment from pBR322 (Sutcliffe,1979) with a 25.3% A+T content and not more than 5 contiguous A+T residues at any place. This fragment has several blocks of G+C (3-10 bp each) and may be construed, in terms of its distribution of A+T and G+C, as the mirror image (in a broad sense) of the 209 bp *Eco*RI-*Pst*I SV40 fragment.

These fragments were chosen for the nuclease protection

experiments not only for their availability and convenient restriction sites but also for the differences in their distribution of A+T and G+C which might be important in assessing the specificity, if any, of the polymerase towards DNA.

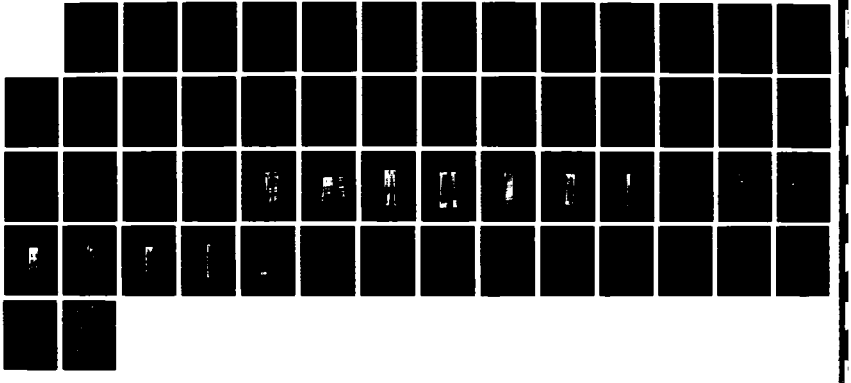
Throughout this paper the term polymerase-DNA 'contact'(s) is used in its broadest sense. It includes all possible chemical interactions between the amino acid side chains of the polymerase and the DNA bases, sugar and phosphate. Thus 'contact' is operationally defined in terms of what is observed on nuclease footprints, and does not signify or connote any specific chemical interaction.

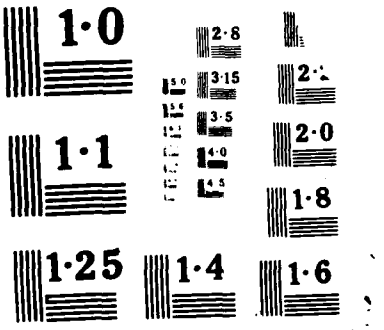
DNaseI footprinting: The 5' end of the *Eco*RI-*Pst*I fragment of SV40 DNA was labeled with ^{32}P (**Materials and Methods**) and increasing amounts of purified polymerase was added to the labeled DNA and the protein-DNA complexes were subjected to DNaseI attack (Galas and Schmitz,1978). Figure 1 shows that at lower concentrations of the polymerase protein (lanes 8 & 9, Fig.1) a small region of protection equivalent to about one turn of the DNA helix (from position approximateley 1800-1810, lanes 8 & 9, Fig 1) occurs. At a higher concentration of the polymerase a large region of protection (~66 bp = 6-7 turns of the DNA helix)is observed (Fig. 1 lane 7; positions1800-1866, Fig. 16 region demarcated 'A'. This apparent region of protection is not entirely contiguous but is punctuated by DNaseI cleavage sites which coincide with the strong DNaseI cleavage sites seen in the absence of the polymerase (lanes 10 & 7, Fig.1). Assuming full binding and

FIG 1 →

AD-A191 557 MOLECULAR TOXICOLOGY OF CHROMATIN(U) CALIFORNIA UNIV SAN FRANCISCO CARDIOVASCULAR RESEARCH INST E KUN 31 DEC 87 AFOSR-TR-88-0009 AFOSR-86-0064 2/2

UNCLASSIFIED F/G 6/11 NL




1·0
1·1
1·25
1·4
1·6
1·8
2·0
2·8
3·15
3·5

polymerase molecules, approximately seven polymerase molecules bind to the DNA at the concentration of the polymerase employed (lane10, Fig.1). At all the concentrations of the polymerase tested, DNaseI protection occurs in blocks equivalent to about one turn of the DNA helix. This observation is consistent with the helical periodicity exhibited by DNaseI cutting sites (Drew and Travers,1985).

Fig 2 →

DNaseI footprinting was also performed on DNA fragments uniquely 3' end-labeled at the *Eco*RI site (Fig. 2A, & Fig16, bottom strand) and the *Pst*I site (Fig. 3, & Fig.16 3' end of the top strand). The results indicate that approximateley ~64 bp of the bottom strand (lane 6, Fig. 2A; & region 'B' Fig.16) and ~85 bp of the top strand from the *Pst*I site (region 'C', Fig.16) are protected by the polymerase from DNaseI attack. There is a 3-6 bp stagger towards the 3' end of the bottom strand of the footprint as compared to the footprint on the top strand (compare region 'A' with region 'B', Fig.16). A similar stagger has been observed for DNaseI footprints in the case of several other DNA-binding proteins (Sawadogo and Roeder,1985; Bohmann et al., 1987; Dynan et al., 1986). A possible explanation for this stagger has been given by Drew,1984; & by Suck and Oefner,1986). The regions protected by the polymerase from DNaseI attack on the top and the bottom strands overlap and the total length of the DNA protected is roughly the same (regions 'A' and 'B', Fig.16). An interesting aspect of the DNaseI cutting pattern of the top strand towards the *Pst*I site is the relatively DNaseI-resistant region seen on naked DNA (positions 1946-1977, Fig. 3; & 'D' in Fig.16). We do not know the reason for the existance of this

Fig 3 →

DNaseI resistant region, but it has been demonstrated that non B-DNA conformations (e.g., Z-DNA) are resistant to DNaseI cleavage (Moller et al., 1984).

The effect of the addition of poly (dI-dC) : poly (dI-dC), a nonspecific polynucleotide competitor, to the polymerase-DNA binding reaction was tested. It was observed that poly (dI-dC) : poly (dI-dC) could compete out the footprint from the 209 bp fragment (lanes 4-6, Fig.1; lanes 8 & 9 Fig. 2A; lanes 3-5, Fig. 3).

In general, we observed that there was decreased overall cleavage by DNaseI in the presence of the polymerase as compared to the cleavage pattern on naked DNA, especially in the case of the DNaseI footprinting with fragments 3' end labeled at the *Pst*I site. Figures 1-3 do not indicate whether the polymerase makes contacts closer to the *Eco*RI or the *Pst*I ends, possibly because smaller fragments than 5-13 bp are either not generated under the conditions described in our DNaseI digestions and / or they are not resolved by these gels. Footprinting experiments with Iron(II)-EDTA, which cleaves at almost every base of the DNA helix could clarify the existence of any terminal contacts.

FIG 4 →

DNaseI footprinting experiments with the three other fragments mentioned above (*Acc*I-*Eco*RI SV40 fragment, Fig. 5; the *Eco*RII 'G' fragment of SV40, not shown; and the *Bam*HI-*Sal*I fragment of pBR322, Fig. 4B) did not reveal footprints similar to the ones observed on the 209 bp *Eco*RI-*Pst*I SV40 fragment. Figure 5 shows that two minor contact points can be detected on the *Acc*I-*Eco*RI fragment of SV40 as indicated by the arrows. It is not clear whether these points of contact are real or fortuitous since similar contacts could not be detected on the bottom strand (data not

Fig 5 → shown). These experiments indicate that the polymerase protein binds specifically to the internal length of the 209 bp *Eco*RI-*Pst*I fragment of SV40 DNA.

To summarise, 1. Poly (ADP-ribose) polymerase protein specifically binds to the 209 bp *Eco*RI-*Pst*I fragment of SV40 DNA. Other DNA fragments tested do not show DNaseI footprints in the presence of the polymerase.

2) The large region of protection (~66-85 bp) from DNaseI attack seen on the 209 bp SV40 fragment and the pattern of cleavage coinciding with the helical twist of the DNA (within ± 2-3 bp) suggests that the DNA lies outside on the surface of the polymerase protein core. This rationale has been employed in the analysis of DNA-DNA gyrase complexes (Lui and Wang,1978; Morrison and Cozzarelli,1981; Kirkegaard and Wang,1981) and DNA-histone octamer complexes (Noll,1974; Lutter, 1978; & Mcghee and Felsenfeld,1980). Our conclusion that segments of DNA lie outside the polymerase protein is in agreement with the electron microscopic data of poly (ADP-ribose) polymerase-coenzymic DNA complexes (de Murcia et al,1983).

Since DNaseI footprints are known to overestimate, by at least 5 bp, the regions occupied by proteins on DNA (Suck and Oefner,1986) we assume that the actual length of DNA in contact with the polymerase protein could be 60-83 bp or less.

3. Poly (dI-dC) : poly (dI-dC) competes out the DNaseI footprint from the 209 bp fragment.

Distamycin A-induced change in the rotational orientation of the DNA in the polymerase-DNA complexes. Portugal and Waring (1987) reported that certain antibiotics such as distamycin A (a minor groove-binding, non-intercalating drug that preferentially binds to A+T rich regions) bind to nucleosomal DNA and alter the rotational orientation of the DNA helix with respect to the octamer of histones. Therefore, it was reasoned that, if the DNA were to lie on the surface of the polymerase protein as indicated by the DNaseI footprint analysis, distamycin A could introduce an alteration in the rotational setting of the DNA on the surface of the polymerase as observed for nucleosomal DNA.

Figure 2B shows that the binding of low amounts of distamycin A to the polymerase-DNA complexes results in increased protection against DNaseI at some regions as compared to the polymerase-DNA comlplexes alone. For example, between position1835 and position1899 several bands are visible in the absence of distamycin A (lane 6, Fig. 2A). If low amounts of distamycin A are added to the polymerase-DNA complexes many of these bands are not observed (lanes 1-4, Fig. 2B). When the concentration of distamycin A is further increased (lanes 5 & 6 Fig. 2B), some new cutting sites appear (at position1801 and at arrows, Fig. 2B) while other cleavage sites disappear (arrows between position1899 &1992, Fig. 2B). Employing the reasoning of Portugal and Waring (1987) this observation suggests that distamycin alters the rotational orientation of the DNA lying on the surface of the polymerase protein as described for the nucleosomal DNA (Portugal and Waring,1987). A similar effect of distamycin A was observed when the 5' end of the *Eco*RIsite (top strand) was labeled (data not

shown).

Micrococcal Nuclease Protection: Digestion of protein-DNA complexes with micrococcal nuclease generally yields a larger region of protection from this nuclease than the digestion of the same protein-DNA complexes with DNaseI. For example, in the case of DNA gyrase-DNA complexes, micrococcal nuclease digestion results in the protection of a DNA fragment about143 ± 3 bp long (Lui and Wang,1978) whereas DNaseI digestion gives fragments which are multiples of ~10 bases (Morisson and Cozzarelli,1981; Kirkegaard and Wang,1981; & Lui and Wang, 1978). This difference has been attributed to the observation that DNaseI recognizes the helical twist of the DNA whereas micrococcal nuclease does not. Therefore, it was of interest to determine the cutting pattern obtained by micrococcal nuclease digestion of the poly (ADP-ribose) polymerase-209 bp SV40 fragment complexes.

FIG 6 →

Figure 6 shows the micrococcal nuclease digestion pattern of the polymerase-209 bp *Eco*RI-*Pst*I fragment of SV40 DNA. At lower concentrations of the polymerase, the length of the DNA protected (~ 64 bases) by the polymerase from micrococcal nuclease is approximately the same as that obtained by digestion with DNaseI, but the number of cleavage sites within the protected region is less than in the case of DNaseI cleavage (compare lane 3, Fig. 2A, with lane 2, Fig. 6). Prominent cutting sites for micrococcal nuclease in the presence of the polymerase occur (arrows, Fig. 6) approximately between position 1789 and position 1802. At higher concentrations of the polymerase, the size of the region protected

from micrococcal nuclease increases (lanes 5-7, Fig. 6). Several cutting sites which occur at lower concentrations of the polymerase (lanes 3 & 4, Fig. 6) disappear at higher concentrations of the polymerase (lane 5-7, Fig. 6). At higher mass ratios of the polymerase : 209 bp fragment, the protected region was entirely contiguous in the case of micrococcal nuclease digestion (lanes 5-7, Fig. 6) but the same region was interrupted by cutting sites for DNaseI (lane 6, Fig. 2A). Micrococcal nuclease protection experiments with 5' *Eco*RI ^{32}P-labeled fragments could not be performed becuase of the pronounced exonuclease (5' phosphodiesterase) activity associated with micrococcal nuclease.

Consistent with the results of DNaseI footprinting, micrococcal nuclease digestion did not indicate binding of the polymerase to the *Bam*HI-*Sal*I fragment of pBR322 (Fig. 4A).

Methylation protection: Exposure of double-stranded DNA to dimethyl sulfate (DMS) results in the specific methylation of the N-7 position of guanine in the major groove and the N-3 position of adenine in the minor groove. Therefore, one of the methods by which the proximity of the contacts between the polymerase protein and the DNA major groove can be assessed is to compare the extent of methylation of the guanines in the polymerase-bound and polymerase-free DNAs. Methylation protection experiments were performed according to the procedure of Siebenlist and Gilbert (1980). Figure 7 shows that in the polymerase-bound DNA, several G residues on the bottom strand are only moderately protected from methylation by DMS, implying that the polymerase makes major groove contacts. We cannot however, rule out the possibility that the

FIG 7 →

polymerase somehow restricts the access of DMS to the G residues without actually penetrating the major groove. The data from these experiments do not permit any conclusions regarding the methylation of adenines in the minor groove since the DMS reaction conditions employed were conducive to G > A cleavage (Maxam and Gilbert, 1980). All the protected Gs (arrows, lane 2, Fig. 7) fall within the regions encompassed by the DNaseI and λ exo footprints. The lack of more complete protection of the G residues is not a result of substantial dissociation of the polymerase from the DNA since DNaseI footprints were still observed even after methylation of the polymerase-DNA complexes with DMS. However this does not rule out the possibility that a minute reorientation of the DNA helix relative to the protein surface may have occured which may not be detectable by DNaseI footprinting.

A comparison between the results of the methylation protection experiment described here for poly (ADP-ribose) polymerase-209 bp fragment and the results of methylation protection experiments with other DNA-binding proteins performed by others could be useful. In the case of DNA gyrase-DNA contacts (Kirkegaard and Wang,1981, report that only two purines show enhanced DMS reactivity) or histone-DNA contacts, little or no protection of the Gs against DMS occurs (Mirzabekov et al.,1977; McGhee and Felsenfeld,1979, report enhanced DMS reactivity of nucleosome DNA at only one 'G' residue), whereas in the cases of the *lac* repressor-operator (Gilbert et al.,1975), the SV40 large T antigen-SV40 replication origin (Ryder et al., 1985) and the promoter-specific transcription factor Sp1- G+C boxes in the SV40

origin promoter-region (Dynan and Tjian, 1983; Kadonaga et al., 1986) complete protection of some G residues against DMS and enhanced DMS reactivity of other G residues has been observed. Therefore, the results of the methylation protection experiments on the poly (ADP-ribose) polymerase-209 bp *Eco*RI-*Pst*I SV40 fragment complex do not strictly resemble any of the above examples but may represent an intermediate category since every G residue within the nuclease footprint is protected from DMS to some extent or the other.

Binding of the polymerase to T4 DNA: The bacteriophage T4 DNA (65.5% A+T) can be naturally glucosylated with bulky glucose residues resulting in the partial (31%) shielding of the major groove (Mathews et al., 1983; Mirzabekov et al,1977). Figure 8 illustrates that poly (ADP-ribose) polymerase does not bind to glucosylated T4 DNA (glu 5-hydroxymethyl cytosine) whereas it can bind to non-glucosylated T4 DNA (5-hydroxymethyl cytosine, HMdC) suggesting that the polymerase makes major groove contacts that are necessary for polymerase binding to T4 DNA. It is however, possible that the glucose residues might by themselves, by an unknown mechanism might interfere with the binding of the polymerase to the DNA. The results of this experiment support, in a general manner, the conclusions from the methylation protection experiment although because of the inherent differences in the binding assay (nitrocellulose filter binding *vs.* DMS footprinting) and the DNAs employed, quantitative comparisons are not possible.

Binding of the polymerase to poly (dA-dT) : poly (dA-dT) and poly (dG-dC) : poly (dG-dC): The polymerase specifically binds to the 209 bp *Eco*RI-*Pst*I fragment and the sequence of this fragment reveals several stretches of A+Ts and a few G+Cs (Fig. 16). The DNaseI footprints, however, do not reveal exclusive binding either to A+Ts or to G+Cs in the sequence. Therefore, it was of interest to find out if the polymerase could bind to poly (dA-dT) : poly (dA-dT) or poly (dG-dC) : poly (dG-dC). Figure 8 shows that the polymerase binds poly (dA-dT) : poly (dA-dT) only moderately (20%) better than poly (dG-dC) : poly (dG-dC) as tested by the nitrocellulose filter binding assay (Sastry and Kun, 1987) indicating that the polymerase binds only with moderately higher affinity to alternating dA-dT stretches.

We have also employed the nitrocellulose filter-binding assay to test if the polymerase protein displayed any affinity towards other nucleic acids, such as, ssRNA, dsRNA, the classical Z-DNA - brominated poly (dG-dC) : poly (dG-dC), and ssDNA (all courtesy of Dr. D.A. Zarling, SRI Inc., Palo Alto, CA). None of these nucleic acids bound the polymerase to any significant extent.

Binding of a 36Kd polypeptide of Poly (ADP-ribose) polymerase to the 209 bp SV40 fragment: In a previous work (Buki and Kun,1987) it was demonstrated that proteolytic cleavage of the polymerase (MW 120,000) with plasmin yielded several polypeptide fragments ranging in size between ~54 Kd and14 Kd (lane 2, Fig. 9). These polypeptides generated by plasmin were electroblotted onto nitrocellulose membranes (see **Materials and Methods**) and the membranes were exposed to the ^{32}P-labeled 209 bp *Eco*RI-*Pst*I

fragment of SV40 DNA (western blotting). Figure 9 (lane 5) demonstrates that only a 36 Kd polypeptide binds strongly to the ^{32}P DNA fragment. Other bands displaying weak affinity for the ^{32}P-DNA fragment are also seen but the 36 Kd polypeptide is the only major band. The 36 Kd band was shown (Buki and Kun,1987) to be a doublet consisting of two polypeptides differing by a single amino-acid. The two peptides were separated from each other by high performance liquid chromatography (described in Buki and Kun, 1987) and run on a polyacrylamide gel (lanes 2 &3, Fig.10). Western blotting revealed that only one peptide (peptide 'A') showed binding to the ^{32}P DNA fragment (lane 4, Fig.10). Furthermore, it was found that binding of a polyclonal antibody (described in Buki and Kun,1987) raised against the whole polymerase protein does not prevent the binding of the ^{32}P DNA fragment to either the whole protein (lane 6, Fig. 9) or to the DNA-binding 36 kd 'A' peptide (lane 7, Fig. 9 & lane 6, Fig.10). The antibody by itself does not bind DNA (data not shown). This experiment indicates that the antibody does not bind to the DNA-binding epitope of the polymerase protein. This finding was important since previously this antibody was only partially characterized in terms of its binding epitopes on the polymerase protein.

FIG 10 →

<u>The 36 Kd peptide generates the same footprint as the whole polymerase protein</u>. The purified 36 Kd 'A' peptide was subjected to DNase1 footprinting with the 209 bp SV40 fragment. It was found that the 36 kd 'A' peptide generated the same footprint as the whole

polymerase protein (compare lane 6 with lane 11, Fig. 2A) suggesting that the DNA binding activity which is responsible for the observed DNaseI footprint pattern (Fig. 2A, lane 6) for the whole enzyme is confined to only a segment of the whole protein.

In figure 5 it was pointed out that two minor contacts were detected when the whole polymerase was employed for DNaseI footprinting. It is possible that these two minor contacts are due to a region of the polymerase that lies outside the 36 Kd segment and these contacts were either absent or present in the unresolved portions of footprint patterns generated by the whole polymerase on the 209 bp SV40 fragment.

Poly (ADP-ribose) polymerase also binds to the ends of DNA: The proposal that poly (ADP-ribose) polymerase binds to the termini of linear DNA (Benjamin and Gill, 1980a; Benjamin and Gill, 1980b & Berger and Petzold, 1985) has never been tested directly. Figures 11-14 demonstrate the results of exonucleolytic digestion of polymerase-DNA complexes.

a) λ exonuclease (5'→3') digestions: Figure 11 (lane 3) shows that in the absence of the polymerase, greater than 90% of the ^{32}P-209 bp fragments labeled at the PstI site are digested to various lengths and λ exo shows several prominent pause sites (see the section entitled 'The 209 bp *Eco*RI-*Pst*I fragment is intrinsically bent' in **Results**). But in the presence of increasing amounts of the polymerase, a majority of the DNA fragments become resistant to λ exonuclease and migrate as full-length molecules (lanes 4 & 5, Fig. 11). Thus the polymerase protein appears to block λ exo activity by binding to the 5' end of the top strand. Even in the presence of high

Fig 11 →

concentrations of the polymerase, a fraction of the DNA molecules are digested to various lengths (lane 5 , Fig. 11). Interestingly, the prominent λ exo pause sites which are present in the absence of the polymerase, are not observed when the polymerase is bound to the DNA (compare lane 3 with lanes 4 & 5, Fig. 11). This result sugests that polymerase binding to the DNA results in a change in the DNA conformation, thus allowing the observed λ exonucleolytic progression beyond the pause sites (which exist in the absence of the polymerase) in the fraction of the DNA molecules susceptible to λ exo. In the presence of the polymerase, a new λ exo pause site (approximately at position1897, Fig. 11; ▼, Fig. 16) is can be seen, perhaps indicating the 5' boundary of the region spanned by the polymerase molecules binding to the top strand. Since the DNA is presumably double stranded and the polymerase also makes contacts on the bottom strand, the influence of this binding to the bottom strand on the creation of the observed λ exo pause site (~ at position 1897) on the top strand cannot be ruled out. The proposed 5' boundary (~ at position 1897) extends beyond the 3' boundary of the DNaseI footprint. This apparent discrepancy may be due to the differences in the mode of recognition of the protein-DNA complexes by λ exo and DNaseI. Similar results were obtained when the 3' end of the *Eco*RI site was ^{32}P labeled. Figure 12 shows that λ exo has only one (or a group of closely spaced unresolved) pause site(s) unlike the top strand where several pause sites are observed (compare lane 3, Fig. 11 with lane 2, Fig.12). In the presence of the polymerase the pause site(s) is again not observed (lanes 3 & 4, Fig. 12), suggesting a polymerase-induced conformational change in the DNA allowing λ

FIG 12 →

exo processivity in a fraction of DNA molecules. A new pause site appears at approximately 1917 (lane 4, Fig. 12) suggesting the 5' boundary of the region spanned by the polymerase on the bottom strand (Fig16 ; ▲). Again, the possibility that polymerase molecules bound to the top strand could influence the creation of a new pause at position 1917 has to be considered. When a 10-fold mass excess of unlabeled DNA fragments was added to the exonuclease assays the blockage of the λ exo activity by the polymerase was alleviated (lanes 6 and 7, Fig. 11 and lanes 5 and 6, Fig. 12), indicating that the polymerase by itself does not irreversibly inactivate the λ exo activity.

b) Exonuclease III digestion: Somewhat different results were obtained with exo III (3'→5' exonuclease) as compared to λ exo (5'→3' exonuclease). Figure 13 (lanes 3 & 4) shows that binding of the polymerase to the 3' end of the top strand completely blocks the exo III activity from the 3' end. Unlike the results of digestion with λ exo, not even a small fraction of the DNA molecules are susceptible to exo III attack in the presence of the polymerase. This could suggest either a) that the polymerase binds more tightly to the 3' ends than to the 5' ends or b) that the mode of recognition of the polymerase-DNA complexes by exo III is different from that of λ exo in the presence of the polymerase. The differences in the extent of digestion observed when the polymerase-DNA complexes were digested with the two nucleases (λ exo & exo III) cannot be attributed to the presence of a PO_4 group on the 5' end (*versus* an OH group on the 3' end) of the DNA restriction fragments because the same results were obtained for λ exo digestions employing dephosphorylated 5' ends (data not shown). The polymerase protein

does not *per se* irreversibly inactivate the exo III activity (lane 2, Fig. 13). As in the case DNaseI footprinting experiments, poly (dI-dC) : poly (dI-dC) competes for polymerase binding to the 3' ends in exo III assays (lane 6, Fig. 13) presumably because the polymerase binds to poly (dI-dC) : poly (dI-dC) (see the following section). One possibility that is not resolved by the exonuclease experiments is that the polymerase could make physical contacts with only one of the ends (either 5' or 3') without contacting the other end and still effectively restrict nuclease access on the non-contacted end.

Binding of the polymerase to the ends of DNA is nonspecific: The binding of the polymerase to the 5' and 3' ends of several restriction fragments and polynucleotides was investigated employing the λ exo and the exo III assays. The results were generally the same as those described for the 209 bp *Eco*RI-*Pst*I fragment of SV40 DNA. For example, figure 14 demonstrates that, although the polymerase does not bind to the internal length of the *Bam*HI-*Sal*I fragment of *pBR*322 as assayed by DNaseI footprinting (Fig. 4) the polymerase still binds to the 3' end of the top strand as indicated by the blockage of exo III activity. Experiments with λ exo showed that the polymerase also binds to the 5' ends of the *Bam*HI-*Sal*I fragment (data not shown). Binding of the polymerase to the 5' or the 3' ends of polynucleotides such as poly (dI-dC) : poly (dI-dC) and poly (dA-dT) : poly (dA-dT) was also observed. These experiments demonstrate that the polymerase binds nonspecifically to the 5' and 3' termini of DNA since DNaseI footprints (such as in Fig. 1-3) are not observed with these restriction fragments and polynucleotides

FIG 14 →

whereas, the exonucleolytic assays could detect polymerase binding.

It is noted that earlier observations by de Murcia et al., (1983) employing electron microscopy revealed that very few polymerase molecules were actually bound to the ends of DNA which is in contradiction to our present findings. The discrepancy could be due to the differences in the two techniques employed (electron microscopy *vs.* nuclease protection) and the DNAs (coenzymic DNA *vs.* purified restriction fragments) used in the two studies.

The 209 bp *Eco*RI-*Pst*I fragment is intrinsically bent: The apparent reduced mobility of certain restriction fragments on high percentage polyacrylamide (5-12% acrylamide) gels, as compared to the expected mobility based on their known lengths, has been attributed to intrinsic DNA bending (Marini et al., 1982; Koo et al., 1986) although other interpretations have been proposed (Diekmann and Lilley,1987). DNA bending has been attributed to the presence of adenine : thymine tracts (Koo et al.,1986) and/or ApA wedges (Ulanovsky and Trifonov,1987; Hagerman,1986) coinciding with the helical periodicity of DNA. A molecular model for the bent junction has been proposed (Selsing et al.,1979).

Applying the "rules" of DNA bending (Koo et al.,1986; Ulanovsky and Trifonov,1987), several loci of potential bending are revealed in the 209 bp *Eco*RI-*Pst*I fragment (Fig. 16; capitalized 'A's). To experimentally verify the presence of DNA bends, we applied the mobility retardation assay for DNA bending. Figure 15A shows that the 209 bp fragment migrates between the 271 bp and 281 bp markers on a 2% agarose gel, whereas on a 5% polyacrylamide gel it runs between the 281 bp and 310 bp markers (Fig. 15B). On a 12%

FIG 15 →

polyacrylamide gel, the 209 bp fragment migrates at approximately 350 bp (Fig. 15C) i.e., with an exaggeration of 167.4%; the ratio of apparent length to the actual length at 4^0C is 1.674. Consistent with the behavior expected of bent DNA fragments (Griffith et al., 1986; Levene et al.,1986) the 209 bp fragment migrates at approximately the actual size on a 5% polyacrylamide gel run at room temperature (22^0C) and at 75V (data not shown).

An interesting aspect of the 209 bp *Eco*RI-*Pst*I fragment is that the prominent λ exo pause sites (at positions ~1950 and ~1912, Fig. 11; and at position ~1898, Fig. 12) occur at or very close to the proposed loci of bending (capitalized 'A' tracts, Fig. 16). Whether this actually means that λ exo recognizes and pauses at bends on the DNA molecule is at present unknown.

4. Discussion

In a previous communication (Sastry and Kun,1987) we reported the cooperative interactions between poly (ADP-ribose) polymerase and histones and the induction of torsional stress as result of polymerase binding to DNA. The present paper, to our knowledge, represents the first effort at studying the mechanism of binding of the polymerase to DNA.

Specificity of the binding of the polymerase to DNA: It is clear from the results of the experiments reported in this paper that mechanistically there are two kinds of contacts that poly (ADP-ribose) polymerase makes with certain types of linear DNA

molecules such as the 209 bp *Eco*RI-*Pst*I fragment of SV40 DNA. 1. Binding of the polymerase to the internal length of the DNA, a reaction shown to be specific for only the 209 bp *Eco*RI-*Pst*I fragment. 2. Binding of the polymerase to the 5' and 3' ends of DNA, a nonspecific reaction observed for several DNAs and polynucleotides.

There are at least two obvious characteristics of the 209 bp fragment which might dictate the specific binding of the polymerase to this restriction fragment : DNA sequence and DNA conformation (or both). Insufficient length as a factor which prevented the polymerase from binding to the other fragments can be ruled out since all other DNA fragments tested are longer than the length of the DNaseI footprint on the 209 bp fragment. The DNaseI footprints of the polymerase on the 209 bp *Eco*RI-*Pst*I fragment reveal that the polymerase protects or makes contacts with both A+T and G+C residues and displays no obvious preference for any particular consensus of bases. The 209 bp fragment is more A+T rich overall than the other DNA fragments tested. Interestingly, the other fragments tested also possess runs of A+Ts, but these fragments do not show DNaseI footprints in the presence of the polymerase. The polymerase on the other hand, binds slightly more effectively to poly (dA-dT) : poly (dA-dT) as tested by the filter-binding assay. Taken together, these observations suggest that for the polymerase to bind the internal length of DNA the presence of higher (>60%) overall A+T content appears to be important although not sufficient.

The 209 bp fragment is intrinsically bent (Fig. 15). DNA bending has been demonstrated to be an important factor which influences the binding of a variety of DNA-binding proteins to DNA

(Drew and Travers, 1985; Poljack and Gralla, 1987 a,b; Ryder et al.,1986; Shuey and Parker, 1986; Gronenborn et al., 1984; Zahn and Blattner, 1987; Bossi and Smith, 1984; Koepsel and Khan, 1986; Radic et al., 1987; & Snyder et al., 1986.). For instance, in the case of the nucleosome octamer the placement of the nucleosome on the DNA (nucleosome phasing) is influenced by the presence of DNA bends (Drew and Travers, 1985; & Travers, 1987) and poly (dA) : poly (dT) which is thought to be a 'conformationally stiff' helix does not allow the formation of nucleosomes on itself (Leslie et al.,1986; Hogan and Austin, 1987; Rhodes, 1979; Prunell, A, 1982; & Kunkel and Martinson, 1981). Furthermore, X-ray diffraction analysis revealed that the DNA which wraps around the histone octamer exists in sharp bends (Richmond et al., 1984). In an earlier report (Sastry and Kun, 1987) we demonstrated that a single octamer of histones could be assembled *in vitro* on to the 209 bp fragment. This supports our contention (drawing analogy from the work of Drew and Travers, 1985; Poljack and Gralla, 1987 a,b) that the 209 bp fragment is 'conformationally flexible'. Although, we have not directly demonstrated the role of DNA bends (with the aid of, for example, 'ring closure experiments' such as those of Zahn and Blattner, 1987) in the case of the association of poly (ADP-ribose) polymerase with DNA, DNA bending (Fig. 15) might be a contributing factor in the observed specificity of binding of the polymerase to the internal length of the 209 bp *Eco*RI-*Pst*I fragment of SV40 DNA (Fig. 1-3). Experiments to directly test this proposal are in progress. It also remains to be seen if polymerase binding to the 209 bp fragment can by itself enhance the already existing intrinsic bend (s) in the 209 bp *Eco*RI-*Pst*I DNA fragment.

A nonspecific competitor, poly (dI-dC) : poly (dI-dC) can compete for binding of the polymerase to the 209 bp fragment (Fig. 1-3, & Fig. 13). Poly (dI-dC) : poly (dI-dC) does not show DNaseI footprints of the polymerase indicating that the polymerase does not bind to the internal length of the polynucleotide but binds to the ends of the polynucleotide (as assayed by the exonuclease digestions). We therefore assume that the polymerase binding to the ends of poly (dI-dC) : poly (dI-dC) explains its ability to act as a competitor.

Proposed mechanism of polymerase binding to DNA: An idealized version of the mechanism of poly (ADP-ribose) polymerase binding to linear DNA could be as follows. The polymerase may first nonspecifically recognize the 5' or the 3' ends of DNA. This event might serve as an 'anchor' for the polymerase protein molecules. Further binding to the internal length of DNA ('wrapping around' of segments of DNA) might depend on DNA sequence (ie., A+T richness) and DNA conformation (i.e., DNA bending). These two events need not necessarily occur separately but may be concerted. The observation that DNaseI protection occurs in blocks equivalent to about one turn of the DNA helix at higher polymerase : DNA mass ratios (lane10, Fig.1), suggests that the binding of the polymerase to the internal length of the DNA may involve spatially close cooperative interactions (reviewed by Ptashne, 1986) between polymerase molecules. In an earlier report (Sastry and Kun, 1987) we showed that the polymerase can also bind to covalently closed circular SV40 DNA i.e., DNA without termini, implying that the binding of

polymerase molecules to DNA termini is not a prerequisite for the polymerase to exhibit binding to the internal length of DNA. In this case polymerase binding might nucleate at an overall A+T rich and / or bent region although this idea has not been tested.

The observation that an isolated 36 Kd polypeptide fragment of the polymerase binds DNA as assayed by western blotting (Figs 9 & 10) and produces footprints indistiguishable from those made by the whole polymerase protein (Fig. 2A) suggests that the DNA-binding domain of the polymerase might be 'extended out' like an 'arm' from the rest of the protein. This suggestion is in agreement with a previous proposal that the DNA and NAD^+ binding domains of the polymerase protein are proteolytically separable (Buki and Kun, 1987; & Kameshita et al., 1985).

Relationship to other work: It has been reported that DNA nicks and breaks, caused by nucleases stimulate the enzymatic activity of the polymerase *in vitro* (Benjamin and Gill,1980a, Benjamin and Gill,1980b) as well as *in vivo* (see Ueda and Hayaishi, 1985, for review). On the other hand, in certain cell types where highly fragmented DNA occurs *in situ* during development, the enzymatic activity of poly (ADP-ribose) polymerase is not correspondingly high (Skidmore et al.,1985). The demonstration that the polymerase can bind nonspecifically to the 5' and/or 3' termini of a variety of DNA molecules (see **"Results"**, Figs. 11-14), implies that the observed stimulation of the enzymatic activity of the polymerase owing to the introduction of nicks and/or breaks into DNA by DNA-damaging agents (as reported by others) may be related to the nonspecific

binding of the polymerse to the termini of DNA at the sites of the nicks or breaks. However, we have not verified utilizing our assay system if the polymerase binds to DNA nicks and breaks in the same fashion as it does to the DNA ends.

A prevailing idea regarding the interaction of poly (ADP-ribose) polymerase with DNA is it that the polymerase does not display any sequence-specific binding to DNA and that any DNA molecule possessing nicks or breaks could stimulate the enzymatic activity of the polymerase (Benjamin and Gill, 1980a; Benjamin and Gill, 1980b; & Berger and Petzold, 1985). The findings presented in this paper and the recent work from our laboratory seem to argue against this line of thought, assuming that the mechanism of interaction of the polymerase with DNA is unchanged by the presence of NAD^+. Firstly, polymerase binding to the ends of DNA has been shown to be nonspecific i.e., all the DNAs tested bind the polymerase at DNA termini. Secondly, polymerase binding to the internal length of the DNA seems to be influenced by the DNA sequence (overall A+T content) and / or conformation (DNA bending). Binding of the polymerase to closed circular DNA (i.e., DNA without nicks or breaks) also occurs (Sastry and Kun, 1987). Data on the kinetics of enzymatic activity with DNA molecules differing in their sequence but possessing the same length and the same number of ends also demonstrate the importance of DNA sequence (Hakam et al., 1987). In view of the foregoing evidence, it is proposed that both DNA sequence and DNA conformation could play an important role in polymerase-binding to certain DNA molecules.

In a preliminary report, Ittel et al., (1985) showed that

micrococcal nuclease digestion of poly (ADP-ribose) polymerase-coenzymic DNA complexes resulted in the protection of approximately 145 bp and 60-90 bp of DNA, depending on the extent of micrococcal nuclease digestion. They did not perform any DNaseI protection experiments nor did they use other nucleases as probes. Co-enzymic DNA is highly fragmented, heterogeneous in size ranging usually between 0.5-5 kb on agarose gels, and remains uncharacterized in terms of its sequence or structure. It is unclear as to whether the same population of coenzymic DNA molecules, in terms of its composition, is obtained when the polymerase is prepared by different methods from different laboratories using different animal tissues. The results of DNaseI and micrococcal nuclease footprinting experiments in the present work show that 64-88 bp of DNA is protected from nuclease attack, which is about the same extent of protection against micrococcal nuclease described by Ittel et al., (1985).

Our earlier finding that polymerase binding to closed circular SV40 DNA induces superhelicity (Sastry and Kun,1987) is in ageement with the present finding that the DNA helix lies outside the polymerase protein.

References

Bauer, P., Hakam, A. and Kun, E. (1986). *Fed. Eur. Biochem. Soc. Letts.* **195.** 331-338.

Benjamin, R. C. & Gill, D. M. (1980)a. *J. Biol. Chem.* **255**. 10502-10508.

Benjamin, R. C. & Gill, D. M. (1980)b. *J. Biol. Chem.* **255**. 10493-10501.

Berger, N. A. & Petzold, S. J. (1985). *Biochemistry.* **24**. 4352-4355.

Bohmann, D., Keller, W., Dole, T., Scholer, H. R., Tebb, G. & Mattaj, I. W. (1987). *Nature*. **325**. 268-272.

Bossi, L. & Smith, D. M. (1984). *Cell.* **39**. 643-652.

Buchman, A. R., Burnett, L. & Berg, P. (1980) In *DNA Tumor Viruses.* (Tooze, J. ed.) pp. 799-829. Cold Spring Harbor Laboratory, N. Y.

Buki,K., Kirsten, E. & Kun, E. (1987). *Anal. Biochem.* in press.

Buki,K. & Kun, E. (1987). *Biochemistry.* Submitted.

de Murcia, G., Jongstra-Bilen, J., Ittel, M. E., Mandel, P. & Delain, E. (1983). *EMBO J.* **2**. 543-548.

Diekmann, S. & Lilley, D. M. J. (1987). *Nucl. Acids. Res.* **15**. 5765-5774.

Drew, H. R. (1984). *J. Mol. Biol.* **176**. 535-557.

Drew, H. R. & Travers, A. A. (1985). *J. Mol. Biol.* **186**. 773-790.

Dynan, W. S. & Tjian, R. (1983). *Cell.* **32**. 669-680.

Dynan, W. S., Tjian, R. & Schimke, R. T. (1986). *Nature.* **319**. 246-248.

Gaal, J. C. & Pearson, C. K. (1985) *Biochem. J.* **230**: 1-18.

Galas, D. J. & Schmitz, A. (1978). *Nucl. Acids. Res.* **5**. 3157-3171.

Gilbert, W., Maxam, A. & Mirzabekov, A. D. (1975). *In Control of Ribosome Synthesis* (Kjeldgaard, N. D. & Maalφe, O., eds). pp. 133-148. Academic Press. New York.

Griffith, J., Bleyman, M., Rauch, C. A., Kitchin, P. A. & Englund, P. T. (1986).
Cell. **46**. 717-724.

Gronenborn, A. M., Nermut, M. V., Eason, P. and Clore, G. M. (1984). *J. Mol. Biol.* **179**: 751-757.

Hagerman, P. J. (1986). *Nature*. **321**. 449-450.

Hakam, A., McLick, J., Buki, K. & Kun, E. (1987). *Fed. Eur. Biochem. Soc. Lett.* **212**. 73-78.

Hashida, T., Oghushi. H., & Yoshihara, K. (1979). *Bichem. Biophys. Res. Comm.* **88**. 305-311.

Hogan, M. E. & Austin, R. H. (1987). *Nature*. **329**. 263-266.

Ittel, M., Jongstra-Bilen, J., Niedergang, C, Mandel, P. & Delain, E. (1985). *In ADP-ribosylation of Proteins* (Althaus, F. R., Hilz, H. & Shall, S. eds) pp. 60-68. Springer-Verlag. Berlin.

Jackowski, G. & Kun, E. (1981) *J. Biol. Chem.* **256**. 3667-3670.

Kadonaga, J. T., Jones, K. A. & Tjian, R. (1986). *Trends in Biochem. Sci.* **11**. 20-23.

Kameshita, I., Matsuda, M., Nishikimi, M., Ushiro, H. & Shizuta, Y. (1985). *J. Biol. Chem.* **261**. 3863-3868.

Kirkegaard, K. & Wang, J. C. (1981). *Cell*. **23**. 721-729.

Koepsel, R. R. & Khan, S. A. (1986). *Science*. **233**. 1316-1318.

Koo, H-S., Wu, H-M. & Crothers, D. M. (1986) *Nature.* **320**. 501-506.

Kun, E., Minaga, T., Kirsten, E., Hakam, A., Jackowski, G., Tseng, Jr., A. and Brooks, M. (1986). *In Biochemical Action of Hormones*,.(Litwack, J., ed) Academic Press, N. Y. Vol. 13, pp. 33-55.

Kun, E. Kirsten, E., Milo, G. E., Kurian, P. and Kumari, H. L. (1983). *Proc. Natl. Acad. Sci. USA* **80**.7219-7223.

Kunkel, G. R. & Martinson, H. G. (1981). *Nucl. Acids. Res.* **9**. 6869-6888.

Leslie, A. G. W., Arnott, S., Chandrasekharan, R. Ratiff, R. L. (1980). *J. Mol. Biol.* **143**. 49-72.

Levene, S. D. , Wu, H-M. and Crothers, D.M. (1986). *Biochemistry.* **14**. 3787-3794.

Lui, L. F. & Wang, J. C. (1978). *Cell.* **15**. 979-984.

Lutter. L. C. (1978). *J. Mol. Biol.* **124**. 391-420.

Maniatis, T., Fritch, E. and Sambrook, J. (1982) *Molecular Cloning - A Laboratory Manual.* Cold Spring Harbor Laboratory, N. Y.

Marini, J. C., Levene, S.D., Crothers, D.M. and Englund, P. T. (1982). *Proc. Natl. Acad. Sci., U.S.A.* **79**. 7664-7668.

Mathews. C. K., Kutter, E. M., Mosig, G., & Berget, P. (eds). (1983). The Bacteriphage T4. Amer. Soc Microbiol. Publ.

Maxam, A. & Gilbert, W. (1980). *Meth. Enzymol.* **65**. 499-560.

McGhee, J. D. & Felsenfeld, G. (1979). *Proc. Natl. Acad. Sci., U.S.A.* **76**. 2133-2137.

McGhee, J. D. & Felsenfeld, G. (1980). *Ann. Rev. Biochem.* **49**. 1115-1156.

Mirzabekov, A. D., San'ko, D. F., Kolchinsky, A. M., & Melnikova, A. F. (1977) *Eur. J. Biochem.* **75**. 379-389.

Moller, A., Nordheim, A., Kozlowski., S. A., Patel, D. J. & Rich, A. (1984). *Biochemistry*. **23**. 54-62.

Morrison, A. & Cozzarelli, N. R. (1981). *Proc. Natl. Acad. Sci., U.S.A.* **78**. 1416-1420.

Noll, M. (1974). *Nucl. Acids. Res.* **1**. 1573-1578.

Oghushi, H., Yoshihara, K. & Kamiya, T. (1980). *J. Biol. Chem.* **255**. 6205-6211.

Poljack, L. G. & Gralla, J.D. (1987). *Nucl. Acids. Res.* **15**. 5433-5442.

Poljack, L. G. & Gralla, J.D. (1987). *Biochemistry.* **26**. 295-303.

Portugal, J. & Waring, M. J. (1987). *Nucl. Acids. Res.* **15**. 885-903.

Prunell, A. (1982). *EMBO. J.* **1**. 173-179.

Ptashne, M. (1986). *Nature.* **322**. 697-701.

Radic, M. Z., Lundgreen, K. & Hamkalo, B. A. (1987). *Cell.* **50**. 1101-1108.

Robbins, K. C. & Summaria, L. (1970). *Meth. Enzymol.* **19**. 184-199.

Rhodes, D. (1979). *Nucl. Acids. Res.* **6**. 1805-1816.

Richmond, T. J., Finch, S. T., Rushton, B., Rhodes, D. and Klug, A. (1984). *Nature.* **311**: 532-537.

Roychaudhary, R. & Wu, R. (1980). *Meth. Enzymol.* **65**. 43-63.

Ryder, K., Vakalopoulou, E., Mertz, R., Mastrangelo, I., Hough, B., Tegtmeyer, P. & Fanning, E. (1986). *Cell.* **42**. 539-548.

Sastry, S. S. & Kun, E. (1987). *J. Biol. Chem.* (Submitted).

Sawadogo, M. & Roeder, R. G. (1985). *Cell.* **43**. 165-175.

Selsing, E., Wells, R. D., Alden, C. J. & Arnott, S. (1979). *J. Biol. Chem.* **254**.5417-5422.

Shuey, D. J. & Parker, C. S. (1986). *Nature.* **323**. 459-461.

Siebenlist, U. & Gilbert, W. (1980). *Proc. Natl. Acad. Sci. U. S. A.* **77**. 122-126.

Skidmore, C. J., Jones, J., Oxberry, J. M., Chaudin, E. & Coumis, M. P. (1985). *In ADP-ribosylation of Proteins* (Althaus, F. R., Hilz, H. & Shall, S. eds.) pp. 116-123. Springer-Verlag. Berlin.

Snyder, M., Buchman, A. R. & Davis, R. W. (1986). *Nature.* **324**. 87-89.

Suck, D. & Oefner, C. (1986). *Nature.* **321**. 620-625.

Sutcliffe, J. G. (1979). *Cold. Spring Harbor. Lab. Symp. Quant. Biol.* **43**. 77-90.

Szewczyk. B. & Kozloff, L. M. (1985). *Anal. Biochem.* **150**. 403-407.

Travers, A. A. (1987). *Trends in Biochem. Sci.* **12**. 108-112.

Tseng, Jr., A., Lee, W. M. F., Kirsten, E., Hakam, A., McLick, J., Buki, K. and Kun, E. (1987). *Proc. Natl Acad. Sci. USA* **84**: 1107-1111.

Ueda, K. & Hayaishi, O. (1985) *Annu. Rev. Biochem.* **54**: 73-100.

Ulanovsky, L. E. & Trifonov, E. N. (1987). *Nature*. **326**. 720-722.

Zahn, K. & Blattner, F. R. (1987). *Science*. **236**. 416-422.

FIGURE 1

FIGURE 2

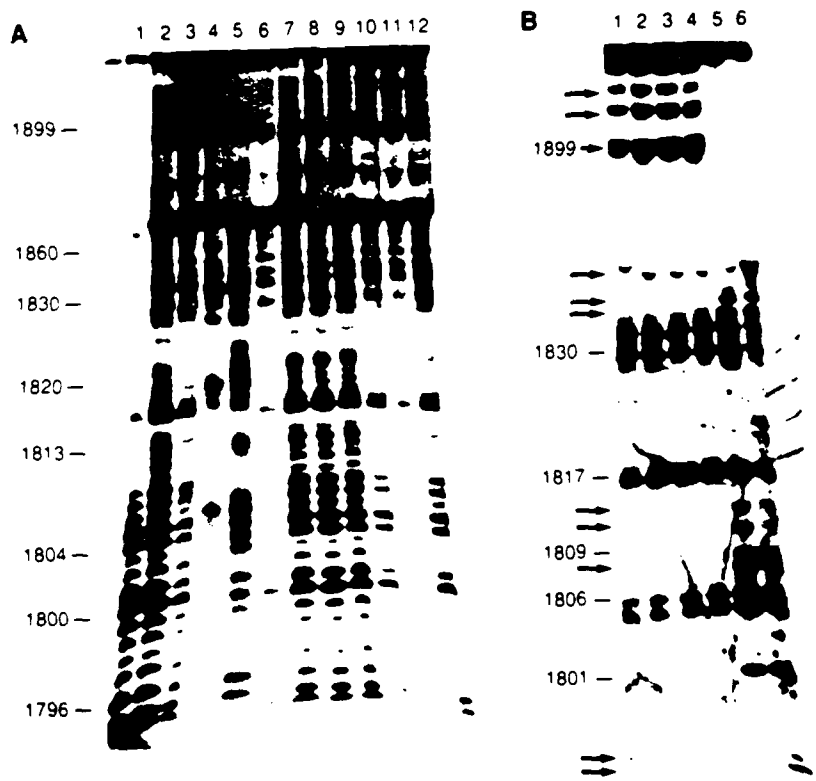

A
1 2 3 4 5 6 7 8 9 10 11 12
1899
1860
1830
1820
1813
1804
1800
1796
B
1 2 3 4 5 6
1899
1830
1817
1809
1806
1801

FIGURE 3

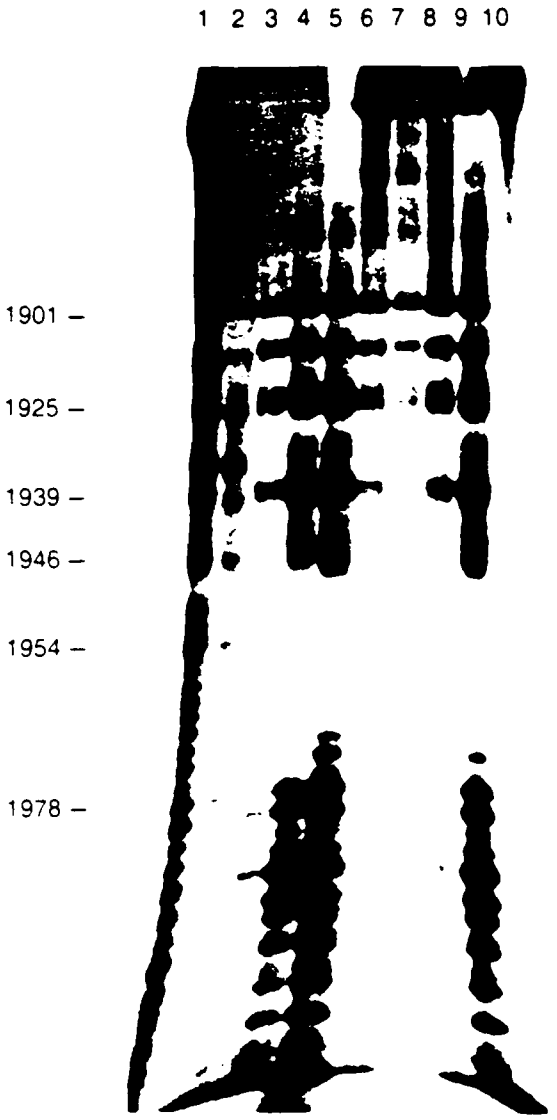


FIGURE 4

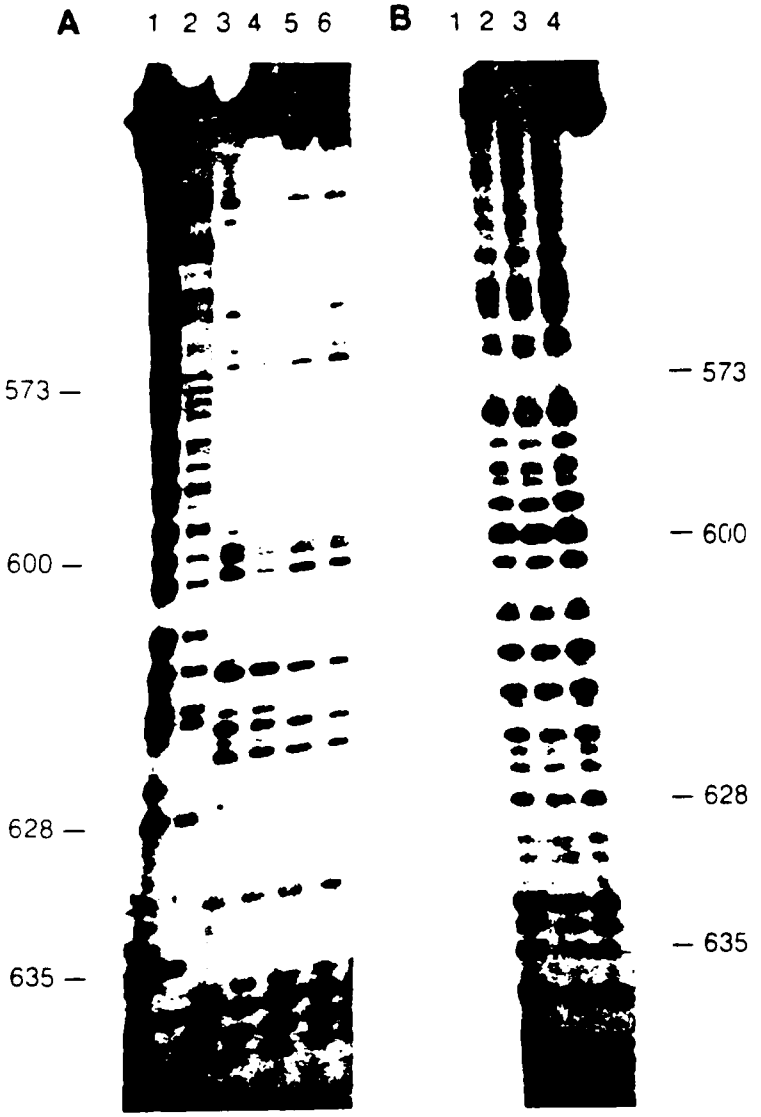


FIGURE 5

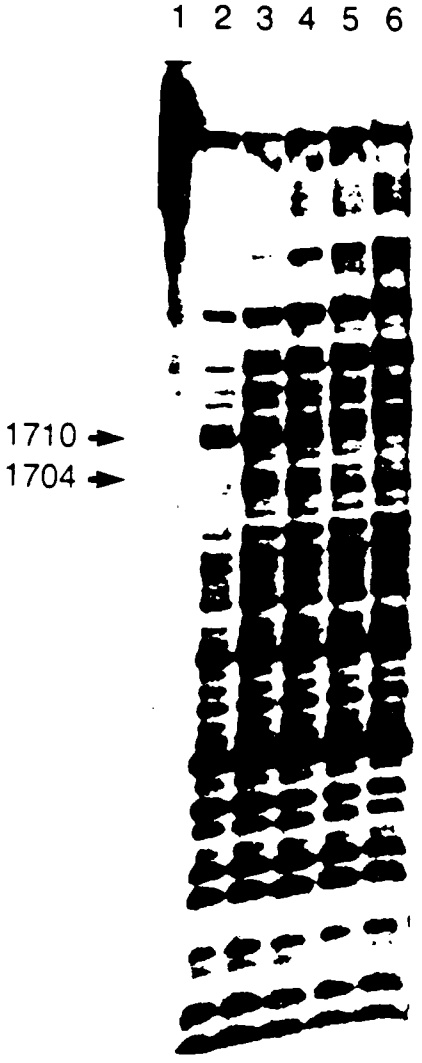


FIGURE 6

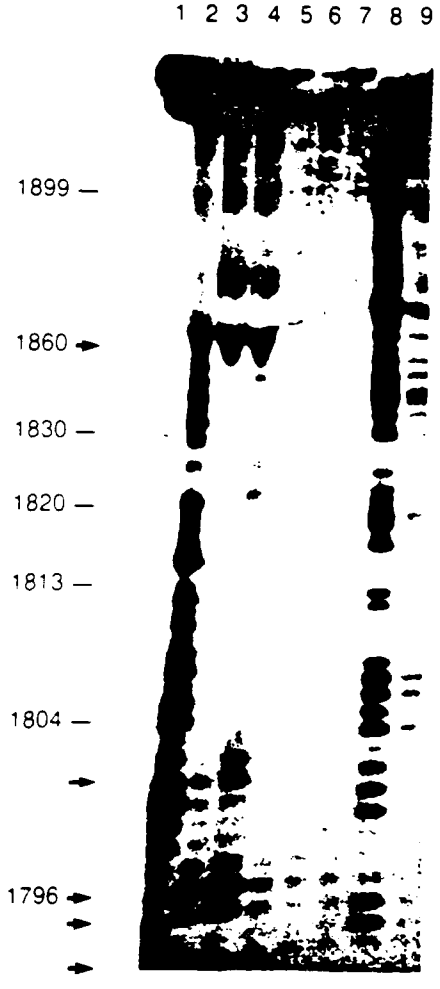


FIGURE 7

FIGURE 8

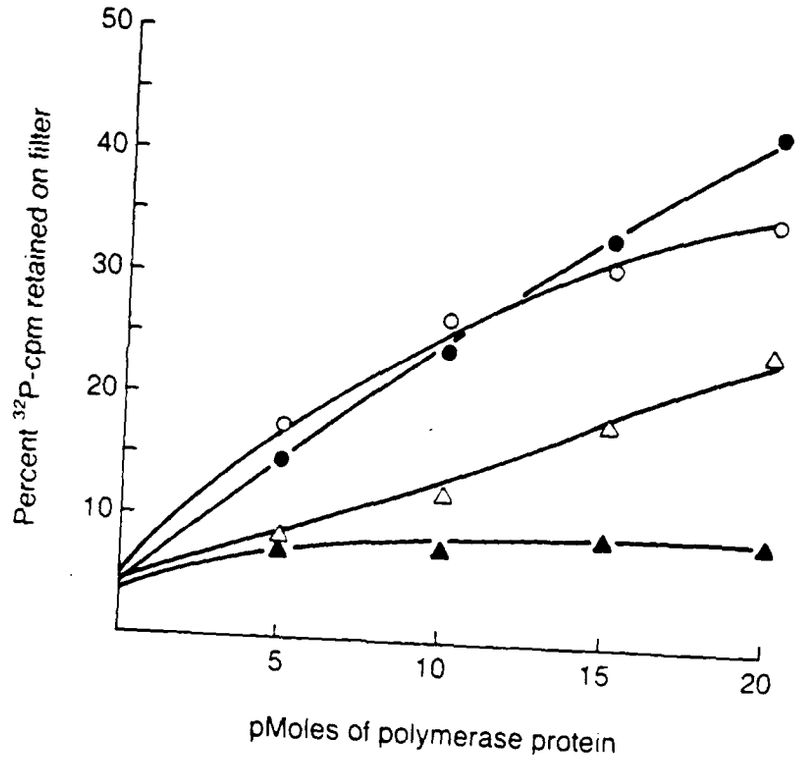

50
40
30
20
10
Percent ^{32}P-cpm retained on filter
5
10
15
20
pMoles of polymerase protein

FIGURE 9

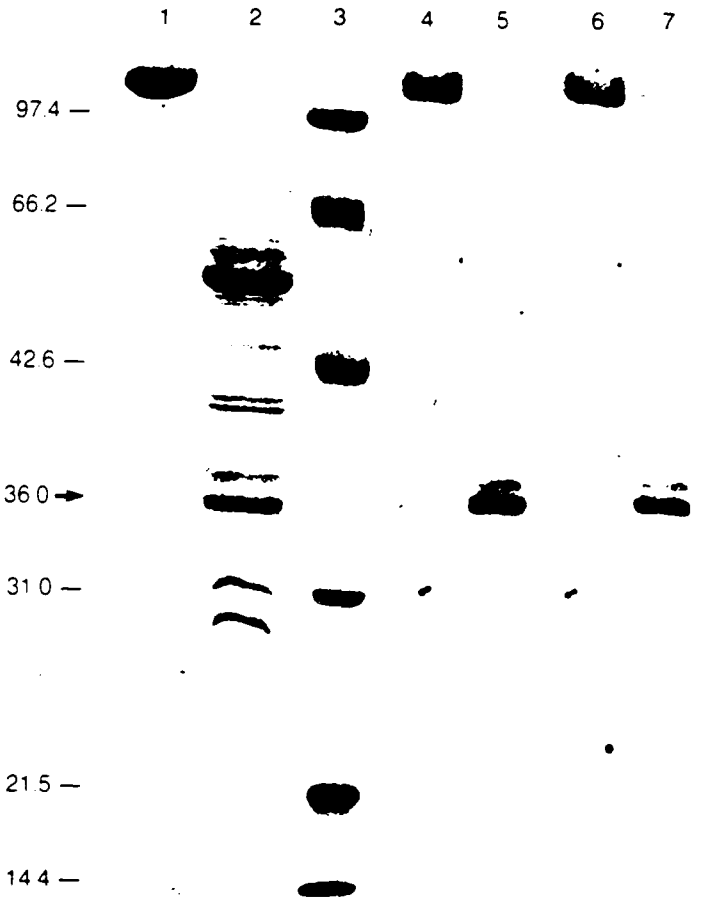


FIGURE 10

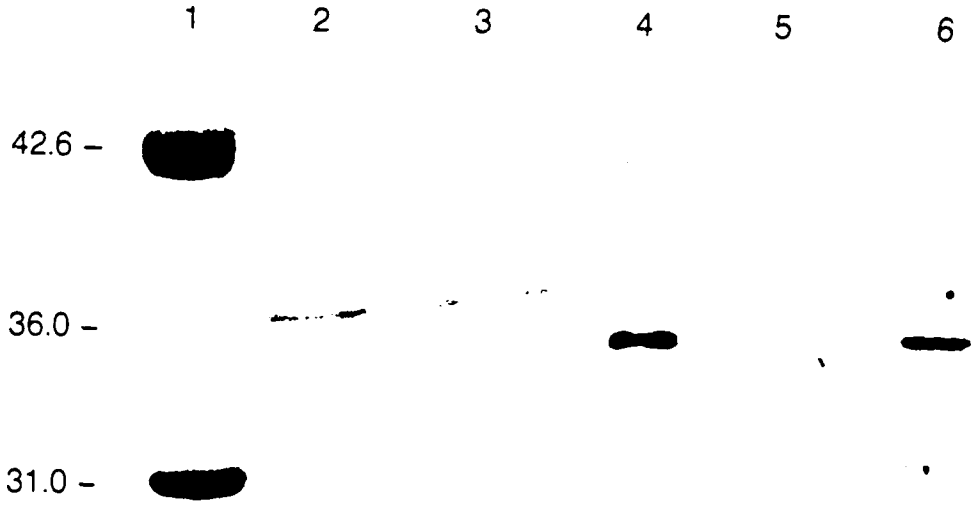


FIGURE 11

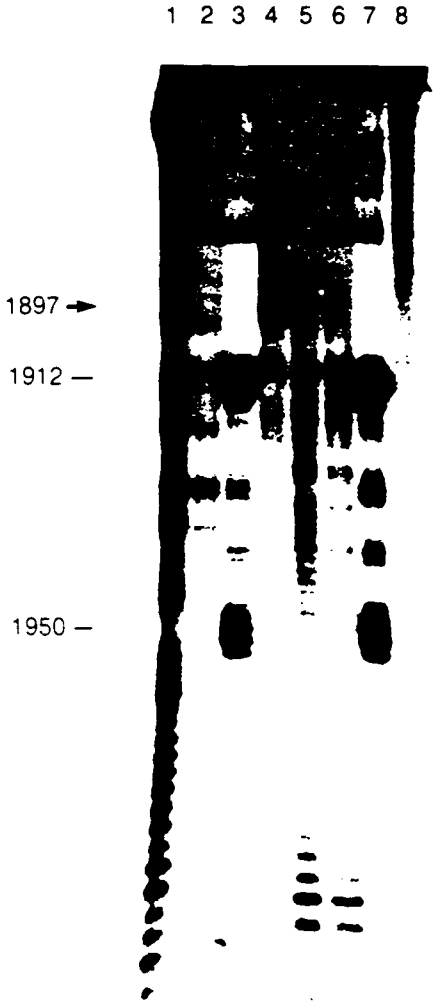


FIGURE 12

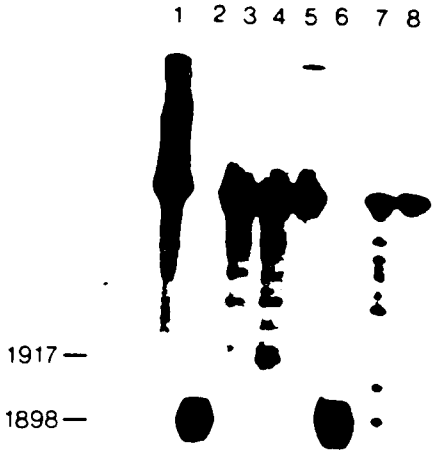


FIGURE 13

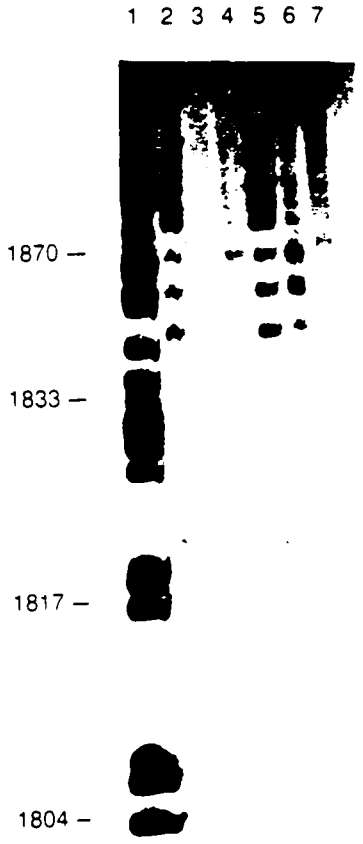


FIGURE 14

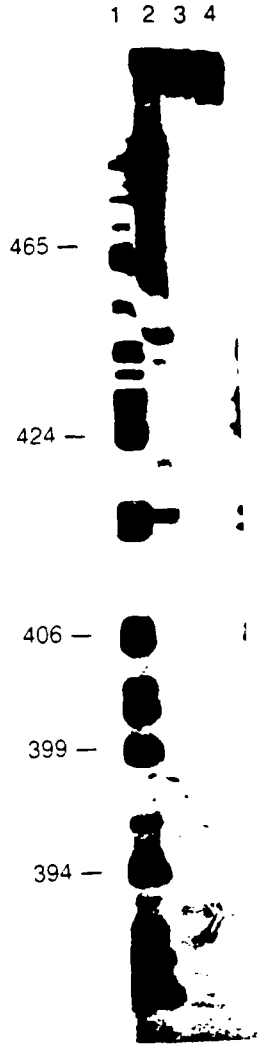


FIGURE 15

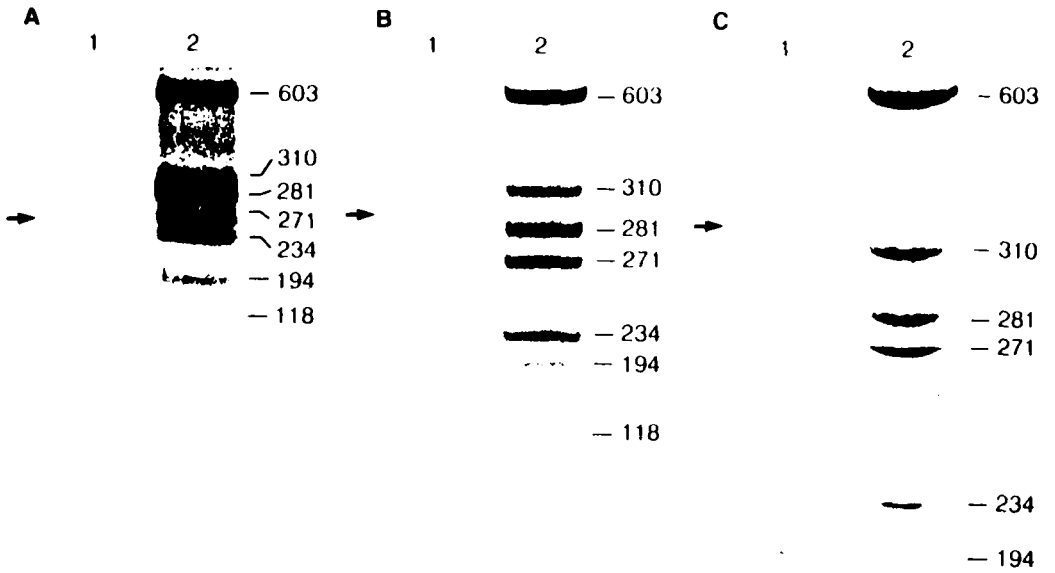

A
1
2
603
310
281
271
234
194
118
B
1
2
603
310
281
271
234
194
118
C
1
2
603
310
281
271
234
194

FIGURE 16

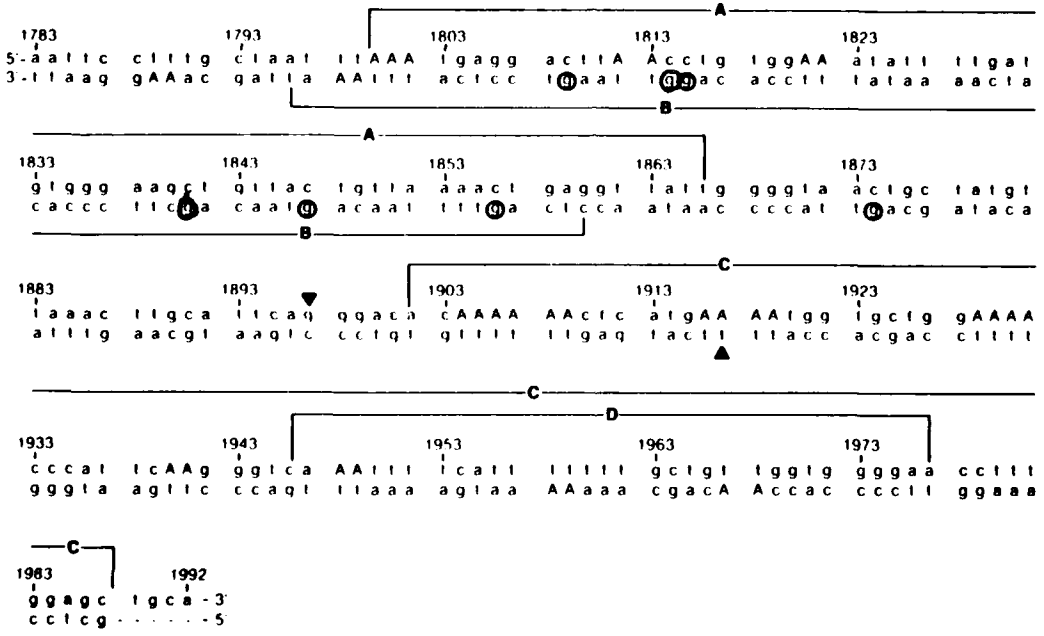

A
1783
1793
1803
1813
1823
5'-aattc ctttg ctaat ttAAA tgagg acttA Acctg tggAA atatt ttgat
3'-ttaag gAAac gatta AAttt actcc tgaat tgtac acctt tataa aacta
B
A
1833
1843
1853
1863
1873
gtggg aagct gttac tgtta aaact gaggt tattg gggta actgc tatgt
caccc ttcga caatg acaat tttga ctcca ataac cccat tgacg ataca
B
C
1883
1893
1903
1913
1923
taaac ttgca ttcag ggaca cAAAA AActc atgAA AAtgg tgctg gAAAA
attig aacgt aagtc cctgt gtttt ttgag tactt ttacc acgac ctttt
C
D
1933
1943
1953
1963
1973
cccat tcAAg ggtca AAttt tcatt ttttt gctgt tggtg gggaa ccttt
gggta agttc ccagt ttaaa agtaa AAaaa cgacA Accac ccctt ggaaa
C
1983
1992
ggagc tgca-3'
cctcg-----5'

Figure legends

FIGURE 1. DNaseI footprinting of poly (ADP-ribose) polymerase with the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment ^{32}P labeled at the 5' *Eco*RI site: Increasing amounts of purified polymerase were added to purified DNA fragment and the protein-DNA complexes subjected to DNaseI cleavage. The products of DNaseI cleavage were run on a sequencing gel. Lane1, C+T markers; lane 2, C markers; lane 3, undigested ^{32}P-DNA; lanes 4, 5 & 6, contained ^{32}P-DNA + 100 ng, 5000 ng, &1000 ng of poly (dI-dC) : poly (dI-dC) respectively + 300 ng of polymerase at each concentration of the competitor polynucleotide; lanes 7,8,9,10, & 11: ^{32}P-DNA + 0, 5, 20, 300 & 60 ng of the polymerase respectively. Each lane contained ~ 50 ng = 2 x 10^4 CPM of ^{32}P-DNA.

FIGURE 2A. DNaseI footprinting of poly (ADP-ribose) polymerase with the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment ^{32}P labeled at the 3' *Eco*RI site:

The footprinting procedure is the same as in figure1. Lane1, ^{32}P-DNA digested for 60 sec in the absence the polymerase; lane 2, ^{32}P-DNA digested for 30 sec in the absence of the polymerase; lane 3, ^{32}P-DNA + 60 ng of the polymerase; lane4, C cleavage markers; lane 5, C+T markers; lane 6, ^{32}P-DNA + 300 ng of polymerase; lane 7, ^{32}P-DNA digested for 30 sec in the absence of the polymerase; lane 8 & 9, ^{32}P-DNA + 1000 ng & 5000 ng of poly (dI-dC) : poly (dI-dC) respectively + 300 ng of polymerase at each concentration of the competitor polynucleotide; lane 10, 11 & 12, ^{32}P-DNA + 20, 300 &

60 ng of the 36 Kd polypeptide of the polymerase respectively.

FIGURE 2B: The effect of distamycin A on polymerase binding to the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment ^{32}P labeled at the 3' *Eco*RI site:lanes 1-6, ^{32}P-DNA + 300 ng of the polymerase + 1 μM, 5 μM, 10 μM, 15 μM, 20 μM, & 40 μM distamycin A respectively. All lanes in figures 2A & 2B contained DNA samples digested for 30 sec except in lane 1 of figure1A. Each lane in both figures contained ~ 50 ng = 2 x 10^4 CPM of ^{32}P-DNA. The significance of the arrows is explained in the text.

FIGURE 3. DNaseI footprinting of poly (ADP-ribose) polymerase with the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment ^{32}P labeled at the 3' *Pst*I site:

The footprinting procedure is the same as in figure1. Lane 1, C+T markers; lane 2, C markers; lanes 3 , 4 & 5, ^{32}P-DNA +100 ng,1000 ng & 5000 ng of poly (dI-dC) : poly (dI-dC) respectively + 300 ng of polymerase at each concentration of the competitor polynucleotide; lanes 6, 7 & 8, ^{32}P-DNA + 20, 300 & 60 ng of polymerase; lane 9, ^{32}P-DNA digested with DNaseI in the absence of the polymerase; lane 10, undigested ^{32}P-DNA. All the samples in each lane were digested for 30 sec. Each lane contained ~ 75 ng = 5 x 10^4 CPM of ^{32}P-DNA.

FIGURE 4A. Micrococcal nuclease footprinting of the polymerase with the *Sal*I-*Bam*HI fragment of *pBR*322 ^{32}P-labeled at the 5' *Sal*I site:

Lane 1, C+T markers; lane 2, C markers; lane 3, ^{32}P-DNA digested in the absence of the polymerase; lane 4, 5 & 6, ^{32}P-DNA + 50 ng, 500 ng & 2000 ng of polymerase respectively. Each lane had ~ 50 ng = 4 x 10^4 CPM except lane 3. Lane 3 was slightly overloaded with ^{32}P-DNA as compared to the other lanes.

FIGURE 4B. DNaseI footprinting of poly (ADP-ribose) polymerase with the *Sal*I-*Bam*HI DNA fragment of *pBR*322 ^{32}P labeled at the 5' *Sal*I site:

Lanes 1 & 2, ^{32}P-DNA + 500 ng & 2000 ng of polymerase respectively; lane 3, ^{32}P-DNA digested in the absence of the polymerase; lane 4, undigested ^{32}P-DNA. Each lane contained ~ 50 ng = 4 x 10^4 CPM.

FIGURE 5. DNaseI footprinting of poly (ADP-ribose) polymerase with the *Acc*I-*Eco*RI SV40 DNA fragment ^{32}P labeled at the 5' *Acc*I site:

Lane 1, undigested ^{32}P-DNA; lane 2 , ^{32}P-DNA digested in the absence the polymerase for 60 sec; lane 3, ^{32}P-DNA digested in the absence the polymerase for 30 sec; lane 4, 5 & 6, ^{32}P-DNA + 500 ng, 1000 ng & 2000 ng of polymerase respectively; Each lane had ~ 50 ng = 4 x 10^4 CPM. G, G+A markers were employed (not shown).

FIGURE 6. Micrococcal nuclease footprinting of poly (ADP-ribose) polymerase with the 209 bp *Eco*RI-PstI SV40 DNA fragment ^{32}P labeled at the 3' *Eco*RI site:

Lane 1, Undigested ^{32}P-DNA; lane 2, ^{32}P-DNA digested in the absence the polymerase; lanes 3-7, ^{32}P-DNA + 60, 20, 200, 300, & 1000 ng of the polymerase respectively; lane 8, C+T markers; lane 9, C markers. Each lane in the figure contained ~ 50 ng = 2×10^4 CPM of ^{32}P-DNA. The significance of the arrows is explained in the text.

FIGURE 7. Methylation protection by poly (ADP-ribose) polymerase of the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment ^{32}P labeled at the 3' *Eco*RI site:

Approximately 200 ng (~1×10^5 CPM) of the ^{32}P-DNA fragment was mixed with ~ 2000 ng of poly (ADP-ribose) polymerase in 100 µl of Na Cacodylate- containing buffer (Siebenlist and Gilbert,1980) and incubated at 25^0C for 15 min. The protein-DNA complexes were then treated with DMS (1 µl of the undiluted stock (10.5 M) supplied by Aldrich Chem. Co), filtered through nitrocellulose filters. The DNA eluted off the filters and the DNA cleaved at modified G residues as described by Siebenlist and Gilbert (1980) and Maxam and Gilbert (1980). Lane 1, ^{32}P-DNA which passed through the nitrocellulose filter i.e., polymerase-free DNA; lane 2, filter-bound ^{32}P-DNA i.e., DNA bound by the polymerase. Each lane contained ~ 10^4 CPM. The arrows indicate Gs that are partially protected from methylation by DMS.

FIGURE 8. Binding of the polymerase to T4 DNA, poly (dA-dT) : poly (dA-dT) and poly (dG-dC) : poly (dG-dC): Increasing amounts of poly (ADP-ribose) polymerase were incubated with nick-translated (with either T4 DNA polymerase for T4 DNA or *E. coli* DNA polymerase for the polynucleotides) T4 DNA (HMdC or glu HMdC), poly (dA-dT) : poly (dA-dT) and poly (dG-dC) : poly (dG-dC) (~ 20-50 ng = 10^5 CPM of each DNA) in 50 µl of binding buffer (see DNaseI digestions, **Materials and methods**) at 25^0C for 20 min. The reaction mixtures were then filtered through nitrocellulose discs, the filter-bound ^{32}P-CPM was counted in a scintillation counter as described in Sastry and Kun (1987). ○ = non-glucosylated T4 DNA; ▲ = glucosylated T4 DNA; ● = poly(dA-dT) : poly (dA-dT); Δ = poly(dG-dC) : poly (dG-dC). Each point in the graph is the average of duplicate reactions. Poly (dA-dT) : poly (dA-dT) and poly (dG-dC) : poly (dG-dC) were of the same length.

FIGURE 9. Binding of a 36 Kd polypeptide fragment of poly (ADP-ribose) polymerase to the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment ^{32}P labeled at the 3' *Eco*RI site: Plasmin-digested polymerase fragments were separated on an SDS-polyacrylamide gel as described earlier (Buki and Kun,1987). See "Western Blotting" in **Materials and methods** for details of the procedure for electroblotting and immunostaining. When the DNA-binding assay was employed, the membrane containing the polypeptides was either directly exposed to ^{32}P-DNA (lanes 4 & 5) or first exposed to the antibody and then bathed in a solution of ^{32}P-DNA (lanes 6 & 7). In both cases the immunostaining steps were not performed. Lane 1,

undigested polymerase (immunostain); lane 2, plasmin-digested polymerase (immunostain); lane 3, protein standards specified in Kd on the left hand side of the figure (Coomassie Brilliant Blue stain), 97.4 = Phosphorylase B, 66.2 = Bovine serum albumin, 42.6 = Ovalbumin; 31.0 = Carbonic anhydrase; 21.5 = Soybean trypsin inhibitor, 14.4 = lysozyme. Protein standards were purchased from BioRad Co (Richmond,CA); lane 4, undigested polymerase bound to ^{32}P-DNA; lane 5, 36 kd polypeptide bound to ^{32}P-DNA; lane 6, Undigested polymerase bound to ^{32}P-DNA after treatment with a solution of the antibody; lane 7, 36 kd polypeptide bound to ^{32}P-DNA after treatment with a solution of the antibody. Lanes 4-7 are autoradiograms. Lanes1, 2, 4-7 contained ~ 3 µg of protein per lane on the gel before electroblotting. Lane 3, contained ~ 1 µg of protein.

FIGURE 10. Binding of only one fragment ('A') of the 36 Kd polypeptide doublet to the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment ^{32}P labeled at the 3' *Eco*RI site: The details of the procedures are given in the legend to figure 9 and in **Materials and methods**. Lane 1, protein standards, 42.6 kd = Ovalbumin, 31.0 kd= Carbonic anhydrase; lane 2, 36 Kd polypeptide 'A' (immunostain); lane 3, 36 Kd polypeptide 'B' (immunostain); lane 4, 36 kd polypeptide 'A' bound to ^{32}P-DNA; lane 5, 36 kd polypeptide 'B' not bound to ^{32}P-DNA; lane 6, 36 kd polypeptide 'A' bound to ^{32}P-DNA after treatment with a solution of the antibody. Lanes 2- 6 contained ~ 2 µg of protein per lane on the gel before electroblotting. Lane1, contained ~ 1 µg of protein. Lanes 2 & 3 were immunostained. Lanes 4-6 are

autoradiograms. Only the relevant portions of the gel or the autoradiograms are shown.

FIGURE 11. λ exonuclease footprinting of poly (ADP-ribose) polymerase with the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment ^{32}P labeled at the 3' *Pst*I site:

Binding assays and λ exonuclease digestions are described in **Materials and methods**. Lane 1, C+T markers; lane 2, C markers; lane 3, ^{32}P-DNA digested in the absence of the polymerase; lane 4 - 6, ^{32}P-DNA + 100, 300, & 60 ng of polymerase respectively; lane 7, ^{32}P-DNA + 300 ng of polymerase + 500 ng of unlabeled 209 bp *Eco*RI-*Pst*I SV40 DNA fragment; lane 8, undigested ^{32}P-DNA. Each lane contained ~ 75 ng ($5x10^4$ CPM)of ^{32}P-DNA fragment.

FIGURE12. λ exonuclease footprinting of poly (ADP-ribose) polymerase with the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment ^{32}P labeled at the 3' *Eco*RI site:

Binding assays and λ exonuclease digestions are described in **Materials and methods**. Lane 1, undigested ^{32}P-DNA; lane 2, ^{32}P-DNA digested in the absence of the polymerase; lane 3 & 4, ^{32}P-DNA + 60, 300 ng of polymerase respectively; lane 5, ^{32}P-DNA + 300 ng of polymerase + 50 ng of unlabeled 209 bp *Eco*RI-*Pst*I SV40 DNA fragment; lane 6, ^{32}P-DNA + 300 ng of polymerase + 500 ng of unlabeled 209 bp *Eco*RI-*Pst*I SV40 DNA fragment; lane 7, C+T markers; lane 8, C markers. Each lane contained ~ 50 ng ($5x10^3$ CPM) of ^{32}P-DNA fragment.

FIGURE13. Exonuclease III footprinting of poly (ADP-ribose) polymerase with the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment ^{32}P labeled at the 5' *Eco*RI site: Binding assays and exonuclease III digestions are described in **Materials and methods**. Lane 1, G markers; lane 2, ^{32}P-DNA + 300 ng of polymerase + 500 ng of unlabeled 209 bp *Eco*RI-*Pst*I SV40 DNA fragment; lane 3 & 4, ^{32}P-DNA + 300, 60 ng of polymerase respectively; lane 5, ^{32}P-DNA digested in the absence of the polymerase; lane 6, ; ^{32}P-DNA + 300ng of polymerase + 1000ng of poly (dI-dC) : poly (dI-dC) as a competitor. lane 7, undigested ^{32}P-DNA. Each lane contained ~ 75 ng (5 x10^4 CPM)of ^{32}P-DNA fragment.

FIGURE 14. Exonuclease III footprinting of the polymerase with the *Bam*HI-*Sal*I fragment of *pBR*322 ^{32}P-labeled at the 5' *Bam*HI site:

Binding assays and exonuclease III digestions are described in **Materials and methods**. Lane 1, G markers; lane 2, ^{32}P-DNA digested in the absence of the polymerase; lane 3 & 4, ^{32}P-DNA + 60, 300 ng of polymerase respectively. Each lane contained ~ 50 ng (5 x10^3 CPM) of ^{32}P-DNA fragment.

FIGURE 15. The 209 bp *Eco*RI-*Pst*I SV40 DNA fragment is intrinsically bent:

Panel A) 2% agarose gel; Panel B) 5% acrylamide gel; and Panel C) 12% acrylamide gel. Lane 1 in each panel shows the 209 bp *Eco*RI-*Pst*I SV40 DNA fragment (arrows) and lane 2 in each panel shows bacteriophage ϕX174 dsDNA cut with *Hae*III serving as markers.

Electrophoresis was performed in the cold (4^0C) at a constant voltage of 0.5V/cm^2 in 1X TBE (Maniatis et al., 1982). Lane 1 in each panel contained 5 ng (~2 x10^3 CPM) of ^{32}P-DNA fragment. Lane 2 in each panel contained 5 ng (~2 x10^4 CPM) of ϕX174 dsDNA *Hae*III markers. Only the relevant portions of the gels are shown.

FIGURE 16. The nucleotide sequence of the 209 bp *Eco*RI-*Pst*I fragment of SV40 DNA and summary of the footprinting data : 'A' = DNaseI footprint region of the polymerase when the 5' *Eco*RI site was labeled; 'B' = DNaseI footprint region of the polymerase when the 3' *Eco*RI site was labeled; 'C' = DNaseI footprint region of the polymerase when the 3' *Pst*I site was labeled; 'D' = The observed DNaseI-resistant region in the absence of the polymerase when the 3' *Pst*I site was labeled. *Circled 'G's are protected from* DMS methylation. ▲ & ▼ are λ exo pause sites in the presence of the polymerase.

END

DATE

FILMED

5-88

DTIC