Vol. 114, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 18, 1983 Pages 99-106

NUCLEOSOMES CONTAIN DNA BINDING PROTEINS THAT RESIST DISSOCIATION BY SODIUM DODECYL SULFATE

Mark Twain Muller

Department of Microbiology
The Ohio State University
Columbus, Ohio 43210

Received May 9, 1983

A new, rapid, and quantitative method has been developed to show that nucleosomes contain non-histone proteins which are not dissociated by an ionic detergent and are firmly, possibly covalently, bound to DNA. The ability of the method to detect and quantitate the binding of proteins known to form stable (covalent) complexes with DNA has been verified using purified topoisomerase I; in addition, the method will measure DNA/protein adducts formed by ultraviolet light. The distribution of detergent resistant proteins in a population of nucleosomes is non-uniform; the mono- through trimers are depleted of these proteins while higher oligomers are enriched. The data suggest that these proteins are not associated with the nucleosome core but more likely with linker regions between core particles.

INTRODUCTION: The binding of a protein ligand to DNA frequently involves relatively weak intermolecular forces (hydrogen bonds, electrostatic attraction) (for review see 1). A characteristic feature of weak multisite interactions between a protein and DNA is the sensitivity of binding to salt concentration (2) and to ionic detergents, such as SDS[1] which may denature the protein and neutralize its cationic binding sites. As a consequence, extraction of animal cell nuclei with high salt (molar range) or detergents removes many chromosomal proteins; however, a number of proteins resist extraction implying that these proteins are very tightly bound to the DNA residue (4-8). Such proteins are thought to be related to the nuclear matrix or chromosomal scaffold. A role of tightly bound proteins in aspects of transcription (10, 11) and DNA replication (3, 12) has been proposed (reviewed in 9). An additional class of chromatin associated proteins which bind

ABBREVIATIONS: SDS, sodium dodecyl sulfate; UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid; topo I, topoisomerase type I; SDS-K^+, the protein/DNA binding method described in this paper.

0006-291X/83 $1.50

strongly (covalently) to the helix are DNA topoisomerases (reviewed in 13); in particular, topoisomerase I has been implicated in transcription (17).

Although it is clear that a variety of proteins exist which bind DNA tenaciously, few of these have been purified to homogeneity. I have developed a method to detect proteins which bind DNA in a detergent (SDS) resistant fashion. The method is rapid and convenient and should allow purification and characterization of proteins which firmly bind DNA. This method can be used in a preparative (to isolate DNA fragments with attached protein) as well as an analytical mode. The distribution of detergent resistant proteins in a population of nucleosomes has been evaluated with this method.

MATERIALS AND METHODS: The SDS-K^+ Method. Reactions were performed in siliconized glass tubes (13 x 100 mm). The standard reaction mixture contained 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 ug BSA/ml (buffer A), labeled DNA (nick translated λ) and variable amounts of purified topo I (14) to give a final volume of 0.1-0.25 ml. The reactions were incubated at 30° for 30 minutes and terminated by the addition of 0.5 ml of buffer A containing 20 ug calf thymus DNA/ml, and 1% SDS (w/v). The mixture was vortexed, warmed to 37° and 0.025 ml of 2.5 M KCl added, followed by brief vortexing and chilling on ice for 5-10 min. Note that by allowing the SDS-K^+ precipitate to form in this way, the rather large precipitate is easier to vortex, wash and resuspend when the preparative method is used (see below). In the analytical method, the 37° warming step is not necessary; however, the reaction should be chilled and the precipitates collected and washed in the following way. Approximately 5 ml of buffer B (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM KCl) were added to precipitated reactions followed by vacuum filtration through glass fiber discs which had been pre-wetted with buffer B (GF/A, GF/C, or Reeve Angel discs from Whatman work equally well). The reaction vessel was then rinsed 3X with 5 ml of buffer B (4°), and each rinse added to the filter. The DNA/protein complexes tend to bind to glass or plastic surfaces; although SDS minimizes this, some non-specific sticking may still occur if the tubes are not rinsed out. The filters were washed with 5 ml of buffer B a total of five times. The filters were then washed with 10 ml of 95% ethanol (4°) followed by 10 ml of 70% ethanol (4°). Filters were dried and radioactivity determined by liquid scintillation spectrometry. Counting efficiencies and reproducibility were significantly improved by using a solubilizer (NCS, Amersham) prior to scintillation counting.

The SDS-K^+ method was also used in a preparative mode. In general, exposure of mixtures of DNA and protein to low salt facilitates binding of the protein to the helix (for review see 1, 2); while this applies to topo I (Muller and Trask, in preparation), it is unlikely that all DNA binding proteins (particularly those that bind with high affinity) behave so predictably. Conditions conducive to binding can easily be established using the analytical SDS-K^+ method and varying mono or divalent salt.

To isolate DNA fragments with attached protein, nucleosomes in buffer A were precipitated with SDS and KCl as described above. The SDS-K^+ precipitate was deposited by centrifugation (10,000 g, for 2 min) and resuspended in 1 ml buffer B. The precipitate was washed in this way a total of 3 times, and the final precipitate resuspended in 0.2 ml of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl. The DNA was precipitated with ethanol (2.5 volumes) and after

resuspending in TE (10 mM Tris-HCl (pH 7.6), 1 mM EDTA), proteinase K was added to removed covalently attached proteins. Protein-free DNA, isolated by SDS-proteinase K and phenol extraction of nucleosomes, was not precipitated by the SDS-K^+ preparative method (data not shown).

Other Procedures: Topoisomerase I was purified as described (14) from chicken erythrocytes. The purified enzyme contained a predominant polypeptide of M_r = 105,000 representing 60-70% of the total. Based upon its ability to covalently bind to DNA (14) this peptide was shown to be topo I. Experiments were performed with enzyme from a single batch using aliquots that had not been subjected to >1 freeze/thaw. Histone H-1 was purchased from Sigma and its purity checked by SDS gel electrophoresis.

Chicken erythrocyte nuclei were prepared as described previously (18). Nucleosomes were prepared by mild digestion of nuclei (to about 10% acid solubility) with micrococcal nuclease. The digestion was terminated by addition of EDTA, the nuclei centrifuged (10,000 g, 2 min), and resuspended in 0.25 mM EDTA (pH 7.2), 0.5 mM PMSF. The released oligonucleosomes were obtained from the supernatant after a second centrifugation step (10,000 g, for 2 min). Agarose gel electrophoresis of DNA was performed as described before (18).

RESULTS AND DISCUSSION: The method reported here allows one to measure the formation of DNA/protein complexes which do not dissociate upon treatment with the ionic detergent SDS. I refer to this as the SDS-K^+ method. Exposure of protein-SDS complexes to K^+ leads to formation of an insoluble potassium-SDS-protein complex which can be removed by low speed centrifugation. Since SDS does not bind double stranded DNA, treatment of protein and DNA with SDS followed by KCl affords a rapid method for selectively precipitating proteins, leaving free DNA in the supernatant. If a protein remains bound to labeled DNA after SDS treatment, this DNA will be detected in the SDS-K^+ precipitate.

To illustrate, the assay was reconstructed using topoisomerase I, purified from chicken erythrocytes (14). We previously demonstrated that a transitory covalent complex is formed between the DNA and avian topo I which can be trapped with SDS (14). Incubation of purified topo I with nick translated DNA (Figure 1) results in precipitation of the DNA with SDS-K^+; the amount of precipitable DNA being proportional to enzyme concentration. The amount of DNA in the SDS-K^+ precipitate corresponds precisely to the amount of DNA shifted to a lower buoyant density in a CsCl gradient (14). In addition, if the reaction products are treated with proteinase K after SDS addition, DNA is not detected in the precipitate.

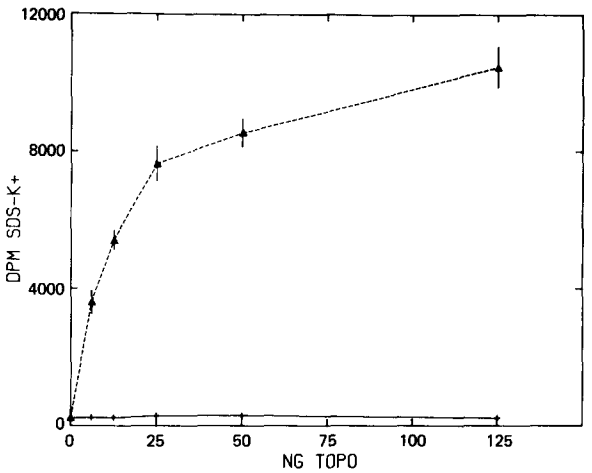

Figure 1. Titration of Topo/DNA Binding by the SDS-K^+ Method. A series of reactions were prepared in buffer A containing a constant amount [^{3}H]-λ DNA (2 ng, 1.4×10^4 dpm) and variable amounts of purified topoisomerase I (14). Reactions (done in quadruplicate) were incubated for 30 min at 30° and terminated by addition of SDS as described in Methods. Prior to addition of KCl, one set of reactions was digested with 100 ug proteinase K/ml, (+——+); another set was not digested with proteinase (▲-----▲). Following a 30 minute incubation (37°), all reactions were processed for SDS-K^+ precipitate DNA as described by the analytical method.

In the absence of topoisomerase, SDS-K^+ treatment did not precipitate free DNA; similarly, proteins which bind electrostatically (inner histones, H-1) did not cause precipitation of the DNA (Table I). Irradiation of histone H-1/DNA complexes with 254 nm light, however, results in very efficient

Table I. DNA/Protein Complexes Detected by SDS-K^+[a]

Protein (Concentration)	DPM, SDS-K^+ Before UV	DPM, SDS-K^+ After UV
Control (no protein)	220 (± 30)	360 (± 21)
+ Topo I (0.06 mg/ml)	14430 (± 454)	11667 (± 598)
+ Histone H-1 (0.02 mg/ml)	820 (± 41)	12200 (± 802)
+ Core Histones (0.1 mg/ml) (H2A, H2B, H3, H4)	396 (± 51)	ND[b]
+ Lysozyme (0.1 mg/ml)	482 (± 29)	ND
+ BSA (1 mg/ml)	246 (± 61)	ND
+ DNA polymerase I (0.2 mg/ml)	297 (± 72)	ND

[a] Reactions (100 ul) contained 2 ng [^{3}H]-λ DNA (1.4×10^4 dpm) plus the indicated proteins. Two sets of reactions were incubated for 30 min at 30°; one set was processed for SDS-K^+ precipitable DNA immediately after incubation at 30°; selected reactions of a second set were placed on parafilm (on ice) and irradiated for 1 h with a 254 nm UV light (G.E. No. G15T8, 15 watt, germicidal lamp) at a distance of 5 cm. The incident power was 1.8×10^{-3} W/cm^2 as measured by a UV dosimeter. Data are averaged from 3 separate experiments.

[b] Not done.

precipitation of DNA by SDS-K^+. It is known that these conditions induce formation of DNA-protein cross-links (19). These results attest to the ability of the SDS-K^+ method to detect DNA fragments with covalently attached protein. Moreover, the ability of the method to detect complexes is not limited to only topoisomerase/DNA intermediates. Thus, the method may be a useful analytical technique to monitor the purification of chromosomal proteins which bind DNA in a detergent resistant complex. These include other topoisomerases (type II; gyrase related enzymes), the sarcosyl resistant proteins associated with nuclear matrix DNA (7) and alkali resistant proteins (15, 16).

The SDS-K^+ method can also be used in a preparative way to isolate DNA fragments with attached protein. Mononucleosomes contain non-histone proteins which cause precipitation of nucleosomal DNA by SDS-K^+; a comparison of SDS-K^+ precipitated and non-precipitated DNA fragments from a population of oligonucleosomes is shown in Figure 2. Treatment with SDS-K^+ caused selective precipitation of longer oligomers, while the mono and dimer sized nucleosomes are depleted in proteins that cause SDS-K^+ precipitation of the DNA. The results in Table I argue that histone H-1 is not responsible for SDS-K^+ precipitation; in addition, selective retention of mono and dimers in the supernate was not observed if the nucleosomes were digested first with proteinase K, indicating that protein is directly responsible (see Fig. 2^a). One explanation for these data is that the efficiency of SDS-K^+ precipitation is somehow dependent upon the size of the DNA bound to the protein. Two pieces of evidence argue against this idea. First, if the efficiency of the SDS-K^+ precipitation is related to DNA fragment size, it is reasonable to assume that the precipitation efficiency would <u>decrease</u> as fragment size increases. This is because a single protein molecule would more readily cause precipitation of a smaller DNA fragment than a larger one; however, just the opposite result is observed in Figure 2. Second, reconstruction experiments with mononucleosomes and purified topo I clearly show that the SDS-K^+ will precipitate DNA fragments of this size (data not shown).

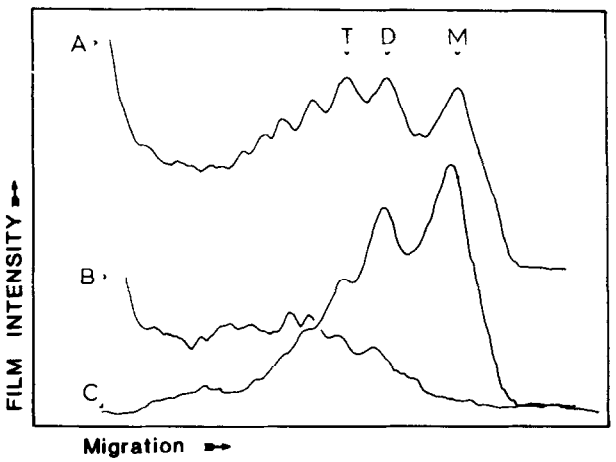

Figure 2. SDS-K^+ Precipitation of DNA in Nucleosomes. The preparative SDS-K^+ method was performed on a population of nucleosome multimers (in buffer A) isolated from chicken erythrocytes as described in Methods. A separate control SDS-K^+ precipitation was carried out on protein free DNA which had first been purified (proteinase K and phenol extraction) from the same population of nucleosomes. Detectable amounts of DNA were not present in the SDS-K^+ precipitate (based upon ethidium bromide staining); DNA from the supernate was purified, ethanol precipitated and resuspended in TE buffer. DNA was also purified from the SDS-K^+ supernate and precipitate of intact nucleosomes followed by ethanol precipitation; in this case the precipitate contained about half as much DNA as in the supernate. The DNA samples were analyzed by agarose gel electrophoresis (18). The gel was stained with ethidium bromide, photographed on a UV light source, and the negative scanned with an LKB soft laser densitometer. A, DNA extracted from the SDS-K^+ supernate of the protein-free DNA control (monomer, dimer, and trimer repeats indicated by M, D, and T respectively); B, DNA derived from the SDS-K^+ precipitate of intact nucleosomes; C, DNA from the SDS-K^+ supernate of intact nucleosomes.

The fact that mono or dinucleosomes are depleted of tightly bound proteins suggests these proteins bind to linkers or alternatively to DNA that is highly sensitive to nucleolytic cleavage such as transcriptionally active chromatin (20). Further studies using cloned genes to probe for transcriptionally active and inactive genes in the SDS-K^+ precipitate are in progress to evaluate the basis of these observations.

The nature of the nucleosomal proteins which are responsible for SDS-K^+ precipitation of DNA is unknown; conceivably, topo I may be present in linker regions. Indeed, this idea is consistent with the binding site size of avian topo I of 20-30 bp (14). When gradient purified dinucleosomes were incubated with superhelical DNA, total relaxation of the plasmid DNA occurred (Figure 3, Lane b) indicating the presence of a topoisomerase. This activity is due to a type I topoisomerase since preincubation of the dimers with monospecific IgG

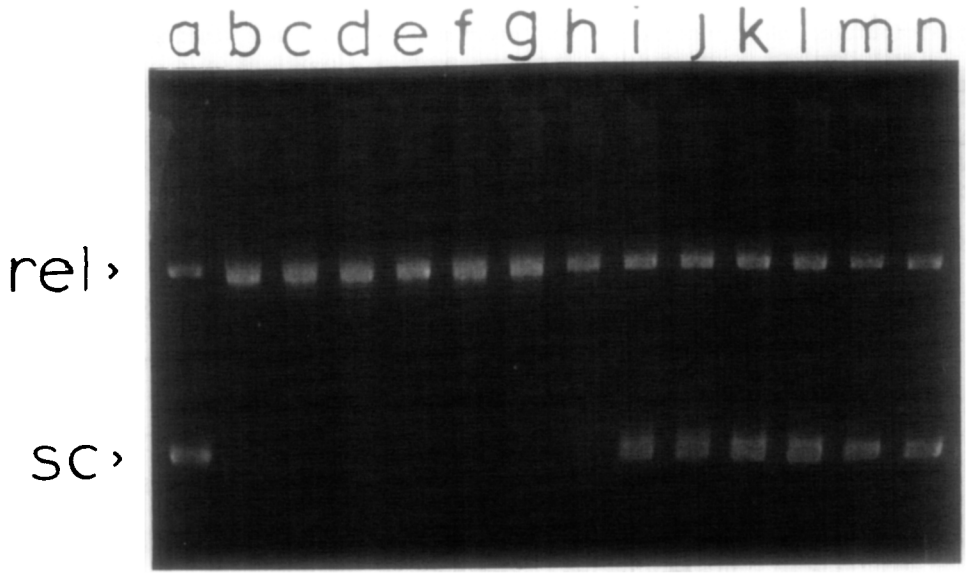

Figure 3. Topoisomerase I activity in Nucleosomes. Dinucleosomes, isolated from sucrose gradient centrifugation of nuclear digests (as described in 21), were preincubated with various antisera for 60 min at 4° in buffer A (116 ug dimers/ml in DNA). Plasmid DNA (superhelical pBR322, 15 ug/ml) was then added and the incubation continued for 12 h at 30°. The reactions were terminated with SDS (1% w/v), digested with proteinase K (56° 30 min) and the DNA analyzed on a 1.4% agarose gel; under the conditions of electrophoresis, the dinucleosome DNA ran off the end of the gel. Lane a, a marker showing superhelical (sc) and relaxed DNA forms (rel); b, reaction containing pBR322 DNA and dinucleosomes. Lanes c-h, dimer nucleosomes preincubated with various amounts of pre-immune IgG prior to addition of plasmid DNA: c, 0.1 ul; d, 0.2 ul; e, 0.5 ul; f, 1 ul; g, 2 ul; h, 5 ul. Lanes i-n, dimer nucleosomes preincubated with various amounts of anti-topoisomerase I IgG prior to addition of plasmid DNA: i, 0.1 ul; j, 0.2 ul; k, 0.5 ul; l, 1 ul; m, 2 ul; n, 5 ul. The protein concentration in the two immunoglobulin fractions was identical (1.8 mg IgG/ml). The details of preparation of anti-topo antiserum and purification of the IgG fraction will be presented elsewhere; however, this IgG reacted with purified topo I by double diffusion tests, and it strongly inhibited the activity of purified topo I (not shown).

directed against purified topo I (14) was shown to inhibit relaxation of plasmid DNA (Figure 3, Lanes c-h). It is reasonable to conclude that topo I is present in nucleosomes and that it is one candidate protein responsible for the observed precipitation of longer nucleosome oligomers in Figure 2; however, I cannot exclude the possibility that additional proteins are present which respond to the SDS-K^+ assay. Recently, the SDS-K^+ method has been used as an assay to purify nuclear proteins, besides topo I, that bind to DNA in a detergent resistant fashion; two proteins have been partially purified using this approach (Hoepfner, R. and Muller, M.T., in preparation). Through the

use of this technology we hope to gain a more comprehensible picture of the distribution and function of an important class of DNA binding proteins in chromatin.

ACKNOWLEDGEMENTS: This work was supported by grant number GM 31640-01 from NIH. The technical assistance of J.R. Spitzner is acknowledged, as is the typing skill of P. Titus.

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