Interaction of Nucleolar Phosphoprotein C23 with Cloned Segments of Rat Ribosomal Deoxyribonucleic Acid†

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ABSTRACT: Protein C23, a predominant nucleolar phosphoprotein and a putative nucleolus organizer protein, was analyzed for its general DNA binding characteristics and for its selectivity in binding plasmid DNAs containing cloned fragments of the genes that code for ribosomal RNA (rDNA). By use of nitrocellulose filter disk assays, the protein bound saturably to nuclear DNA with a relatively high affinity. Binding was maximal at low ionic strength (0–0.1 M KCl) with progressively decreasing binding at or above 0.2 M. In competition assays protein C23 showed a marked preference for linear single-stranded vs. double-stranded DNA and little or no affinity for ribosomal RNA. The relative affinities of rDNA sequences for protein C23 were determined with cloned fragments spanning 15.8 kilobases (kb) of DNA starting approximately 3.7 kb upstream from the initiation site for 45S preribosomal RNA to near the 3′ end of the sequence coding for 28S RNA. Of the five linearized plasmids tested, only one (pKW1) was an effective competitor for ^{32}P-labeled nuclear DNA. As measured by the concentration of competing DNA required to achieve 50% competition, pKW1 was approximately 20-fold more effective than the second best competitor. The DNA insert in pKW1 is a 3.5-kb sequence which is located in the nontranscribed spacer region less than 0.5 kb upstream from the initiation site for 45S preribosomal RNA. These results suggest that protein C23 has a preference for binding DNA sequences in the nontranscribed spacer of rDNA.

The major known functions of the nucleolus are the synthesis and processing of preribosomal RNA and the assembly of ribosomes (Busch & Smetana, 1970). Numerous proteins appear to participate in the process of ribosome biogenesis (Warner 1974, 1979; Kumar & Subramanian, 1975; Prestayko et al., 1974) or contribute to maintaining the structure of the nucleolus (Olson & Busch, 1978). However, little is known about the structure or function of the individual nucleolar proteins. Recently, a major nucleolar protein, designated C23 by Orrick et al. (1973), was isolated (Mamrack et al., 1979; Tsutsui et al., 1980) and partially characterized structurally (Mamrack et al., 1979). This protein has a molecular weight of approximately 110 000 and contains several phosphorylated highly acidic regions; e.g., one 42-residue tryptic peptide from protein C23 contains 25 carboxyl groups (Mamrack et al., 1979). These highly negatively charged regions have been proposed to interact with basic regions of histones or ribosomal proteins (Mamrack et al., 1979; Olson, 1983).

Recently, by immunological methods, protein C23 was found to be predominantly localized to the nucleolus (Olson et al., 1981). However, it appears to be distributed among several fractions of the nucleolus. For example, a portion of the protein is readily extractable with low ionic strength buffers (Rothblum et al., 1977), whereas another fraction is found associated with preribosomal RNP particles (Prestayko et al., 1974; Olson et al., 1974b). A third portion of C23 appears to be tightly bound to chromatin. The residual or chromatin-associated form was previously designated C18 (Yeoman et al., 1973; Olson et al., 1975). However, more recent studies have shown that proteins C18 and C23 have very similar physical characteristics and contain identical amino-terminal sequences (Rao et al., 1982). Protein C23 preferentially stains with silver on polyacrylamide gels (Lischwe et al., 1979) as do nucleoli (Busch et al., 1979) and nucleolus organizer regions (NORs) of chromosomes (Howell et al., 1975; Goodpasture & Bloom, 1975). Furthermore, protein C23 has been found at the NORs by immunochemical methods (Lischwe et al., 1981). Taken together, these observations suggest that protein C23 interacts with chromatin was well as with preribosomal RNP particles in the nucleolus, and it may play a role in organizing the chromatin and RNP components of the nucleolus.

The specificity of location and the proposed organizer role of protein C23 suggest that it recognizes and preferentially interacts with other nucleolar macromolecules. Although these macromolecules could be other nucleolar proteins or nucleic acids, we have chosen to examine the interaction of protein C23 with DNA as the first in a series of studies on the macromolecular interactions of nucleolar proteins. These studies were initiated to determine whether protein C23 is capable of interacting with DNA, and if so, whether there is any sequence preference in the DNA binding. The studies reported here indicate that protein C23 contains DNA binding activity and that there is preference for certain sequences in the nontranscribed spacer that occurs between the repeated genes which code for ribosomal RNA.

Materials and Methods

Animals, Cells, and Cell Fractions. Novikoff hepatoma ascites cells which were grown in male Sprague-Dawley rats (Charles River, Wilmington, MA) were harvested 6 days after transplantation. Nucleoli were isolated from the Novikoff hepatoma cells by the magnesium–sucrose method (Rothblum et al., 1977). Nuclei were prepared by a method based on that of Chauveau et al. (1956). Briefly washed Novikoff hepatoma cells were homogenized in 2 M sucrose containing 10 mM Tris[1] (pH 7.5), 12 mM $MgCl_2$, and 0.1 mM PMSF[1] and then centrifuged at 28000*g* for 60 min. The pellet of this centrifugation was resuspended in 0.88 M sucrose containing 10 mM

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; bp, base pairs; kb, kilobases; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TE, 0.01 M Tris and 0.001 M EDTA, pH 7.6; Me_2SO, dimethyl sulfoxide; rDNA, DNA sequences coding for ribosomal RNA; rRNA, ribosomal RNA.

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Tris (pH 7.5), 12 mM $MgCl_2$, and 0.1 mM PMSF and then centrifuged at 1200*g* for 20 min. This pellet was used for DNA extraction.

Protein Preparations. The protein C23 used in these studies was obtained from nucleoli of Novikoff hepatoma ascites cells. Immediately after isolation, the nucleoli were extracted with 0.4 N H_2SO_4, and the extracted protein was precipitated with ethanol as previously described (Olson et al., 1974a). Protein C23 was then purified from the precipitated acid-soluble protein by either of two methods: (1) the preparative polyacrylamide gel electrophoresis–hydroxylapatite procedure of Mamrack et al. (1979) or (2) a modification of the method of Tsutsui et al. (1980) which has recently been described (Rao et al., 1982). In method 2, the acid-soluble protein was fractionated on columns of Sephacryl S-200 followed by purification of C23 containing fractions on Sephadex G-150 columns. The protein preparations were of the same level of purity as previously reported (Mamrack et al., 1979; Olson et al., 1981), i.e., the protein migrated essentially as a single spot on two-dimensional electrophoresis. No detectable differences in DNA binding characteristics were found between batches of protein C23 prepared by either method. However, since method 2 yielded greater quantities of protein, it was used for isolating protein for most of these studies.

DNA Preparations and Cloned Fragments of rDNA. Nuclear DNA was extracted from Novikoff hepatoma nuclei according to the method of Kavenoff & Zimm (1973). Final purification of the DNA was achieved by banding to equilibrium in a CsCl gradient. After dialysis the A_{260}/A_{280} ratios were between 1.8 and 1.9 and the A_{260}/A_{230} ratios were between 2.3 and 2.5. The DNA was labeled with ^{32}P by nick translation (Rigby et al., 1977) by using [α-^{32}P]dCTP (obtained from New England Nuclear) without added deoxyribonuclease I. All enzymes were obtained from Bethesda Research Laboratories.

Recombinant DNA molecules were comprised of plasmid pBR322 and various fragments of the genes for rat ribosomal RNA. The original samples of pDF4 and pDF8 (Fuke et al., 1981) and pDF15 and pDF20 (M. Fuke, personal communication) were a generous gift of Dr. Motohiro Fuke. Plasmids pKW1 and pKW2 were obtained by subcloning *Eco*RI/*Bam*HI and *Bam*HI/*Hin*dIII fragments of pDF20 into pBR322 that was also cleaved with the same pair of restriction endonucleases. The plasmids were used to transform *Escherichia coli* strain HB101 (Wensink et al., 1974), amplified in the presence of chloramphenicol, and purified from detergent lysates by banding on two successive CsCl gradients containing ethidium bromide (Clewell & Helinski, 1969). For binding assays all plasmids were linearized by limit digestion with *Eco*RI, followed by several extractions with phenol and then ether and finally ethanol precipitation. In some experiments protruding single-stranded ends of the *Eco*RI-digested fragments were "filled in" by incubation with the Klenow fragment of DNA polymerase I in the presence of all four deoxyribonucleoside triphosphates (Klenow & Henningsen, 1970).

DNA Binding Assays. DNA binding by protein C23 was measured by the nitrocellulose filter disk assay (Jones & Berg, 1966; Riggs et al., 1970) by using ^{32}P-labeled whole nuclear DNA. Prior to the assay the nitrocellulose filters (BA 85, from Schleicher & Schuell, Keene, NH) were soaked in 0.5 M KOH for 15 min and then washed exhaustively with distilled water and finally with binding buffer. Binding reactions were run in 0.25 mL of a binding buffer containing 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM Tris-HCl (pH 7.2) 0.1 M KCl, 0.1 mM PMSF, 0.01% bovine serum albumin (Sigma RIA grade),

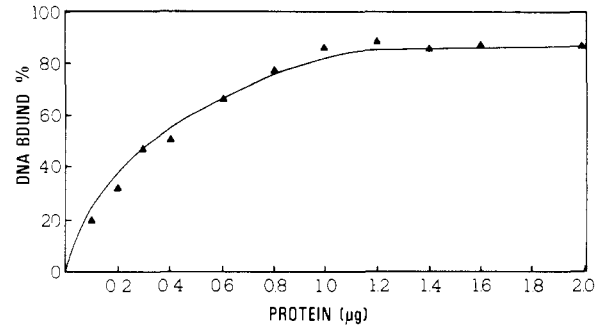

FIGURE 1: Direct equilibrium binding curve of protein C23 interacting with ^{32}P-labeled whole nuclear DNA. Increasing amounts of protein were added to binding buffer containing 0.1 μg of DNA in a total volume of 0.25 mL as described under Materials and Methods.

and 5% Me_2SO with a DNA content of 50–100 ng. In some experiments the KCl concentration was varied between 0 and 1 M. Protein C23 stock solutions in 0.9 M acetic acid were adjusted to pH 7.2 with a concentrated solution of Tris base, and aliquots were then diluted appropriately with binding buffer. Reactions were initiated by addition of protein C23 in binding buffer. In competition assays the competing DNA was mixed with the labeled DNA in the binding buffer prior to initiation of the reaction. Immediately prior to performing binding assays the plasmids were heated at 65 °C for 3 min and quick cooled in ice so that, for restricted plasmid DNAs that were not treated with DNA polymerase I, it was possible to eliminate any intra- or intermolecular reassociation of cohesive ends generated by *Eco*RI. All reactions were carried out at 30 °C for 20 min at which time the reaction was stopped by filtration through the nitrocellulose membranes. The filters were then washed twice with 2-mL portions of the binding buffer. After filtration the filters were dissolved in 0.5 mL of 0.5 N HCl plus 1 mL of ethyl acetate prior to addition of 4 mL of Aquasol II scintillation fluid. The samples were counted in a Beckman LS 9000 scintillation counter. In some experiments the *E. coli* single-stranded DNA binding protein, SSB (obtained as a generous gift from Dr. Kenneth Williams), was substituted for protein C23 in either the direct binding or competition assays essentially as described above.

Results

DNA Binding Characteristics and Assay Conditions. The nitrocellulose filter disk assay was used for the quantitation of DNA binding to protein (Jones & Berg, 1966; Riggs et al., 1970). The assay employed nuclear DNA which was ^{32}P-labeled by nick translation (Rigby et al., 1977). The ^{32}P-labeled DNAs typically had specific activities of (1–3) $\times 10^7$ cpm/μg. Most assays were performed under standard assay conditions at pH 7.2 in which the binding buffer contained 0.1 M KCl. Figure 1 indicates that when DNA binding was assayed under standard conditions using a nuclear DNA concentration of 0.4 μg/mL and increasing concentrations of protein, half-saturation was achieved at a protein concentration of approximately 1.2 μg/mL (assay volume = 0.25 mL). This corresponds to a ratio of 3 μg of protein/μg of DNA. By use of the half-saturation protein concentration (assuming a 1:1 molar ratio of DNA:protein) and the molarity of the DNA fragments (estimated at 15 000 base pairs by agarose gel electrophoresis) the dissociation constant was estimated to be approximately 10^{-8} M. At saturating concentrations, protein C23 was capable of binding 80–90% of the nuclear DNA in the assay mixture (Figure 1). In other experiments using ^{32}P-labeled nucleolar DNA, binding curves essentially identical with those using whole nuclear DNA were obtained. Similar binding curves

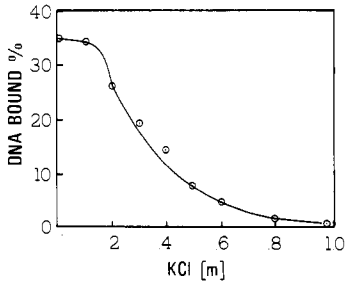

FIGURE 2: Effect of ionic strength on DNA–protein complex formation. Protein C23 (0.3 μg) was added to 0.1 μg of whole nuclear ^{32}P-labeled DNA in 0.25 mL of binding buffer containing various concentrations of NaCl. Samples were processed as described under Materials and Methods.

Table I: Binding of Various Sized DNAs by Protein C23[a]

treatment	approximate mean size (base pairs)	bound (%)
none	>23 000	73.9
sheared with 30-gauge needle[b]	15 000	71.7
sonication,[c] 20 s at 2.5 setting	14 000	77.8
sonication, 20 s at 4.5 setting	500	78.3
sonication, 60 s at 5.0 setting	200–300	82.2

[a] Incubation mixtures (0.25 mL) contained 0.05 μg of ^{32}P-labeled whole nuclear DNA and 2 μg of protein C23 in binding buffer under standard conditions (see Materials and Methods). DNA sizes were estimated by electrophoresis in agarose or polyacrylamide gels as described. [b] Obtained under conditions described in the text. [c] Performed using a Heat Systems Model W-375 ultrasonic generator equipped with a standard tapered microtip on a 0.5-in. disruption horn. Sonication was done at 0–5 °C at a DNA concentration of 1.67 μg/mL in TE buffer.

were also obtained by using protein purified either by the preparative gel electrophoresis–hydroxylapatite method (Mamrack et al., 1979) or by the modified Tsustui method (Rao et al., 1982).

In order to optimize binding conditions various parameters such as pH, ionic strength, reaction time, and DNA size were examined. Protein C23 bound DNA over a broad range of pH from 5.5 to 8, with no apparent maximum or minimum activity over that range. A pH value (7.2) in the physiological range was chosen for standard assay conditions. In assays performed with protein concentrations below saturation, DNA binding activity was highly sensitive to ionic strength with maximal binding at 0–0.1 M KCl (Figure 2). At salt concentrations greater than 0.1 M the binding activity dropped as the ionic strength was increased; binding was decreased by approximately 50% at 0.3 M and was negligible at 0.8 M. Thus, assays were performed at low ionic strength, generally at or near 0.1 M KCl.

The binding reaction took place quickly under standard assay conditions, with binding reaching a plateau by 10–15 min. However, assay times of less than 15 min resulted in poor reproducibility. When the binding reaction time was extended to 20 min before filtering, scatter in the data was significantly reduced.

Various sizes of DNA were obtained by shearing or sonicating ^{32}P-labeled nuclear DNA. The average size of this DNA varied from 200–300 base pairs up to more than 23 000 base pairs as estimated by electrophoresis in agarose (Helling et al., 1974) or polyacrylamide (Maniatis et al., 1975) gels. Similar amounts of DNA were bound to protein C23 under standard conditions and saturating levels of protein regardless of the size of DNA (Table I). Although the binding did not appear to be DNA size dependent, subsequent experiments

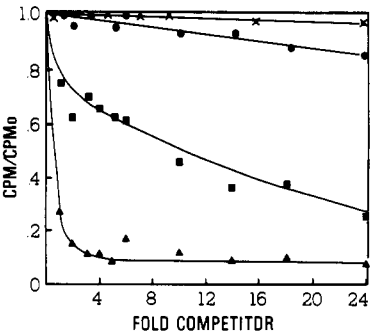

FIGURE 3: Competition of linear duplex or single-stranded pBR322 plasmid DNA or ribosomal RNA with binding of ^{32}P-labeled whole nuclear DNA by protein C23. Assay mixtures contained 0.05 μg of ^{32}P-labeled whole nuclear DNA, 0.3 μg of protein C23, and 0–1.2 μg of competing DNA or RNA in 0.25 mL of binding buffer. The linearized plasmid DNA was obtained by digestion with *Eco*RI and endonuclease. Single-stranded DNA was prepared by heating linear duplex DNA at 100 °C for 3 min and quick cooling in ice. In some experiments the linearized DNA was treated with the Klenow fragment of DNA polymerase I as described under Materials and Methods. An equimolar mixture of 18S and 28S ribosomal RNA (0–1.5 μg) was used for the RNA competition experiments. Abscissa (cpm/cpm$_0$), ratio of cpm bound in the presence of competititor to cpm bound in the absence of competitor; (■) linear duplex DNA; (▲) single-stranded DNA; (×) linear duplex DNA treated with Klenow fragment of DNA polymerase I; (●) ribosomal RNA.

were standardized with regard to DNA length. DNA was hydrodynamically sheared by 10 passages through a 30-gauge needle at 0–5 °C in TE buffer using maximum thumb pressure resulting in fragments with a mean length of about 15 kb.

Binding of Single-Stranded DNA by Protein C23. Preliminary studies suggested that protein C23 preferred to bind single-stranded nuclear DNA compared to double-stranded DNA. This was confirmed by equilibrium competition experiments using ^{32}P-labeled double-stranded nuclear DNA in competition with unlabeled single-stranded or double-stranded DNA from plasmid pBR322 for protein C23. The double-stranded DNA was obtained by cleavage of the plasmid by *Eco*RI: Gel electrophoresis confirmed that all detectable DNA was in the form of linear molecules that were 4.3 kb in length. Single-stranded DNA was obtained by heat denaturation of linearized plasmid DNA. Figure 3 shows that linearized single-stranded pBR322 DNA was a more effective competitor for nuclear DNA than double-stranded pBR322 DNA. The double-stranded DNA required 8–10-fold more DNA than did single-stranded DNA to reach a level of 50% competition. However, since digestion with *Eco*RI endonuclease produces a protruding single-stranded tetranucleotide sequence at both ends of the linearized plasmid, these may have contributed to the binding of protein C23 to duplex DNA. To test this possibility the linearized plasmid was subjected to treatment by the Klenow fragment of DNA polymerase I in the presence of all four nucleoside triphosphates to fill in the single-stranded regions. When this material was used as a competitor, there was little, if any, competition (Figure 3). In other experiments (not shown) the circular supercoiled plasmid DNA was also a very poor competitor. Thus, protein C23 has a marked preference for single-stranded DNA.

Protein C23 has been found associated with nucleolar preribosomal RNP particles (Prestayko et al., 1974; Olson et al., 1974b), raising the possibility that it may interact directly with preribosomal RNA. In addition, since the protein prefers single-stranded DNA, it may also interact with RNA which is largely single-stranded. Figure 3 indicates that when a mixture of 18S and 28S ribosomal RNA was used as a competitor against ^{32}P-labeled whole nuclear DNA, little com-

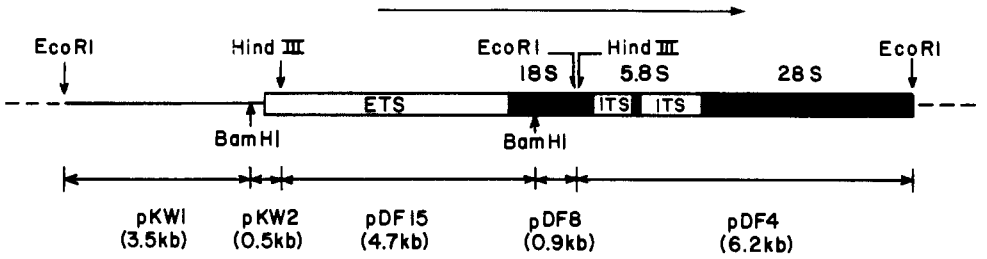

FIGURE 4: Composite map of rat rDNA clones. The map shows the restriction fragments used in the protein C23 binding assays. The boxed-in region designates those regions transcribed as 45S pre-rRNA. Shaded areas indicate the sequences for rRNAs; unshaded areas indicate the external transcribed spacer (ETS) and the internal transcribed spacer (ITS). Arrow designates direction of transcription (5′ to 3′).

petition occurred. Thus, it is unlikely that protein C23 has any significant affinity for cytoplasmic ribosomal RNA.

Binding of Cloned rDNA Fragments. Our initial inability to detect differences between the binding of nucleolar and nuclear DNA to protein C23 and combined with the presence of a single-stranded DNA binding activity suggested that the binding was nonspecific. However, preferential binding of C23 to certain sequences of limited abundance may have been obscured by nonspecific binding to highly abundant sites. Therefore, the binding of protein C23 to uniquely nucleolar sequences, i.e., the genes for ribosomal RNA, was tested.

The DNAs used for competition assays were derived from five fragments which span nearly the entire length of the transcribed region of 45S preribosomal RNA plus a large portion of the upstream spacer region (Figure 4). All DNA fragments were originally part of a single insert designated rat rRNA gene-4 cloned in bacteriophage Charon 4A (Fuke et al., 1981) and subsequently subcloned in plasmid BR322. Beginning at the 5′ end of the map (Figure 4) the fragments have the following characteristics: Plasmid pKW1 has a 3.5-kb insert containing DNA exclusively from the nontranscribed spacer region. Plasmid pKW2 has the adjacent 0.5-kb fragment and contains the reported initiation site for transcription of 45S pre-rRNA. About half of this fragment is from the nontranscribed spacer region, and the other half is from the externally transcribed spacer region. Plasmid pDF15 has a 4.7-kb insert that contains most of the externally transcribed spacer region and about 0.5 kb of the 5′ end of the gene coding for 18S rRNA. Plasmid pDF8 contains 0.9 kb from the center of the 18S rRNA gene. Finally, plasmid pDF4 has a 6.2-kb insert that encompasses the 3′ end of 18S rRNA gene plus the internal transcribed spacers, the 5.8S gene, and most (approximately 4 out of 4.5 kb) of the DNA coding for 28S rRNA.

To measure the relative effectiveness of the cloned rDNA fragments to bind protein C23, competition assays identical with those shown in Figure 3 were used. This permitted the use of only one labeled DNA, in this case nuclear DNA, and eliminated artifacts which may be generated in separately labeling each of the cloned fragments. Since it was previously shown that circular supercoiled DNA bound poorly to protein C23, the plasmid DNAs were linearized with restriction *Eco*RI endonuclease. Figure 5 shows that on an equal mass basis plasmid pKW1 most effectively competed with ^{32}P-labeled nuclear DNA for binding protein C23. Less than 1-fold mass excess was required to achieve 50% competition. The next most effective competitor was plasmid pKW2. With this plasmid 50% competition was achieved at approximately 1.5-fold mass excess competing DNA. Plasmids pDF15 and pDF8 were next and approximately equal in their ability to compete for protein C23, with 50% competition at 6- and 8-fold excess, respectively. Plasmid pDF4 was the poorest competitor. Thus, the ability to compete for protein C23

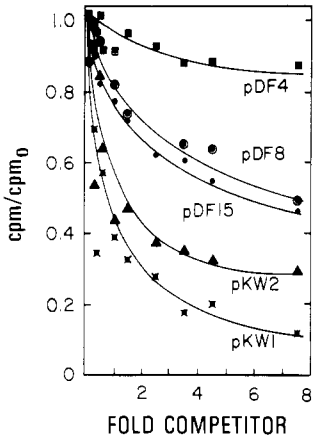

FIGURE 5: Equilibrium competition assays with various cloned fragments of rDNA. Assays were performed as under Materials and Methods with 0.05 μg of ^{32}-P-labeled nuclear DNA, 0.3 μg of purified protein C23, and various concentrations of competing DNAs as plasmids containing the DNA inserts as indicated (see map in Figure 4). All plasmids were linearized by digestion with *Eco*RI restriction endonuclease. Results are expressed as the ratio of cpm bound in the presence of competitor to cpm bound in the absence of competitor.

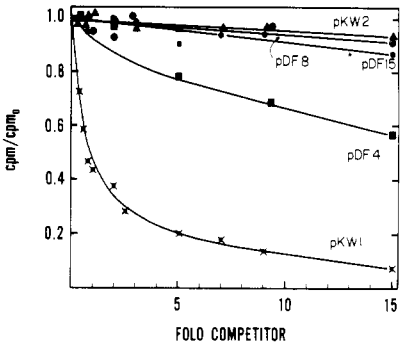

FIGURE 6: Equilibrium competition assays of blunt-ended DNA from plasmids containing rDNA fragment inserts. The plasmids were linearized with *Eco*RI endonuclease and then treated with the Klenow fragment of DNA polymerase I. Assays were performed and data expressed as in Figure 5.

approximately followed the 5′ to 3′ order of the fragments as they are transcribed in the genome with those closest to the nontranscribed spacer being the best competitors.

Since protein C23 contains single-stranded DNA binding activity, this may obscure our ability to fully detect preferential binding to specific double-stranded DNA sequences; i.e., the protruding single-stranded tetranucleotide sequences at the ends of the *Eco*RI-cleaved plasmid might be responsible for a substantial part of the binding of the recombinant plasmids by protein C23. To test this possibility the series of competition assays shown in Figure 5 was repeated with plasmids which had been treated with the Klenow fragment of DNA polymerase I to fill in potential single-stranded regions and to produce

blunt ends. When plasmids treated in this way were assayed for competition with ^{32}P-labeled nuclear DNA in binding protein C23, only pKW1 retained its ability to compete (Figure 6). As with untreated pKW1, the blunt end containing plasmid achieved 50% competition at approximately a 1 to 1 ratio of pKW1 to nuclear DNA. Upon treatment with the Klenow fragment, plasmids pKW2, pDF15, and pDF8 essentially lost all of their ability to compete. Plasmid pDF4 retained a similar level of competitiveness as the untreated sample, but it did not reach 50% competition even at 15-fold excess. (Note that Figure 6 includes higher levels of competing DNA than does Figure 5.) Plasmid pDF4 is 1.35 times larger than pKW1, taking into account the sizes of the inserts plus the pBR322 vector sequences of 4.36 kb (Sutcliffe, 1978). Thus, when equal weights of each plasmid were used, there were proportionately more copies of the pKW1 insert available for binding. However, the difference in competitive ability between the two plasmids is approximately 20-fold (Figure 6% calculated by extrapolation). Thus, the difference in molar ratios alone cannot account for the difference in competition that was observed.

To confirm that single-stranded regions were not responsible for the binding of pKW1 to protein C23, the plasmids digested with *Eco*RI were also treated with S1 nuclease under conditions known to excise single-stranded DNA (Wiegand et al., 1975). A competition curve of the S1 nuclease treated plasmid pKW1 was essentially the same as the curves for pKW1 shown in Figures 5 and 6 (data now shown). Thus, the selectivity of binding of plasmid pKW1 by protein C23 appears to be independent of single-stranded regions of DNA, while binding by the other plasmids prior to treatment with Klenow fragment of DNA polymerase I appears to be largely, if not exclusively, dependent upon single-stranded DNA binding activity.

Additional experiments were done to determine whether plasmid pKW1 contained transiently single-stranded regions (e.g., A-T rich sequences). For this purpose, SSB, the helix-destabilizing and single-stranded DNA binding protein from *E. coli* (Williams & Konigsberg, 1981), was substituted for protein C23 in the binding assay. In preliminary experiments the SSB–DNA binding curves were similar to the C23–DNA binding curve in Figure 1, although saturation was achieved at somewhat lower molar ratios of protein to DNA. Since SSB has affinity essentially only for single-stranded DNA, its binding must be due to single-stranded regions in the nuclear DNA that were introduced during its isolation and in vivo labeling (nick translation). The similarity in binding curves further supports the suggestion that protein C23 is also largely binding single-stranded regions of the ^{32}P-labeled nuclear DNA. Plasmids pKW1 and pDF15, which had been treated with the Klenow fragment of DNA polymerase I, were tested for their ability to compete for the binding of ^{32}P-labeled nuclear DNA by SSB. These two plasmids were chosen because of their differential ability to compete for protein C23 (Figure 6) and for their similarity in insert size (Figure 4). If pKW1 contained potentially single-stranded regions it should compete with nuclear DNA in a manner similar to the results shown in Figure 6. However, Figure 7 shows that neither plasmid was an effective competitor, indicating that under the binding conditions used in this study neither plasmid contained single-stranded regions. Therefore, it is unlikely that protein C23 binds pKW1 by virtue of helix destabilization properties.

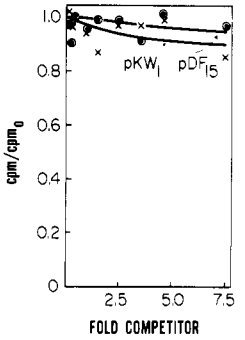

FIGURE 7: Equilibrium competition assays of blunt-ended DNA from plasmids containing rDNA fragment inserts using *E. coli* single-stranded DNA binding protein (SSB) instead of protein C23. Assays were performed as under Materials and Methods with 0.05 μg of ^{32}P-labeled nuclear DNA, 0.01 μg of purified SSB, and various concentrations of competing plasmids which were treated as in Figure 6. The data were expressed as in Figures 5 and 6.

Discussion

The organization and interaction of proteins and nucleic acids in the nucleolus are poorly understood. Protein C23 is a major nucleolar protein (Orrick et al., 1973), which has properties that suggest that it may play an organizational role in the nucleolus (Mamrack et al., 1979; Lischwe et al., 1979). Since protein C23 is predominantly localized to the nucleolus (Olson et al., 1981), these studies were initiated to determine whether the protein recognizes uniquely nucleolar DNA sequences, i.e., the genes for ribosomal RNA. The data presented here show that, in addition to an affinity for single-stranded DNA, protein C23 exhibits preferential binding to a cloned 3.5-kb DNA fragment that is adjacent to and extends upstream from the transcriptional initiation site for 45S pre-rRNA. Furthermore, protein C23 does not appear to have significant affinity for cytoplasmic ribosomal RNA.

At this point we do not know the basis for the interaction between pKW1 and protein C23. Since the cloned insert is a relatively long sequence (3.5 kg), protection experiments (Tijan, 1978; Weideli et al., 1980) or further studies on smaller subcloned fragments will be required to delineate the minimum length and the precise location of the region which innteracts with the protein. However, at least two alternatives may be possible regarding the nature of the binding site on the DNA: (1) the binding of protein C23 may be dependent on transiently single-stranded regions of A-T rich sequences of DNA, or (2) the binding may be at a discrete nucleotide sequence. With regard to the first alternative, it is known that there are A-T rich regions upstream from the transcriptional initiation site in rDNA (Bach et al., 1981). Therefore, it is possible that protein C23 binds these A-T rich regions by virtue of its affinity for single-stranded DNA. On the other hand, experiments utilizing either a single-stranded DNA binding protein (SSB) or nonspecific DNA binding proteins (histones) support the suggestion that the C23–DNA interaction may have some degree of sequence specificity. Substitution of the *E. coli* single-stranded DNA binding protein SSB for protein C23 in the direct binding assay indicated that the labeled nuclear DNA used in the assay contained single-stranded regions. Since the DNA binding saturation curves of SSB and C23 were similar, it is likely that protein C23 binds largely to single-stranded regions in the labeled nuclear DNA. However, plasmid pKW1 was an effective competitor against the apparent single-stranded DNA binding activity in protein C23, even when the plasmid was "filled" in by the Klenow fragment of DNA polymerase I or treated with S1 nuclease. Furthermore, it was shown that when SSB was substituted for C23 in the competition experiments, pKW1 was not an effective competitor against nuclear DNA binding by SSB. Therefore, it is unlikely that pKW1 contains even transiently single-stranded regions. This further supports the second

alternative that protein C23 preferentially binds certain double-stranded sequences.

In additional experiments histones were substituted for protein C23 in the plasmid competition experiments, such as those shown in Figure 6. Again, the differential competition of pKW1 vs. pDF15 was not seen; i.e., both plasmids competed equally well for binding of C23 (data not shown). Thus, protein C23 appears to have an affinity for pKW1 that is not exhibited by certain nonspecific DNA binding proteins, such as histones. From the above data we tentatively conclude that protein C23 has some degree of double-stranded DNA sequence dependent preference.

Although protein C23 has been found associated with preribosomal RNP particles (Prestayko et al., 1974; Olson et al., 1974a,b), these studies indicate that it has virtually no affinity for cytoplasmic ribosomal RNA. Alternatively, it may bind to regions of 45S pre-rRNA that are removed during the maturation process of 5.8S, 18S, and 28S rRNA. Warner (1974) suggested that this may be the role of the "nucleolar stable proteins" of the nucleolar preribosomal particles.

Daskal et al. (1980) have presented evidence that the silver-staining regions of nucleoli are in the fibrillar components which contain the nascently transcribed preribosomal RNA. Since proteins C23 and B23 are the major silver-staining proteins of the nucleolus (Lischwe et al., 1979), it was suggested by Daskal et al. (1980), and later confirmed by immunochemical localization, that protein C23 is at the fibrillar centers of nucleoli (Lischwe et al., 1981). Thus, it is conceivable that protein C23 is part of the transcriptional complex or located in promoter regions of the rDNA spacers. If so, it may function as part of the transcriptional process by helix destabilization through single-stranded binding activity. The nontranscribed spacer regions at the 5′ side of rDNA in *Tetrahymena* contain DNase I hypersensitive sites (Borchsenius et al., 1981) which may result from single-stranded regions (Larsen & Weintraub, 1982). Groudine & Weintraub (1982) suggest that the DNase I hypersensitive sites associated with active genes result from single-stranded regions which may be stabilized and prepetuated by single-stranded DNA binding proteins. If this hypothesis is correct, protein C23 would be a candidate for a single-stranded DNA binding protein with that function in the nucleolar transcriptional system.

Since protein C23 is a relatively large protein, it is likely that only a portion of the protein interacts with DNA. Preliminary studies indicate that certain large fragments of the protein retain the ability to bind DNA. It is conceivable that the highly acidic regions of protein C23 (Mamrack et al., 1979; Rao et al., 1982) simultaneously interact with histones on nucleosomes to further destabilize the DNA in localized regions of chromatin. Further studies on the interaction of protein C23 with intact chromatin should help answer this question and may provide clues toward the understanding of a precise function of this protein.

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Covalent and Noncovalent Interactions of Aflatoxin with Defined Deoxyribonucleic Acid Sequences†

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ABSTRACT: The major stable reaction product of activated aflatoxin B_1 (AFB1) with DNA is the N^7-guanine adduct. By using a simple extension of the Maxam–Gilbert sequencing technique on defined DNA sequences modified by activated AFB1, we have shown [Muench, K. F., Misra, R. M., & Humayun, M. Z. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6–10] that the induction of alkali labile sites is strongly and predictably influenced by the nucleotide sequence context in double-stranded DNA. In this paper, we present data that show the following: (a) sequence-specific effects are abolished in single-stranded DNA while at the same time the overall reactivity of guanine (G) residues is strongly suppressed when compared to double-stranded DNA; (b) in single-stranded DNA capable of forming intrastrand hairpin stem–loop structures, AFB1 reacts strongly with base-paired G residues in a sequence-specified manner but not as efficiently with non-base-paired G residues; (c) certain chemicals related in structure to AFB1, and the intercalating dye ethidium bromide, inhibit the reaction of AFB1 with DNA. These data are consistent with the possibility that a sequence-specific precovalent association between double-stranded DNA and AFB1 is a factor in the observed specificity in covalent reactions. We also present data on another kind of covalent AFB1 binding, namely, the formation of photoadducts. Previous work showed that AFB1, in analogy with related coumaryl chemicals, the psoralens, can be photoactivated, resulting in stable adduct formation with DNA. We show here that a significant reaction is with G residues, presumably at the N^7 position.

Aflatoxin B_1 (AFB1)[1] is a mycotoxin produced by a number of strains of the fungal genus *Aspergillus* found as a natural contaminant of the food chain (Busby & Wogan, 1979). Experimentally, AFB1 is a highly toxic substance, a powerful mutagen, and the most potent liver carcinogen described to date. In chemical terms, AFB1 is a highly substituted coumarin (Figure 1). The 8,9 double bond (previously called the 2,3 double bond) has been shown to be essential for the toxic, mutagenic, and carcinogenic properties of AFB1, since AFB2, which differs from AFB1 in not having this double bond, is less active by about 2 orders of magnitude. AFB1 requires activation before significant reaction with biological macromolecules, and activation is believed to proceed via epoxidation of the 8,9 double bond (Essignmann et al., 1977; Lin et al., 1977). Although the putative epoxide has never been isolated due to presumed reactivity, there is compelling evidence for its involvement. Activated AFB1 can react with proteins, RNA, and DNA (Miller & Miller, 1977) although the critical macromolecule is assumed to be DNA. AFB1 can be activated in vitro either by liver microsomal preparations [e.g., D'Andrea & Haseltine (1978)] or by oxidation with a mild organic oxidant like chloroperoxybenzoic acid (Martin & Garner, 1977). Recently, it has been shown that near-ultraviolet irradiation induces photobinding of AFB1 to DNA (Shieh & Song, 1980). The major stable adduct with DNA formed by the epoxide mechanism is the N^7-guanine (N^7-G) adduct (Figure 1), which alone (together with its secondary derivatives) may account for more than 90% of AFB1 stably associated with DNA. There is no direct evidence for cause–effect correlations between N^7-G modification by AFB1 and mutagenesis and carcinogenesis. However, AFB1 modification does affect the template function of DNA, as predicted for bulky DNA modifications; and since N^7-G modification is the predominant stable effect of the reaction of AFB1, biological significance has been attributed to this lesion (Miller & Miller, 1977). Moreover, the N^7 modification results in weakening the N-glycosidic bond at modified G residues such that both spontaneous and enzymatic depurination and strand scission are likely to be promoted (Singer, 1975). Both of these events are known to have biological consequences (Singer, 1975; Schaaper & Loeb, 1981).

An interesting, but largely unexplored, question on DNA modification by chemical carcinogens is the effect of nucleotide sequence environment of the target site. With reference to aflatoxin, one can ask whether all G residues react similarly on a random basis or whether the bases surrounding a given

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[1] Abbreviations: AFB1, AFB2, AFG1, and AFG2, aflatoxin B_1, B_2, G_1, and G_2; 8-MOP, 8-methoxypsoralen; Me_2SO_4, dimethyl sulfate (DMS in figures); DMF, dimethylformamide; DS, double stranded; SS, single stranded; CT DNA, calf thymus DNA; CPB, chloroperoxybenzoic acid; bp, base pair(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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