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BOVINE PANCREATIC DNASE I BINDS VERY TIGHTLY TO DNA FRAGMENTS AND MAY BE MISTAKEN FOR PUTATIVE ENDOGENOUS NUCLEAR PROTEINS COVALENTLY BOUND TO DNA

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Using published methods for the isolation of nuclear proteins tightly bound to DNA, and resistant to removal by SDS or 16–BAC detergent and urea, several new protein bands in the region of 55 kd and 62 kd on SDS gel and 43 kd and 70 kd on 16–BAC gel electrophoresis were identified in extracts of avian erythroid nuclei. These bands were radiolabelled by subjecting the DNA–protein complexes to nick–translation in the presence of [^{32}P]–dCTP, followed by prolonged digestion with excess bovine DNase I. Amino acid sequence analysis shows that these bands contain DNase I. These results indicate that DNase I can form stable complexes with DNA, and suggest that DNase I–DNA complexes may be mistakenly identified as nuclear proteins covalently bound to DNA.

It has been recognized for over 30 years that covalently linked DNA–protein complexes exist (1). Many examples of stable DNA–protein complexes have been documented in bacteria and viruses, but to date, no analogous eukaryotic cellular compounds have been unequivocally shown to exist (1). Transient covalent linkage to DNA has been demonstrated for eukaryotic topoisomerase I and II (2), and it has been demonstrated that DNA may be linked to the nuclear matrix by bonds that are resistant to protein denaturants such as cesium chloride and SDS (3–11). Moreover, a peptide linked by an ester bond between DNA phosphates and an hydroxyamino acid, probably tyrosine, has been shown to be present in proteinase–treated nuclear DNA (6,7) and in nuclear matrices prepared by high salt and urea extraction (8). However, the identity of the proteins involved in nuclear attachment has not been established. Using several different techniques, investigators have consistently reported finding proteins of $M_r \simeq 50$ kd and 60 kd (4,10–12). Most methods for isolation have utilized DNase I to remove DNA from the complex or to isotopically–labelled short DNA fragments tightly bound to protein (and thus protected from complete DNase I digestion) by nick translation (3,4,6–12).

Abbreviations

16–BAC, benzyldimethyl–n–hexadecylammonium; PVDF, polyvinylidene difluoride; kd, kilodaltons; DNase I, bovine pancreatic deoxyribonuclease I (EC 3.1.21.1).

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We have studied stable DNA–protein complexes isolated from adult chicken reticulocyte chromatin and DNase I–treated DNA. Our results demonstrate that bovine pancreatic DNase I can bind very tightly to small fragments of DNA to form discrete electrophoretic bands. This finding is discussed in light of previous reports concerning the identity of putative proteins that bind covalently to eukaryotic DNA.

Methods

Isolation of DNA–Protein Complexes: DNA–protein complexes were isolated from adult avian reticulocyte nuclei. Reticulocytes were collected from birds treated with 1–acetylphenylhydrazine (20 mg/kg/day X 3 to 4 days) and nuclei were prepared as previously described (13). DNA tightly bound to protein were isolated by the SDS/K^+–precipitation method (14,15). Briefly, nuclei digested with micrococcal nuclease for up to 30 min (13) were resuspended in 1% SDS, 10 mM Tris–HCl pH 8.0, 1 mM EDTA at 50°C. Insoluble material was removed by 30s centrifugation at 12000 g at room temperature. The supernatent was isolated and made 100 mM with KCl and warmed to 50°C to clear. After incubation on ice for 10 min, SDS/K^+ was pelleted by centrifugation at 12000 g for 10 min at 4°C. The pellet was washed twice with 100 mM KCl, 10 mM Tris–HCl pH 7.5, 1mM EDTA by warming to 50°C to resuspend, followed by incubation on ice and centrifugation as before. DNA–protein complexes were isolated by three extractions with phenol (1:1 v/v) (6). In some experiments, SDS/K^+–pelleted samples were fractionated by gel filtration on Sepharose–6B in 0.1% SDS, 100 mM NaCl, 10 mM Tris–HCl pH 7.5, 1mM EDTA (5), with collection of the void volume peak for further study.

Radiolabelling of DNA by Prolonged Nick–Translation: The nick–translation labelling of protein–DNA complexes and DNA was carried out as described previously (9,10). Radiolabelled samples, with DNase I–digested oligonucleotides removed by repetitive gel filtration, were submitted to SDS–polyacrylamide gel electrophoresis (16).

16–BAC Gel Electrophoresis: As an alternative to repetitive gel filtration, electrophoresis in 16–BAC as described by MacFarlane (17) was used. This method has the advantage that nuclei acids precipitate or migrate in an opposite direction from proteins, and obviates the need for separation of the products of nick–translation from radiolabelled proteins prior to electrophoresis. Briefly, after DNase I digestion, samples were made. 1.5% (w/v) 16–BAC, 22.5% (w/v) urea, 2.5% (v/v) b–mercaptoethanol and 5% (v/v) glycerol, and sonicated in a 40 watt ultrasonic cleaning bath. Samples were applied to a 1.5 X 32 cm slab gel prepared as described elsewhere (17). After electrophoresis, the gel was stained with Coomassie Blue (0.1% w/v), dried, and submitted for autoradiography (17).

Amino Acid Sequencing: After preparative SDS–gel electrophoresis (1.5 mM X 16 cm X 16 cm, 12% acrylamide), protein bands were visualized by immersion in 100 mM KCl. The excised bands were soaked in several changes of warm 0.025 M Tris, 0.192 M glycine, 0.1% SDS until clear, and then electro–transferred to a PVDF–based membrane (Immobilon, Millipore) in the same buffer. The 55 kd protein band (p55) was submitted for amino acid sequencing by a gas–phase (Beckman) by standard technique (18).

Results

Several methods for isolating DNA covalently bound to DNA have been described (3,4,11,15). We chose to use SDS/K^+–precipitation as this method is simple, rapid, and easily scaled–up for preparative purposes (15). To determine the identity of the protein(s) tightly bound to DNA, SDS/K^+–precipitated DNA was extracted repetitively with phenol and chloroform:isoamyl alcohol to remove contaminating unbound proteins (6), and then incubated with DNase I to release bound proteins. Results from this approach are shown in

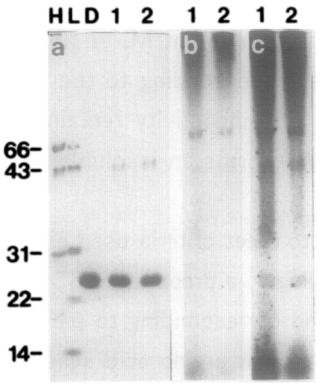

Figure 1: Analysis of Proteins Labelled by Nick–Translation and Exhaustive DNaseI Digestion. Avian reticulocyte genomic DNA (1 and 1) or DNA–protein complexes (1 and 2) were used as substrates in the nick–translation reaction using [^{32}P]–dCTP, followed by exhaustive digestion with DNaseI, as previously described (9, 10, and see Methods). The samples were directly submitted to 16–BAC gel electrophoresis. a) Commassie blue stained gel. The autoradiogram of this gel is shown after: b) short exposure; c) longer exposure. H, high molecular weight markers; L, low molecular weight markers; D, DNaseI alone.

Fig. 1. Note that protein bands of apparent $M_r \simeq 25$ kd (p25), 43 kd (p43) and 70 kd (p70) were found using 16–BAC gel electrophoresis, with band p25 corresponding to DNase I. In the absence of DNase I, or when DNase I was added to samples during boiling in 1% SDS, 2.5 mM EDTA, no additional bands were detected. Using SDS gel electrophoresis, p43 had a $M_r \simeq 55$ kd (p55); and p70 a $M_r \simeq 62$ kd (data not shown). Note also that bands were detectable, in roughly equal amounts, whether or not avian DNA was treated with proteinase K prior to extraction. It has been reported that the protein(s) covalently linked to DNA can be only partially degraded by proteinase treatment (6,7,9).

To confirm that the protein bands identified by gel electrophoresis were indeed proteins bound tightly to DNA prior to nuclease treatment, samples of proteinase–treated DNA and DNA containing DNA–protein complexes were radiolabelled by the nick–translation reaction (9,10). DNA not protected by protein was removed by a final DNase I incubation (see ref. 9 for discussion of this method). The products of this reaction were then analyzed by autoradiography of 16–BAC gels (Fig. 1) or, by autoradiography of SDS

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                1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16
p55             ? -Lys-Ile-Ala-Ala-Phe-Asn-Ile-Arg- ? -Phe-Gly-Glu-Thr- ? -Met
DNaseI    Leu-Lys-Ile-Ala-Ala-Phe-Asn-Ile-Arg-Thr-Phe-Gly-Glu-Thr-Lys-Met
(Bovine)
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Figure 2: Amino Acid Sequence of the 55 kd Protein. The protein was isolated by SDS–gel electrophoresis of avian reticulocyte DNA–protein complexes treated with DNaseI (see Methods). The sequence of bovine pancreatic DNaseI is from ref. 20.

gels using samples isolated by repetitive gel filtration (data not shown). Radiolabelled bands corresponding to p43 and p70 were found after brief exposure of the 16–BAC gel. In addition, a faint band at p25 was also detected after longer exposure (Fig. 1). Radiolabelled bands were present in SDS gels at apparent $M_r \simeq 100$, kd (p100) and, faintly, at $M_r \simeq 62$ kd. A band in the 16–BAC gel corresponding to the p100 band in SDS gels is, if present, obscured by the large quantity of radioactivity remaining at the origin, presumably due to incompletely digested DNA. DNA forms an insoluble pellet in 16–BAC layering buffer and does not enter the gel (17).

The band p43 appeared to meet criteria used elsewhere to identify protein covalently bound to DNA (3–11). Accordingly, a preparative SDS gel was run with the DNA–protein sample used in Fig. 1. The band corresponding to p43 (p55) was electroblotted to a PVDF membrane, and directly submitted for amino acid sequence analysis. Results are shown in fig. 2. As can be seen, the sequence corresponds exactly with that published for DNase I (20).

To determine if any of the bands present after nick–translation labelling may represent avian cellular proteins tightly bound to DNA, isolated protein–DNA samples were radioiodinated using a method documented to label only protein (6). After repetitive phenol

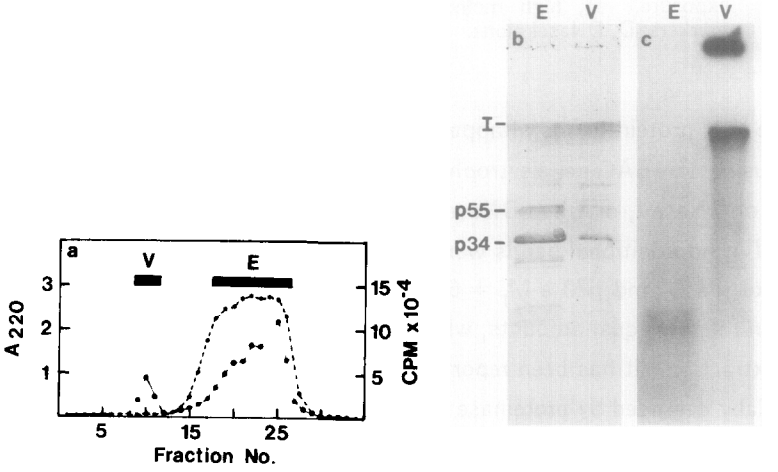

Figure 3: ^{125}I–Labelled Avian Reticulocyte DNA–Protein Complexes Digested with DNaseI Do Not Enter SDS Stacking Gels. DNA–protein complexes were extracted from 80 mg avian reticulocyte nuclei and purified by repetitive phenol extractions (see Methods). After iodination of a small aliquot with I^{125} as previously described (6), the radioiodinated and unlabelled DNA–protein complex was ethanol precipitated. The dryed pellet was resuspended in 5 ml of 50 mM NaCl, 10 mM Tris–HCl pH7.5, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2 mM PMSF, and DNaseI (1.5 mg; Sigma) was added. After incubation for 2 h at 37°C, the sample was made 5 mM with EDTA and 1% with SDS. The sample was then applied to a 5 x 25 cm sepharose 6B column and eluted with 0.1% SDS, 100 mM NaCl; 10 mM Tris–HCl pH 7.5, 1 mM EDTA. Fractions of 2 ml were collected. a) Elution profile of the column; cpm x 10^{-4}/fraction (–●–), A_{220}/fraction (–●–). Fractions in the void volume (V) or eluted volume (E) were collated and concentrated by pressure ultrafiltration (30 kd cut–off membrane, Pharmacia). Equal volumes of the concentrated samples were subjected to SDS gel electrophoresis; b) Coomassie blue stained gel; c) autoradiogram of the gel. The bands for native DNaseI (p34) and p53 are indicated.

extraction, samples were incubated with DNase I (see Methods). After incubation, the sample was fractionated by gel filtration. The void volume containing a peak of radioactivity, and the later eluting A_{220} peaks were collated separately, concentrated by ethanol precipitation and submitted for SDS gel electrophoresis. As shown in Fig. 3, DNase I and higher molecular weight bands were not radiolabelled. Instead, incorporated radioactivity was chiefly associated with the material in the void fraction that did not enter the acrylamide stacking gel. A similar finding has been previously reported for protein, covalently bound to DNA, isolated from nuclear matrix preparations of rat sperm and Guerin ascites tumor cells (8).

Discussion

The results show that bovine pancreatic DNase I can bind tightly to DNA to form a complex that is resistant to denaturation by ionic detergents, urea, β–mercaptoethanol and boiling. Several distinct gel electrophoretic bands of DNase I–DNA complexes were found. These bands are probably generated by DNase I binding progressively longer DNA fragment lengths, as the radioactivity of the dCTP–labelled complexes, relative to stainable protein, increased with decreasing gel mobility. The crystallographic structure of an octanucleotide DNA–DNase I complex has been determined (21). These studies have shown that DNase I binds to the minor groove of the DNA molecule over a length of 6 bp plus two adjacent phosphates (21). The interactions between enzyme and DNA appear to be chiefly hydrogen bonds and Vander Waal's contacts; covalent linkage has not been reported (21,22). Although our studies do not document that the linkage between DNase I and DNA is covalent, this possibility is suggested by the stability of the complex to the variety of protein denaturants employed.

DNase I has been almost universally used in the purification of proteins from nuclear matrices and from DNA–protein complexes (3,4,6–12). In these studies, nuclear proteins of molecular weights ranging from 20 kd to over 100 kd have been reported to be very tightly or covalently attached to eukaryotic DNA (3–11). Two major protein bands, of $M_r \simeq 50$ kd and 60 kd, have been described in several studies (3–5,9–12). With the exception of vimentin, $M_r = 57$ kd (11), the putative DNA attachment proteins have not been identified. Using previously published methods for isolating nuclear protein–DNA complexes, major DNase I–DNA complexes were found with $M_r \simeq 55$ kd and 63 kd (in SDS gels) in the studies reported here. These DNase I–DNA complexes may be mistaken for putative cellular DNA attachment proteins, particularly in those studies in which prelabelling of nuclear proteins, prior to the addition of DNase I, was not carried out (3,4,9,10,12).

In the studies reported here, radiolabelled protein bands were not detected in 12% acrylamide SDS–gels when protein–DNA complexes were labelled with ^{125}I prior to exposure to DNase I. Instead, radioactivity was chiefly associated with material that was retained in the 3% acrylamide SDS–stacking gel. A nuclear matrix protein covalently bound to eukaryotic DNA that did not enter an SDS–stacking gel has been previously reported (8). This protein was bound to DNA by a tyrosine phosphate linkage (6,8), and peptide mapping

indicated that this protein was not topoisomerase I or II (8). We have found that a cloned 480 bp DNA fragment that spans the nuclear matrix attachment region in the avian β–globin gene enhancer (3,19) becomes very tightly bound to a non–topoisomerase protein when incubated with avian nuclear matrices (19; and Zenk, Gindr, Bennett, Brotherton, manuscript submitted). This 480 bp DNA–protein complex also fails to enter a 3% SDS–acrylamide gel (Zenk, Ginder, Brotherton, manuscript in preparation). These results suggest that the major matrix protein(s) that bind very tightly to DNA fail to resolve in standard SDS gels and have yet to be identified. The failure to enter even low porosity gels may indicate low solubility in SDS, a large hydration shell (perhaps secondary to glycosylation (23)), or large protein chain size. These possibilities have not yet been addressed.

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