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DNA binding proteins of rat thigh muscle: Purification and characterization of an endonuclease

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Abstract. Two major DNA binding proteins of molecular weights 34,000 and 38,000 have been identified in the 30,000 *g* supernatant (S-30) fraction of rat thigh muscle extracts. The presence of 38 KD DNA binding protein in the muscle S-30 could be demonstrated only if Triton X-100 treated extracts were used for Afinity chromatography suggesting that this protein may be a membrane associated DNA binding protein. The 38 KD DNA binding protein differed from the 34 KD DNA binding protein also in its chromatographic behaviour in DE-52 columns in which the 38 KD protein was retained, while the 34 KD protein came out in the flow-through in an electrophoretically pure form. The 34 KD DNA binding protein can also be purified by precipitation with $MgCl_2$. Incubation of 0·15 M NaCl eluates (containing the 38 KD and/or 34 KD DNA binding protein) in the presence of 100 mM Mg^{2+} resulted in the specific precipitation of the 34 KD protein. Prolonged incubation (30 days) of the 0·15 M NaCl eluates containing the two DNA binding proteins at 4°C led to the preferential degradation of the 34 KD DNA binding protein. Nitrocellulose filter binding assays indicated selective binding of purified 34 KD protein to ss DNA. Purified 34 KD DNA binding protein cleaved pBR 322 supercoiled DNA, and electrophoresis of the cleavage products in agarose gels revealed a major DNA band corresponding to the circular form of DNA.

Keywords. Afinity chromatography; DNA binding protein; endonuclease cleavage of pBR 322 DNA.

Introduction

As a result of the realization of the importance of DNA endonucleases in DNA metabolism, especially in the processes of initiation of DNA synthesis and repair, several attempts have been made to isolate and characterize these proteins. Afinity chromatography of proteins on DNA-cellulose columns has proven to be a valuable technique for the purification of endonucleases and other DNA binding proteins. Restriction enzymes and other endonucleases bind to double-stranded regions or to localized single-stranded stretches of double-stranded and superhelical DNAs while S-1nuclease binds to single-stranded DNAs. Although numerous endonucleases have been isolated from prokaryotic cells and their biological functions established, investigations on eukaryotic endonucleases were of recent origin. DNA-binding

Abbreviations used: DBP, DNA binding protein; *ß*ME, *ß*-mercaptoethanol; PMSF, phenyl methyl sulphonyl fluoride; SDS, sodium dodecyl sulphate; PAGE, Polyacrylamide gel electrophoresis.

proteins with topoisomerase activities have been reported to be present in rat liver (Me Conaughy *et al.,* 1981; Ross *et al.,* 1983). Machray and Bonner (1981) have shown the conversion of super-helical pBR 322 DNA into linear form by a chromatin associated DNA binding protein (DBP). A DBP with endonucleolytic activity specific for apurinic and apyrimidinic DNA has been isolated from human placenta (Shaper *et al.,* 1982). Another endonuclease from placenta was shown to cleave pBR 322 DNA to linear form (Premeela *et al.,* 1984).

A 34 KD DBP was found to be present in large quantities in Yoshida ascites tumour cells (Rajakumar and Shanmugam, 1983). Since this tumour is of connective tissue origin, an attempt was made in search of similar DBPs in normal connective tissues. The results presented here show the presence of two DBPs of molecular weight 38,000 and 34,000 in rat thigh muscle of which the latter protein has been purified and studied in detail. The purified 34 KD protein is shown to possess endonuclease activity capable of converting supercoiled pBR 322 DNA into circular form. The muscle enzyme differs from the above mentioned eukaryotic endonucleases in that it produces a single cut circular DNA as a major cleavage product.

Materials and methods

Calf thymus DNA, Sigma cell (R) type-100 cellulose, Phosphorylase a, bovine serum albumin, human γ-globulin, ovalbumin, cytochrome C, Tris and Dowex were the products of Sigma Chemical Co., St. Louis, Missouri, USA. Acrylamide and bis-acrylamide were the products of Bio-Rad. DEAE-cellulose (DE-52) and phosphocellulose were obtained from Whatman, Co, UK. Nitrocellulose membrane filters were the products of Schelicher and Schuell inc., Germany. [^{32}P]-orthophosphoric acid (carrier-free) was obtained from Bhabha Atomic Research Centre, Bombay.

Isolation of [^{32}*P*] *-labelled Escherichia coli DNA*

[^{32}P]-DNA was extracted from *E. coli* using phenol-chloroform-isoamylalcohol according to the modified method of Marmur (1961). The isolated DNA was dissolved in 1 mM NaCl and passed through a column of Dowex-1 × 4 to remove anionic contaminants. The flow-through was collected and precipitated with ethanol. The precipitate was dissolved in 1 mM NaCl for use in further studies. DNA was denatured by heating at 100°C for 10 min followed by rapid cooling.

Preparation of cytoplasmic factions

All operations were carried out at 0-4°C unless otherwise mentioned. For the preparation of muscle extract, thigh muscle of albino rats was minced and then suspended in 10 volumes of sterile water. The suspension was homogenized thrice in a Sorvall homogenizer with the speed setting at 5, for 30 sec each time. The suspension was made 20 mM Tris pH 8· 1, 1 mM EDTA, 1 mM *β*-mercaptoethanol (*β*ME) and 1 mM phenyl methyl sulphonyl fluoride (PMSF). The homogenate was centrifuged at 30,000 *g* and the supernatant was used for the analysis of DBPs.

In some preparations, 1 % Triton X-100 was used in the extraction procedure for the preparation of S-30.

DNA-cellulose chromatography

Denatured DNA-cellulose column chromatography was carried out as described earlier (Shanmugam *et al.,* 1975; Raj akumar and Shanmugam, 1983, 1984). Muscle S-30 containing approximately 60-70 mg protein was chromatographed in a 0·8 × 4 cm denatured DNA-cellulose column. After washing the column extensively with buffer A (20 mM Tris pH 8·1, 1 mM EDTA, 1 mM βME and 1 mM PMSF) containing 50 mM NaCl, the bound proteins were eluted with the above buffer containing 0·15 M NaCl.

DEAE-cellulose chromatography

The 0·15 M NaCl eluate of DNA-cellulose column was dialysed extensively against buffer A containing 50 mM NaCl for 12 h with two changes of buffer. A column (1·5 × 8 cm) was packed with pre-equilibriated DEAA-cellulose and then washed with 100 ml of the column buffer containing 50 mM NaCl. The dialysed 0·15 M NaCl eluate from DNA-cellulose column was loaded onto the column at the flow rate of 10 ml/h. The flow-through was collected separately. The column was washed with 50 ml of column buffer and this was pooled with the flow-through. The bound proteins were then eluted using the column buffer containing 0· 15, 0·4 and 2 M NaCl.

The pooled flow-through and the wash fractions were concentrated by dialysis against sucrose and then precipitated with 100 % ammonium sulphate. The precipitate was collected by centrifugation at 30,000 *g* for 15 min and dissolved in a minimal quantity of column buffer.

Filter binding assay

Nitrocellulose filter binding assay was performed according to the procedure of Tsai and Green (1973). Before use, the filters were boiled in water and then soaked in filter binding buffer (20 mM Tris-HCl, pH 8, 1 mM EDTA, 2 mM βME and 5 % glycerol) for at least 12 h. For a typical assay, 2 μg of [^{32}P]-*E. coli* DNA (1,200 cpm) and appropriate concentrations of protein, in a final volume of 100 μl, was incubated at 37°C for 30 min. The assay mixture was slowly filtered through prewashed filters at a flow rate of 0·5 ml/min. The filters were washed thrice with 3 ml of buffer containing 1 % dimethyl sulphoxide, dried and counted.

Endonuclease assay

Endonuclease activity was monitored according to the modified procedures of Clough (1979). The assay mixture (20μl) contained 2 μl of enzyme sample, 5 mM $MgCl_2$, 5 μg of pBR 322 DNA, 10 mM βME and 50 mM Tris pH 8·3. All samples were incubated at 37°C for 30 min. The reaction was stopped by heating the samples at 60°C for 5 min. After the addition of glycerol (10 %) and bromophenol blue (0·002 %), the samples were loaded on 1 % agarose gels and electrophoresed (Premeela *et al.,* 1984).

Electrophoresis of proteins

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out as described earlier (Rajakumar and Shanmugam, 1983).

Results and discussion

DN A-cellulose chromatography of muscle proteins

DNA-cellulose chromatography of muscle S-30 extracts on ss DNA-cellulose columns and subsequent elution of bound proteins with 0·15 M NaCl removed most of the DBPs from the column. The 0·15 M NaCl eluate contained 2·5 % of the total proteins used for chromatography. Figure 1 shows a gel electrophoretic profile of DBPs present in the 0·15 M NaCl eluate. A 38 KD and a 34 KD protein were the major DBPs in the 0·15 M NaCl eluates obtained from the denatured DNA-cellulose column (figure 1, lane C). These two DBPs were observed only if a Triton X-100-treated S-30 was used for chromatography. As seen in figure 1, lane D, when the cytoplasmic fraction was prepared without using Triton X-100, only the 34 KD protein was seen in the 0·15 M

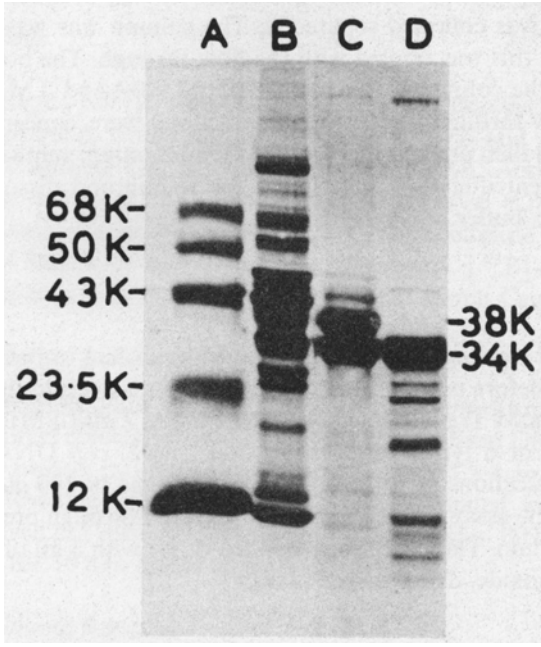

Figure 1. SDS-PAGE of muscle DBPs. Muscle extract was prepared with or without using Triton X-100 and chromatographed on heterologous denatured DNA-cellulose columns. The bound proteins were eluted and electrophoresed in 10-18% Polyacrylamide gradient gels. Lane A, molecular weight markers; lane B, S-30 prepared using Triton X-100; lane C, DBPs eluted with 0·15 M NaCl from denatured DNA-cellulose column in which S-30 prepared in the presence of Triton X-100 was chromatographed; lane D, DBPs, eluted from denatured DNA-cellulose column in which S-30 prepared in the absence of Triton X-100 was chromatographed.

NaCl eluates of denatured DNA-cellulose columns. These results suggest that the 38 KD DBP may be a membrane-associated protein.

Effect of Mg^{2+} on the muscle DBPs

When the 0·15 M NaCl eluate containing the 34 KD and 38 KD proteins were incubated with 100 mM Mg^{2+} at 37°C for 18–24 h, a milky white precipitate was formed. After centrifugation and electrophoresis, the precipitate was found to contain the 34 KD DBP and was nearly 95 % pure (figure 2). Similar results were obtained when 0·15 M NaCl eluate containing only the 34 KD DBP was used for Mg^{2+} precipitation. Incubation of the DBPs with Mg^{2+} at 4°C for longer periods (1 month) resulted in the preferential degradation of the 34 KD DBP to smaller polypeptides (figure 2B).

Purification of the muscle 34 *KD DBP*

In the purification profile shown in figure 3, 0·15 M NaCl eluates of the ss DNA-cellulose columns (in which muscle extracts prepared in the presence of Triton X-100 were chromatographed) were used. These eluates contained both the 34 KD and 38 KD DBPs. When these eluates were chromatographed on DEAE-cellulose columns, the flow-through had the 34 KD DBP in a pure form (figure 3). Similarly, when the 0·15 M NaCl eluate containing mainly the 34 KD DBP (obtained from extracts prepared in the

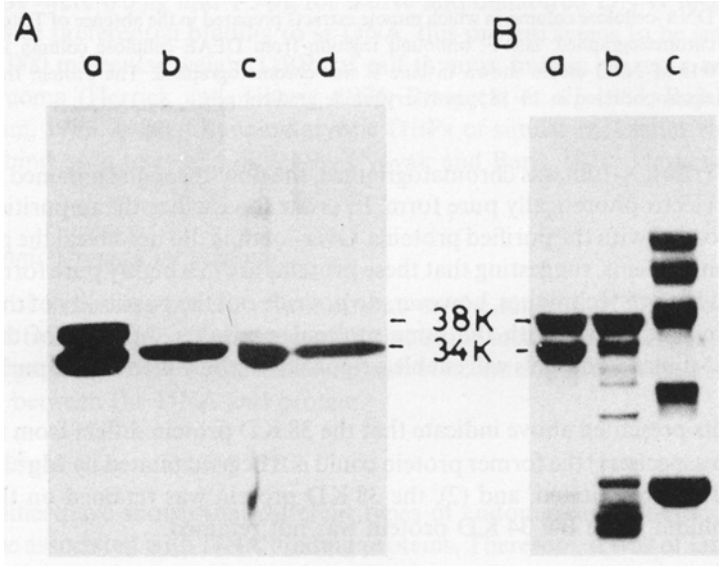

Figure 2. Mg^{2+} precipitation of DNA-binding proteins. The 0·15 M NaCl eluates from ss DNA-cellulose columns were adjusted to 100 mM Mg^{2+} using $MgCl_2$ and were incubated at 37°C for 18–24h. A. Lanes a and c are unincubated controls; lanes b and d, Mg^{2+}-precipitated samples. The DBPs used for lanes a and b were obtained from Triton X-100 treated extracts while the DBPs used for lanes c and d were derived from S-30 prepared in the absence of the detergent. B. Shows the effect of prolonged (1 month) incubation of DBPs in 100 mM Mg^{2+} at 4°C. Lane a, unincubated 0·15 M NaCl eluate and lane b, incubated sample. For this experiment, the DBPs were obtained from Triton X-100 treated S-30, c, Molecular weight markers.

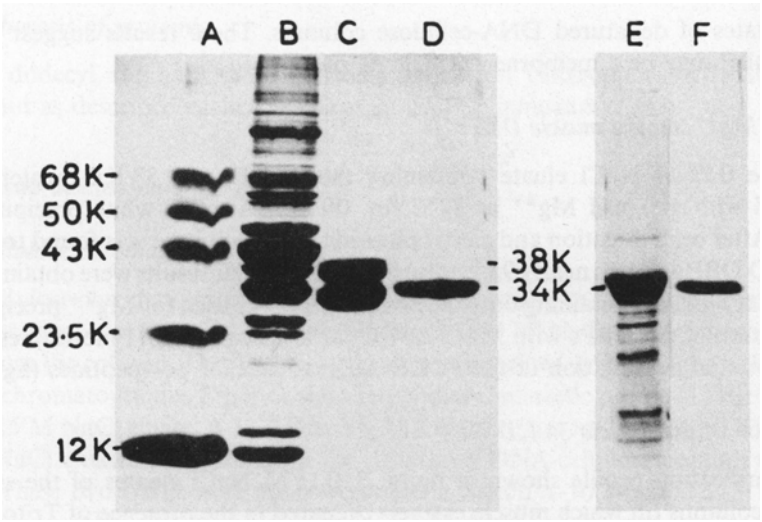

Figure 3. Purification of 34 KD DBP. Lane A, molecular weight markers; lane B, total proteins of muscle extract prepared using Triton X-100; lane C, 0·15 M NaCl eluate from ss DNA-cellulose column in which S-30 prepared in the presence of Triton X-100 was chromatographed; lane D, unbound fraction from DEAE-cellulose column in which the 0·15 M NaCl eluate shown in lane C was chromatographed; lane E, 0·15 M NaCl eluate from ss DNA-cellulose column in which muscle extracts prepared in the absence of Triton X-100 was chromatographed; lane F, unbound fraction from DEAE-cellulose column in which the 0·15 M NaCl eluate shown in lane E was chromatographed. The protein fractions were electrophoresed in 10–18% Polyacrylamide gradient gels.

absence of Triton X-100) was chromatographed, the flow-through contained the 34 KD DBP in an electrophoretically pure form. In order to visualize the impurities, the gels were over-loaded with the purified proteins. Over-loading did not reveal the presence of contaminant proteins, suggesting that these proteins are in a highly pure form (figure 3, lanes D and F). Our techniques, however, do not rule out the possibility of the presence of contaminant proteins with the same molecular weights. Analysis of the purified proteins in 2-dimensional gels will enable a rigorous establishment ofthe purity ofthese proteins.

The results presented above indicate that the 38 KD protein differs from the 34 KD DBP in two aspects: (1) the former protein could not be precipitated by M g^{2+}, while the latter could be precipitated, and (2), the 38 KD protein was retained on the DEAE-cellulose column while the 34 KD protein was not retained.

Specificity of binding

The binding specificity of the 34 KD DBP to either native or denatured DNA was determined using nitrocellulose filter binding assay according to the procedures of Tsai and Green (1973). Native or denatured DNA (2 μg) was incubated with increasing amounts of the 34 KD DBP for 30 min and assayed. The results presented in figure 4A indicate that the muscle 34 KD DBP preferentially bound to ss DNA. The protein

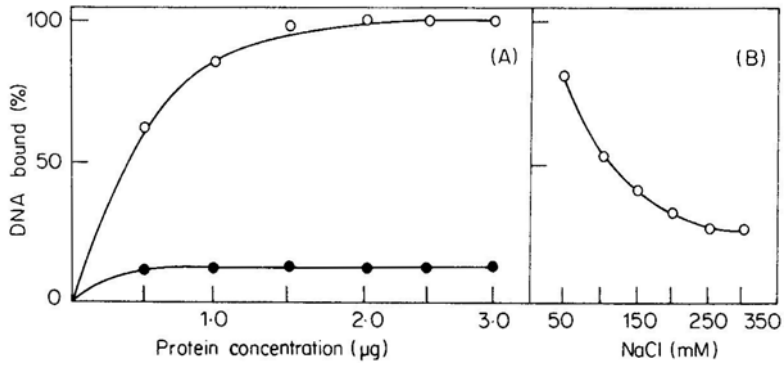

Figure 4. Nitrocellulose filter binding assay of 34 KD DBP. **A.** Increasing quantities of the muscle 34 KD DBP were incubated with 2 μg of native(●)or denatured (O) [^{32}P] -DNA (1200cpm) from *E. coli.* The DNA-protein complexes were separated by filtration on nitrocellulose membrane filters and counted in aqueous scintillation fluid. **B.** Effect of increasing concentrations of NaCl on the binding of the muscle 34 KD DBP. Filter binding assay was done with 1·5 μg of 34 KD DBP, 2 μg of heat denatured [^{32}P] -DNA (1200 cpm) from *E. coli* and indicated amounts of NaCl.

retained 10% of the native DNA and 95 % of the ss DNA. The saturation levels of the muscle DBP were 0·5 μg and 1·5 μg for native and denatured DNAs respectively.

In terms of preferential binding to ss DNA, this protein seems to be similar to the 33,000-34,000 molecular weight DBPs of calf thymus, human placenta and Yoshida ascites sarcoma (Herrick and Alberts, 1976; Premeela *et al.,* 1984; Rajakumar and Shanmugam, 1983, 1984). Other eukaryotic DBPs of similar molecular weights were shown to bind both to ss and ds DNAs (Novak and Baril, 1978; Mather and Hotta, 1977).

Effect of ionic strength on binding

The effect of increasing concentration of ions on the binding of DBP to DNA, was determined by altering the ionic concentrations in the incubation medium. The results shown in figure 4B indicate that increasing salt concentrations decreased the complex formation between the DNA and protein.

Endonuclease activity of 34 *KD DBP*

Recent studies have shown that different types of endonuclease activities have been found to be associated with DNA binding proteins. Therefore, it was of interest to see whether the purified 34 KD DBP of muscle possessed any endonucleolytic activity. For endonuclease assays, pBR 322 DNA was used as substrate and the cleavage products were analysed by electrophoresis in agarose gels. The results presented in figure 5, lane A indicate the presence of 3 bands corresponding to multimeric, circular and superhelical forms in the control samples incubated in the absence of the 34 KD protein. However, DNA samples incubated with the 34 KD protein showed a steep increase in the amount of the circular form with concomitant decrease in the supercoiled form, (figure 5, lanes B and D). These results suggest that the 34 KD

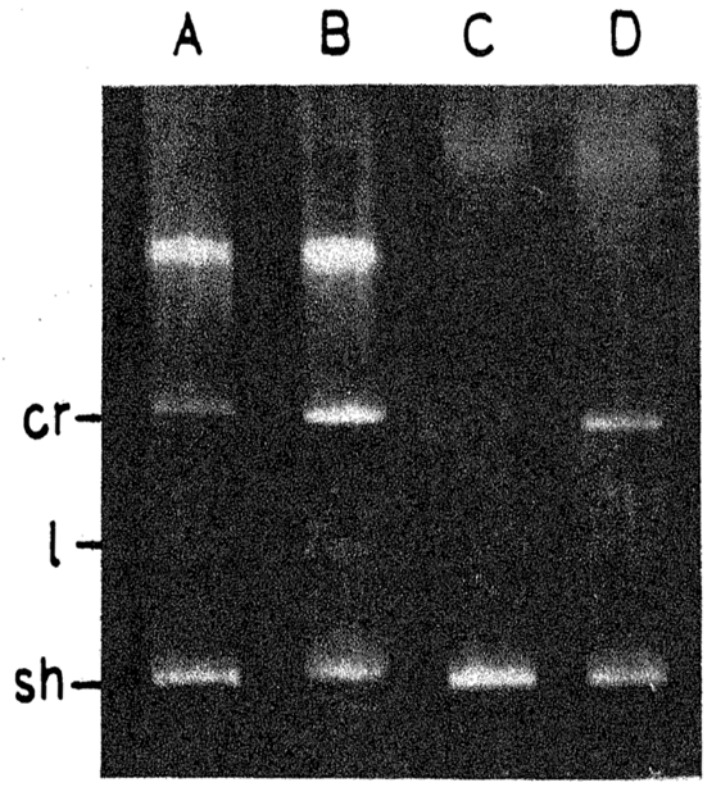

Figure 5. Endonucleolytic cleavage of native and heat denatured pBR 322 DNAs by muscle 34 KD DBP. Native (lane B) and denatured (lane D) pBR 322 DNA (5 μg) were incubated with 0·5 μg of muscle 34 KD DBP for 30 min at 37°C. Lanes A and C respectively contained native and denatured pBR 322 DNAs incubated in the absence of 34 KD DBPs. After the assay, the samples were electrophoresed in 1 % agarose gels for 5 h at 60v. m, multimer; Cr, relaxed circular; 1, linear; Sh, super helical.

protein possesses endonucleolytic activity capable of converting the super-helical DNA into circular form.

When the endonuclease activity was studied as a function of increasing time (figure 6), for a period of 15, 30, 45 and 60 min, the following results were obtained: (i) the conversion of supercoiled DNA into the circular form gradually increased with the time of incubation, (ii) the optimal time for maximal enzyme activity was 30 min; and (iii) no additional degradation products were seen following longer periods of incubation.

Endonuclease activity increased with the increase in protein. 3 μg of the 34 KD protein was able to completely convert the pBR 322 DNA into the circular form. No additional degradation products were seen when increased amounts of protein were used (figure 7).

The results presented in figures 5-7 show that the 34,000 molecular weight DBP possesses endonuclease activity. In contrast to the action of other eukaryotic endonucleases which produced linear DNAs from supercoiled DNAs (Machray and Bonner, 1981; Premeela *et al.,* 1984), the muscle enzyme produced circular DNAs from superhelical pBR 322 DNA suggesting that the endonuclease activity cleaved the DNA by introducing a single cut in one of the strands of the DNA. The fact that increased time of incubation (figure 6) or higher concentrations of DBP (figure 7) failed to

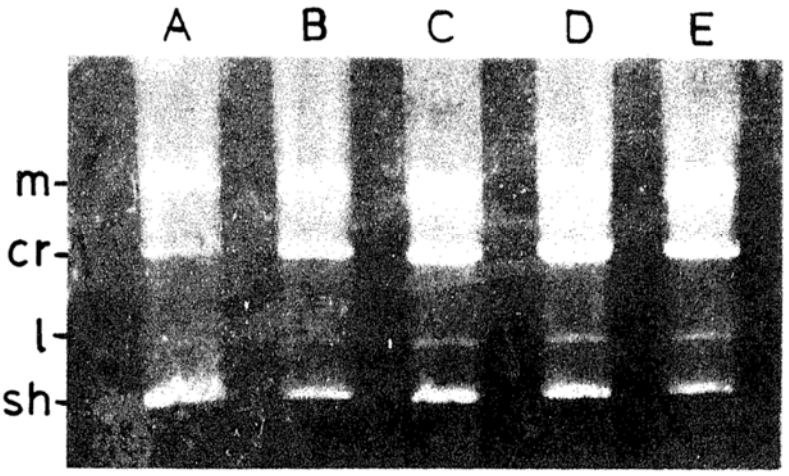

Figure 6. Time course of cleavage of native pBR 322 DNA by muscle 34 KD DBP. Aliquots containing 5 μg pBR 322 native DNA were incubated with 1 μg of the purified protein for the desired periods and electrophoresed in 1 % agarose gels for 5 h at 60v. Lane A, control pBR 322 DNA, lanes, B, C, D and E contained pBR 322 DNAs incubated with muscle 34 KD DBP for 15, 30, 45 and 60 min respectively.

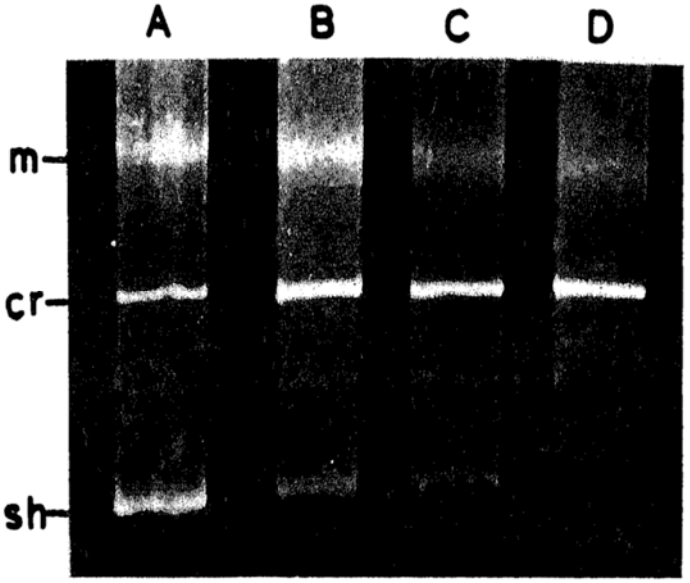

Figure 7. Effect of increasing concentrations of the DBPs on endonuclease activity. Aliquots containing 5 μg of pBR 322 DNA were incubated with increasing concentrations of muscle 34 KD DBP for 30 min and electrophoresed in 1 % agarose gels. Lane A, control pBR 322 DNA; lanes B, C and D contained samples incubated with 1, 2 and 3 μg of proteins respectively.

produce additional bands other than the single-nick circular form, indicates the absence of contaminating nucleases.

In the light of the implication of the role of DNA endonucleases in the initiation of DNA replication and repair (Cleaver, 1974; Linn, 1982; Brown *et al.,* 1982), it will be of interest to know the function of the muscle endonuclease in DNA metabolism. Studies

aimed at a detailed characterization of the muscle endonuclease, including the determination of cleavage site(s) are in progress.

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