Purification of a DNA-binding protein from Xenopus laevis unfertilized eggs

G. Carrara, S. Gattoni, D. Mercanti and G. P. Tocchini-Valentini

Laboratory of Cell Biology - C.N.R., Via Romagnosi, 18A, 00196 Rome, Italy

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ABSTRACT

A DNA-binding protein from Xenopus laevis unfertilized eggs has been purified to apparent homogeneity. It is a heat stable, lysine-rich protein and has a molecular weight corresponding to 8,200 daltons, measured by sodium dodecyl sulphate gel electrophoresis. The protein, which is active in a monomeric form, stimulates DNA polymerase α, and binds to single and double stranded DNA. One egg contains about $4 \times 10^2$ molecules (minimum estimate) of the protein; since we calculate that $4 \times 10^8$ molecules are sufficient to cover the entire genome (haploid complement), there is much more protein than is needed to cover chromosomal DNA.

INTRODUCTION

Xenopus laevis oocytes and eggs provide an interesting system in which to study various aspects of DNA metabolism.

During oogenesis genetic recombination takes place (1). Young oocytes selectively replicate ribosomal DNA genes (2), whereas older oocytes are unable to carry out any kind of DNA replication (3). In fertilized eggs chromosomal DNA replication occurs at an exceptionally high rate. Autoradiographic studies have shown that the exceptional rate of DNA synthesis is explained by an unusually large number of origins of replication (4). Presumably the repertoire of proteins required for DNA replication are synthesized during oogenesis and stered in unfertilized eggs.

We recently described a cell-free system derived from oocytes capable of producing a variety of structures from supercoiled SV 40 + DNA, such as: figure 8 dimers, catenated dimers, circular dimers, catenated trimers, Cairns' structures, complex multimers and circular monomers.
with tails (5). The system is soluble and one can therefore attempt the isolation of the various components and the identification of their relative roles in the formation of the complex DNA. We have characterized a series of activities present in the extract, such as DNA polymerase (6-8), an endonuclease, and a DNA relaxing protein (9). We have applied DNA-cellulose chromatography, a simple and successful method for identifying a variety of proteins involved in DNA metabolism, to the fractionation of an extract derived from *Xenopus laevis* eggs. In this paper we describe the purification and characterization of a small, heat stable protein (molecular weight 8,200) contained in the fractions eluted from DNA-cellulose at 0.5 M salt. The protein binds to native and to denaturated DNA and stimulates DNA polymerase α. We refer to this protein as *Xenopus laevis* DNA-binding protein one (X-DBP 1). We have calculated that each egg has at least 4 x 10^12 molecules of X-DBP 1.

**MATERIALS AND METHODS**

a) Materials

Deoxynucleoside 5'-triphosphates were purchased from Schwartz Mann, Orangeburg, N.Y.  [3H] deoxyguanosine 5'-triphosphate (7.4 Ci/mmol) and [3H] methyldeoxythymidine 5'-triphosphate (48 Ci/mmol) were purchased from New England Nuclear. Munktell 410 cellulose powder was obtained from Bio-Rad, Richmond, California. Pancreatic deoxyribonuclease (ribonuclease free) and calf thymus DNA were obtained from Worthington Biochemicals Corp.. Proteins used as molecular weight markers, for molecular weight determination by gel filtration methods, were purchased from Schwartz Mann. DNA polymerase α from HeLa cells was the kind gift of Dr. S. Spadari and Dr. A. Weissbach (10).

b) Methods

"Activated" calf thymus DNA. "Activated" calf thymus DNA was prepared by subjecting DNA to a limited digestion. 100 mg of calf thymus DNA was incubated at 37°C for 15 min with 4 μg of RNAase-free pancreatic DNAase in 20 ml of 50 mM-Tris.HCl(pH 7.5), 10 mM-Mg acetate. The reaction was stopped by adding NaCl to a final concentration of 0.5 M and
heating for 30 min at 60°C; the DNA was phenol-extracted and, after centrifugation at 10,000 g for 10 min, the upper phase was ether-extracted.

**DNA-cellulose.** DNA-cellulose was prepared according to the combined methods of Litman (11) and Alberts and Herrick (12): a solution of denatured calf thymus DNA was mixed with cellulose, prepared according to Alberts and Herrick, and lyophilized. The material was then suspended in absolute ethanol and U.V. irradiated according to Litman. About 4 mg of DNA per gram of cellulose remained bound after washing three times with 1 mM-NaCl.

**Gel electrophoresis.** Polyacrylamide gel electrophoresis of proteins in presence of sodium dodecyl sulphate was performed according to the method of Weber and Osborn (13).

**Protein determination.** Protein concentration was determined according to the method of Lowry et al. (15) using bovine serum albumin as the standard.

**Amino acid composition.** Lyophilized samples were hydrolyzed at 120°C in 1 ml of 6 HCl for 48 and 72 hours. Analysis was performed in a Beckman model 121 automatic amino acid analyzer.

**Sephadex chromatography.** An aliquot of X-DBP 1 was dialyzed against 50 mM-Tris-HCl (pH 7.5), 1 mM-EDTA, 1.4 mM-2-mercaptoethanol, 100 mM-NaCl, centrifuged at 10,000 g for 10 min and loaded on a Sephadex column (G-75, Superfine; 1 x 150 cm) which was calibrated with five different protein markers before and after the run with the dialyzed X-DBP 1. The column was eluted at a flow rate of 4 ml/hr. The fractions were assayed for DNA polymerase activity and for the capacity to stimulate *Xenopus laevis* DNA polymerase α.

**DNA polymerase α preparation and assay.** *Xenopus laevis* DNA polymerase from stage 6 oocytes (7) was prepared according to Tatò et al. (6). The
reaction mixture, in a final volume of 0.1 ml, contained 50 mM-Tris.HCl (pH 7.5), 6 mM-dithiothreitol, 6 mM-Mg acetate, 10 μM-dATP, dCTP, dTTP, 3.5 μM $[^3H]$dGTP (8,000 cpm/pmol) and 7 μg of "activated" calf thymus DNA. The reactions were run at 37°C for 30 min and the trichloroacetic acid precipitable radioactivity was collected on glass filters.

Purification of X-DBP 1. Unfertilized Xenopus laevis eggs were obtained according to Gurdon (16) and dejelled according to Dawid (17). Eggs were finally washed with buffer A [50 mM-Tris.HCl (pH 7.5), 1 mM-EDTA, 1.4 mM-2-mercaptoethanol, 10% glycerol (v/v) and 50 mM-NaCl] and stored at -20°C. All operations were carried out at 4°C. Eggs were resuspended in 2 volumes of buffer A, containing 0.3% Triton X-100, and homogenized. The homogenate was centrifuged at 10,000 g for 10 min. Floating lipids were discarded and the supernatant was centrifuged at 100,000 g for 60 min. The high speed supernatant was either immediately used or stored at -20°C.

The high speed supernatant (S-100) derived from 40 ml of Xenopus laevis eggs was applied on a 50 ml DNA-cellulose column equilibrated with buffer A. After washing with 3 volumes of buffer A, the column was eluted at a flow rate of 50 ml/hr, with buffer B [50 mM-Tris.HCl (pH 7.5), 1 mM-EDTA, 1.4 mM-2-mercaptoethanol, 10% glycerol (v/v)] containing 150 and 500 mM-NaCl. Fractions of the 500 mM-NaCl step, which showed absorbance at 280 nm, were pooled and dialyzed for 3 hours using acetylated dialysis tubing, against two 1-liter volumes of buffer B. The dialyzed material (Fraction I) was applied to a 30 ml DEAE cellulose (Whatman 52) column equilibrated with buffer B. The flow-through was assayed for DNA polymerase activity and for the capacity to stimulate Xenopus laevis DNA polymerase α. The fractions which were found to stimulate DNA polymerase α were combined (Fraction II) and applied to a 11 ml phosphocellulose (Whatman P11) column equilibrated with buffer B. After washing with 3 volumes of the same buffer, the column was eluted with 100 ml of a linear gradient of 0-2M NaCl. The fractions of the gradient (3 ml/fraction) were assayed for DNA polymerase activity and for the capacity to stimulate Xe-
The fractions which stimulated DNA polymerase were dialyzed against buffer B (Fraction III). Fraction III, when analyzed by sodium dodecyl sulphate gel electrophoresis, showed a single band (see Results); the protein contained in Fraction III is referred to in this paper as X-DBP 1.

**DNA polymerase stimulation assay.** Reaction mixtures, in a final volume of 0.1 ml, contained: 50 mM-Tris. HCl (pH 7.5), 6 mM-dithiothreitol, 6 mM-Mg acetate, 10 μM-dATP, dCTP, dTTP, 3.5 μM [3H]dGTP (8,000 cpm/pmol) and 7 μg of "activated" calf thymus DNA, 10 μl of DNA polymerase diluted in buffer B containing 1 mg/ml of bovine serum albumin (0.05-0.1 pmoles incorporated at 37°C for 30 min), and 40 μl of Fraction II or Fraction III. The reactions were run at 37°C for 30 min and the trichloroacetic acid precipitable radioactivity was collected on glass filters.

**Nuclease assay.** X-DBP 1 (2 μg) was assayed for endonuclease activity in an incubation mixture (0.1 ml) containing 50 mM-Tris. HCl (pH 7.5), 6 mM-dithiothreitol, 6 mM-Mg acetate, 10 μM (each) dCTP, dATP, dTTP, 1.6 μg of supercoiled SV 40 [3H]DNA (5,000 cpm/μg). Incubation was at 37°C for 30 min. The reaction was stopped by adding sodium dodecyl sulphate, EDTA, NaCl and NaOH to final concentrations of 0.6%, 20 mM, 0.5 M and 160 mM respectively. The complete mixture was layered on a linear 5-20% alkaline sucrose gradient and centrifuged in a SW 41 Spinco rotor at 35,000 rpm at 21°C for 4 hours. Fractions were collected, trichloroacetic acid precipitated and counted.

X-DBP 1 (2 μg) was assayed also for exonuclease activity with 1.2 μg of E. Coli [3H]DNA (4,000 cpm/μg) in the same incubation mixture described above. After 30 min at 37°C, acid precipitable radioactivity was measured.

**Trypsin digestion.** X-DBP 1 (50 μg) was incubated in 100 μl of buffer B with 0.5 μg of trypsin-TPCK for 30 min at 37°C. Aliquots of the incubation mixture (5 μl) were diluted 20 times and assayed according to the
DNA polymerase stimulation assay described above. Control experiment showed that trypsin, at the dilution considered, does not affect DNA polymerase activity.

**DNA binding assay.** The retention of single stranded and double stranded E. Coli \(^3\)H DNA on cellulose nitrate filters in the presence of the protein was assayed according to Banks and Spanos (18). The nitrocellulose filters were boiled 20 min in water and soaked overnight in BD buffer [20 mM-Tris.HCl (pH 8.0), 1 mM-EDTA, 2 mM-2-mercaptoethanol, 50 mM-NaCl, 5% glycerol and 1% dimethylsulphoxide] before use. Native (1.3 /µg) and denatured (1.3 /µg) E. Coli \(^3\)H DNA were incubated with X-DBP 1 (0 to 13.5 /µg) in 20 mM-Tris.HCl (pH 8.0), 1 mM-EDTA, 2 mM-2-mercaptoethanol, 50 mM-NaCl and 5% glycerol (total volume 0.2 ml) at 30°C for 5 min. The solution was then diluted to 3 ml with cold BD buffer and immediately filtered through a nitrocellulose filter, prewashed with 3 ml of BD buffer, at a flow rate of 5 ml/min. The filter was washed with 3 ml of BD buffer and the radioactivity determined in a toluene-based scintillation fluid.

**RESULTS**

**Purification of the protein.** The purification of X-DBP 1 entails DNA-cellulose chromatography of an S-100 supernatant derived from eggs, followed by DEAE cellulose and phosphocellulose chromatography.

The protein was assayed for its ability to stimulate *Xenopus laevis* DNA polymerase α. The stimulatory activity is recovered in the flow-through of the DEAE cellulose column (Fraction II). This fraction also shows DNA polymerase activity. The separation of the DNA polymerase activity from X-DBP 1 is achieved by phosphocellulose chromatography (Fig.1). X-DBP 1 elutes around 850 mM-NaCl (Fraction III). We observed a major peak of activity, usually accompanied by several minor peaks; the pattern of the minor peaks varied with each experiment and because of this variability we cannot conclude that they indicate heterogeneity of X-DBP 1.

Figure 2 shows sodium dodecyl sulphate polyacrylamide gels of Frac-
Fig. 1: Phosphocellulose chromatography of Fraction II. 40 μl from each fraction was assayed for DNA polymerase activity (----) and for DNA polymerase stimulation activity (----) (see Material and Methods).

Sequences II and III. Fraction III presents a single band whose electrophoretic mobility corresponds to a molecular weight of 8,200; the mobility of the band is not affected by 2-mercaptoethanol reduction.

X-DBP 1 does not show endo or exonucleases activities; 2 μg of X-DBP 1 did not convert supercoiled SV 40 [3H] DNA to the open form, after incubation for 30 min at 37°C. The same amount of X-DBP 1, when assayed for exonuclease activity, does not release any acid soluble counts, after 30 min of incubation at 37°C with radioactive E. coli DNA.

The Xenopus laevis protein was found to bind to both native and denatured E. coli [3H]DNA (Fig. 3) when assayed by the binding reaction on nitrocellulose filters (see Materials and Methods). This finding distinguishes our protein from the unwinding proteins isolated from several prokaryotic (19-24) and eukaryotic organisms (18) (25-28), because the unwinding proteins bind to denatured but not to native DNA.

Size of the protein in the native form. Since sodium dodecyl sulphate electrophoresis does not give any information about whether the native protein
exists in a multimeric form, Sephadex chromatography was performed (Fig. 4) as described in Materials and Methods, to determine the molecular weight of X-DBP 1 in its native form. The difference of the molecular weights determined by gel filtration (molecular weight 6,500) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (molecular weight 8,200) may be due to some affinity of the protein for the Sephadex.

**Amino acid composition.** The amino acid composition of X-DBP 1 (Table 1) indicates that our protein is lysine-rich and lacks cysteine and tryptophan.
Fig. 3: Binding of X-DBP 1 to single and double stranded DNA. Solutions containing 1.3 μg of E. coli [3H]DNA (4,100 cpm) and varying amounts of X-DBP 1 (expressed in μg on the abscissa) were incubated at 30°C for 5 min in 0.2 ml final volume. After the incubation, the reaction mixtures were diluted to 3 ml with cold BD buffer and filtered at a flow rate of 5 ml/min. In the absence of the binding protein, less than 1% of the DNA in the assay was retained by the filter. Single stranded DNA x—x double stranded DNA ••.

Moreover the protein does not contain significant amounts of tyrosine, phenylalanine, threonine, methionine, isoleucine and leucine. It should be noted that the alanine content is low compared to the lysine content.

The lysine-arginine ratio of X-DBP 1 is 2.2, a value which is very different from that characteristic of four out of five major histone species (29). The histone H2b shows a lysine-arginine ratio of 2.6, but its lysine-alanine ratio is 1.5, whereas the lysine-alanine ratio of X-DBP 1 is 7.1. X-DBP 1 cannot be a fragment of a histone, because no hypothetical fragment of 70 amino acids from the molecule of any of the five major histone species has an amino acid composition compatible with that of X-DBP 1.

The amino acid composition of X-DBP 1 explains the high affinity of the protein for DNA and phosphocellulose.

Effect of X-DBP 1 on in vitro DNA synthesis by Xenopus laevis DNA polymerase. The major DNA polymerase activity present in oocytes and eggs
is the DNA polymerase\(\alpha\), a high molecular weight enzyme capable of using "activated" DNA as template, unable to utilize poly (rA). oligo (dT), and sensitive to N-ethylmaleimide. This enzyme is localized in the nucleus of the oocytes (7). X-DBP 1 stimulates incorporation of deoxytriphosphates catalyzed by *Xenopus laevis* DNA polymerase\(\alpha\) in a standard assay (see Materials and Methods). We have observed up to ten-fold stimulation. Figure 5 (B) shows that at relatively high concentrations, increasing amounts of the protein present in the assay produce a gradual decrease in the stimulation. For every protein concentration tested, the reaction was linear for 30 min (Fig. 5 A). This result indicates that the protein influences the final extent of DNA synthesis.

The effect of X-DBP 1 on DNA synthesis by *Xenopus laevis* DNA polymerase\(\alpha\) remains unchanged when the protein is preincubated for 5 min at
Table 1: Amino acid composition. Lyophilized samples of X-DBP 1 were hydrolyzed at 120°C in 1 ml of 6 N HC1 for 48 and 72 hours. The data presented are mean values from independent determinations of three different preparations. Lack of tryptophan was deduced from the absence of colour in the staining reaction performed on chromatographic paper with Erlich's reagent (37), and of fluorescent emission specific for this amino acid. Lack of cysteine was deduced from the absence of cysteic acid in the amino acid analysis carried out after performic acid oxidation (38) and acid hydrolysis in 6 N HC1 at 110°C for 24 hr. Tyrosine, phenylalanine, threonine, methionine, isoleucine and leucine were not detected in significant amounts.

90°C and then cooled before the assay, showing that X-DBP 1 is a heat stable protein.

On the contrary the stimulatory activity of X-DBP 1 is abolished by treatment with trypsin.

It is clear that the stimulation is not due to contaminating nucleases present in X-DBP 1, since appropriate tests described above showed there are not such activities.

Although the mechanism by which X-DBP 1 stimulates the incorporation of deoxytriphosphates is unknown, we have used this property to follow the protein during purification.
Fig. 5: Incorporation kinetics in a standard DNA polymerase stimulation assay. The reaction components, at concentrations described in Materials and Methods, were incubated in a total volume of 0.4 ml, in the presence of increasing concentrations of X-DBP 1 a) 0.0 μg/ml; b) 80 μg/ml; c) 60 μg/ml; d) 20 μg/ml; e) 40 μg/ml. 75 μl aliquots were taken at the times noted and precipitated with trichloroacetic acid. The same data are plotted against time in (A), and against the amount of protein contained in the 75 μl aliquot in (B).

When DNA polymeraseα from HeLa cells was used instead of the *Xenopus laevis* enzyme, X-DBP 1 was found to stimulate the incorporation, and results analogous to those shown in Figure 5 (A and B) were obtained. This last finding indicates that the stimulation of in vitro DNA synthesis does not show specificity, in the sense that the protein from *Xenopus laevis* acts even if the DNA polymeraseα originates from a different organism. We have not yet investigated whether the protein stimulates specifically only DNA polymeraseα or also other cellular DNA polymerases.

We have investigated the possibility that X-DBP 1 could have an effect on in vitro reactions catalyzed by other *Xenopus laevis* enzymes which use DNA as substrate.
We have chosen to study the effect of X-DBP 1 on the reactions catalyzed by a DNA relaxing protein and by an endonuclease that have been recently purified from *Xenopus laevis* extracts (9). We have found that X-DBP 1 has no effect on the reaction catalyzed by the DNA relaxing protein. The DNA relaxing protein was assayed using the method described by Keller (30); the method is based on the observation that supercoiled DNA migrates in agarose gel electrophoresis more rapidly than covalently closed DNA, free of superhelical turns. X-DBP 1 also has no effect on the reaction catalyzed by the *Xenopus laevis* endonuclease. The amount of open circular and of full length linear SV 40 DNA produced from the supercoiled form by the endonuclease is the same both in the presence and in the absence of the protein.

**DISCUSSION**

In this paper we describe a DNA binding protein purified to apparent homogeneity from *Xenopus laevis* eggs, named X-DBP 1. The molecular weight of the pure protein is 8,200, determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. X-DBP 1 stimulates *in vitro* DNA synthesis catalyzed by DNA polymerase α and the stimulatory activity was found to correspond to a molecular weight of 6,500 using Sephadex chromatography; therefore multimeric forms of X-DBP 1 do not seem to occur to a significant extent in the conditions we used. DNA polymerase α is the major DNA polymerase in *Xenopus laevis* oocytes and eggs and in oocytes is localized in the nucleus (7); a number of observations suggest that DNA polymerase α has a role in DNA replication (31).

The amino acid composition of X-DBP 1 shows that we are not dealing with a fragment of a histone molecule. If 70 amino acid fragments of the molecules of any of the five major histone species are hypothetically considered, the amino acid composition derived from the known sequences is incompatible with the possibility of X-DBP 1 being a fragment derived by cleavage.

A calculation of the number of molecules of X-DBP 1 per egg is rather difficult, because we did not assay for the protein in crude extracts. By as-
assuming 100% recovery, one egg contains 0.05 μg of X-DBP 1. This gives a figure of $4 \times 10^{12}$ molecules per cell. \textit{Xenopus laevis} eggs contain, beside chromosomal DNA, mitochondrial DNA and amplified ribosomal DNA. If one takes into consideration only the chromosomal DNA, since the haploid complement corresponds to 3 pg, we are dealing with $3 \times 10^9$ base pairs (32). Ten nucleotides can span a distance of 34 Å in double stranded DNA, and assuming that X-DBP 1 is 30 Å long, one molecule would cover 8 base pairs.

Therefore $4 \times 10^8$ molecules of X-DBP 1 are enough to cover the entire genome. We must therefore conclude that there is a large excess of X-DBP 1 with respect to chromosomal DNA in the egg. Even if we take into account mitochondrial and amplified ribosomal DNA, there is still an excess of X-DBP 1 of at least one order of magnitude.

It is interesting at this point to consider that there is usually a relationship between the synthesis of DNA binding proteins and the intracellular quantity of the form of the DNA to which the protein binds. For example, it is unequivocally demonstrated that there is a direct correlation between gene 32 synthesis and the quantity of single stranded intracellular DNA (33).

In the majority of cells, histones are synthesized coordinately with DNA synthesis (34). However, Adamson and Woodland (35) have demonstrated that in \textit{Xenopus laevis} oocytes, even if DNA replication had not occurred for several months, histones were being synthesized. The same authors estimated that in activated or fertilized eggs, histones were synthesized at a much higher rate (about 500 times) than is immediately required. It is possible that during oogenesis a large amount of X-DBP 1 is also synthesized and stored in order to provide for the early cleavage stages, which are characterized by a switch from the complete absence of cell division and DNA synthesis to an extremely rapid rate of cell division (15 min or less).

We must however consider the possibility that the protein is not in fact bound to DNA in a physiological environment and that the DNA binding
properties could simply be a manifestation of its amino acid composition.

On leave of absence from Institute of Virology - University of Rome

On leave of absence from Institute of General Pathology - Catholic University of Rome

Abbreviations used: + simian virus 40; ++ tosyl-phenilalanylchloromethyl-ketone.

REFERENCES