

ISOLATION OF A NUCLEIC ACID
UNWINDING PROTEIN FROM
PHYSARUM POLYCEPHALUM

by

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PREFACE

This thesis is an attempt to begin to understand one of the structural complexities of DNA replication. Our purpose at the beginning of this study was to show that proteins which disturbed the secondary structure of nucleic acids could be correlated with DNA replication.

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ABSTRACT

Recently the DNA-binding phosphoproteins of Physarum polycephalum have been characterized during the cell cycle. Increased amounts of certain polypeptides which bind tightly to single stranded DNA during S and M phases of the cell cycle were reported. Other work suggests that proteins which bind tightly to single stranded DNA have the ability to destabilize the DNA helix and may be involved in DNA replication. This study reports the isolation of a single stranded DNA binding protein which will destabilize a DNA-like helix. The protein was isolated from Physarum polycephalum, and indirect evidence is presented to suggest it is one of the polypeptides which has been found to increase during and just prior to DNA replication in Physarum. This is the first report of temporal correlation between helix destabilization (or "unwinding") activity and DNA replication in normal cycling cells.

INTRODUCTION

Physarum polycephalum is an acellular myxomycete, or slime mold, which is of interest to the cell biologist for several reasons (reviewed by Rusch 1970). It is a simple eukaryote whose genome is small and separable into two components by isopycnic density gradient centrifugation. One component is main band DNA, the other is a satellite DNA which has been shown to be the ribosomal RNA gene (Mohberg and Rusch 1971). Physarum has two growth stages, a vegetative sycytial diploid stage and a haploid amoeboid stage. In addition, Physarum enters a quiescent state called spherulation when nutrients are withheld. Physarum may be experimentally induced to differentiate from one form to another so that cellular gene regulatory mechanisms may be studied.

Of special interest to this study is the fact that Physarum may be grown in bulk in semi-defined liquid media in a shaker culture or as a surface macroplasmodium cultured on a media-soaked filter paper. A macroplasmodium may be prepared by placing a microplasmodial pellet on filter paper and adding media thirty to sixty minutes later. While the microplasmodia are in close opposition to one another on the dry filter paper, they fuse. The nuclei in the resulting macroplasmodium become enclosed in a common cytoplasm. They undergo a partially synchronous mitosis four and one-half to five hours later. From this time until the organism can no longer be supplied with fresh media to all parts of the cell,

the nuclei of the macroplasmodium will undergo a synchronous mitosis every eight to ten hours. Nuclear mitoses may be monitored by nuclear morphology with the phase contrast microscope. Therefore, cellular extracts from the same cell may be prepared at specific time points throughout one and even two cell cycles.

The Physarum Cell Cycle

The Physarum cell cycle is somewhat atypical from other eukaryotes (reviewed by Schiebel 1973). DNA synthesis or S phase immediately follows mitosis. During mitosis, the nuclear membrane remains intact, and since Physarum is a syncytium, there is no cytokinesis following mitosis. Nevertheless, the mitotic synchrony of Physarum has been exploited to obtain information about the biochemical regulation of the eukaryotic cell cycle.

The last two hours of G_2 before mitosis is a time in which several biochemical processes increase. Cyclic nucleotides such as cAMP and cGMP increase during this time (Lovely and Threllfall 1976). Cyclic AMP activates a protein kinase which catalyzes histone H1 phosphorylation (Bradbury et al. 1973) and the phosphate content of this nuclear protein increases concomitantly with cAMP levels. The precise function of these fluctuations of regulatory compounds is uncertain. Bradbury suggests that histone H1 phosphorylation may be necessary for chromosome condensation during mitosis. This hypothesis may explain other fluctuations in Physarum metabolism during this period. Alternatively the cell may be

producing compounds necessary for DNA and RNA synthesis, both of which occur immediately after mitosis.

S phase is a portion of the cell cycle in which DNA related metabolism increases. Protein synthesis, cGMP levels, and RNA transcription increase. Many of these increases may be supportive of DNA replication occurring during this time. Several lines of evidence suggest that in order for the DNA of Physarum to replicate, protein synthesis and RNA transcription must also be occurring simultaneously in the cell (Cummings and Rusch 1966, Muldoon et al. 1970, Fouquet et al. 1975). One specific class of proteins whose synthesis has long been known to be coupled to DNA synthesis in eukaryotes is the histones (Jackson, Granner and Chalkley 1975). Histones are structural proteins of the eukaryotic chromosome. They are known to disassociate from the DNA before it is replicated and then reassociate with the molecule after replication (Jackson et al. 1975). The reassociation seems to be random with both old and new histone binding equally to old and new DNA. Histones, therefore, serve to package the new DNA and seem to take no part in its synthesis. However, histones are not the only proteins synthesized during S. DNA replication may be coupled to protein synthesis because specific protein factors are needed to initiate, maintain, and regulate DNA synthesis.

An intriguing problem for investigators of the mechanisms of eukaryotic DNA replication is the identification of specific proteins involved in the replication of DNA. Physarum DNA

replication is an attractive model system to study in this effort, not only because large amounts of cellular material may be obtained at specific times during S phase, but because Physarum DNA replication seems to occur in a regulated sequence.

Physarum DNA Replication

Physarum DNA synthesis (measured by tritiated thymidine incorporation) increases rapidly after telephase. It maintains a constant rate for ninety minutes and then declines for ninety minutes to the low levels observed during G_2 (Braun, Mittermayer and Rusch 1965, Braun and Willi 1969). Using bromo deoxyuridine and H^3 thymidine labels, Braun and Willi (1969) showed that DNA replicated during a particular segment of the cell cycle was replicated during that particular segment of the subsequent cell cycle. Muldoon et al. (1970) later suggested that there were at least ten replicative units which were initiated sequentially during S phase, and that each unit depended on a different protein synthetic event. He drew his conclusions from an experiment in which he treated Physarum with cycloheximide at small time intervals throughout S. He then measured the percentage of the genome which had been replicated after the addition of cycloheximide. Instead of the percent of DNA replicated increasing linearly with each time point, replication of the genome proceeded in ten discrete steps. Using a BUdR density label at each of the ten replication steps, Muldoon further found that the DNA strand replicated during

a particular interval was replicated during the same interval of subsequent S phase. Subsequent experiments (Evans, Littman, Evans and Brewer 1976) have found that cycloheximide causes an increase in TTP pools and thus a decrease in TTP specific activity. This work suggested that although DNA synthesis is inhibited by cycloheximide, the ten discrete replicative steps that Muldoon et al. found may need further investigation.

Most recent work concerning the role of proteins in Physarum DNA replication have involved more extensive use of inhibitors and the analysis of intermediate DNA products. Brewer (1972) and Brewer, Evans and Evans (1974) analyzed newly made DNA on sucrose density gradients. He found 4×10^7 dalton subunits which were synthesized at a rate of 1.5×10^5 daltons per minute at each replication fork. The initiation of these subunits were asynchronous. The majority were initiated within the first thirty to forty minutes of S but some required two hours for initiation (these late replication subunits were not satellite DNA). Accumulation of 3-4 S ("Okazaki") type fragments of newly made DNA was not found in initial analyses of Physarum replication intermediates, but later studies (Funderud and Haugli 1977b) have shown such low molecular weight DNA fragments. These fragments are quickly ligated into larger 4×10^7 dalton subunits, which eventually ligate to form the Physarum genome.

Each step of the Physarum replication process (i.e., initiation of 3-4 S fragments, their ligation to replicon length segments, and the ligation of replicons) is sensitive to cycloheximide

treatment (Funderud and Haugli 1977b). This evidence strongly suggests that newly synthesized proteins are necessary for DNA replication in Physarum. Cell free systems are being developed to identify the protein factors involved in DNA replication in Physarum (Brewer and Ting 1975, Funderud and Haugli 1977a). Similar studies in other eukaryotes (Seki and Mueller 1976, Müllbacher and Ralph 1977, Planck and Mueller 1977) indicate that diffusible cytoplasmic factors are necessary for initiation and ligation of "Okazaki" type fragments. Although Physarum cell free DNA replication systems have produced products similar to DNA produced in vivo, identification of stimulatory factors has yet to be published. There is evidence from in vivo experiments which indicates that factors controlling DNA replication are diffusible from cytoplasm to nucleus.

Early work by Guttess and Guttess (1968) has shown that if two plasmodia whose cell cycles are out of phase are fused, mitosis will occur in the fused plasmodium after a time which is the average of the times until the predicted mitoses of the original two plasmodia. A recent extension of this technique (Wille and Kauffman 1975) has fused a late S plasmodium with an early S plasmodium. The nuclei of the early S plasmodia had been labeled in the previous cell cycle with H^3 thymidine during late S. When the late S and the early S plasmodia were fused, BUdR was added to induce a density shift in DNA made at that time. The plasmodium was harvested before the expected time of late DNA synthesis and

the DNA was analyzed on cesium chloride isopycnic density gradients. The BUdR label was found in the H^3 labeled DNA peak indicating that late S factors had migrated to the early S nuclei and caused premature replication of late S DNA regions.

DNA Replication in Other Eukaryotes

DNA replication in other eukaryotic organisms is also dependent on diffusible cytoplasmic factors. Most eukaryotes share similar mechanisms of DNA replication. DNA replication is initiated at multiple growing points which are bidirectional (Huberman and Riggs 1968, McFarlane and Callan 1973, Kuebbing, Diaz and Werner 1976, van't Hoff 1976). It proceeds at each replication fork at a rate of .5 - 1.3 micrometers per minute. DNA polymerase initially polymerizes short DNA fragments which are later ligated into longer segments. Growing points which are close to each other begin almost simultaneously while distant growing points are more asynchronously initiated (Hand 1975). There are from 25-145 μm between growing points (McFarlane and Callan 1976). Information from cell free DNA replication systems indicates that each step of DNA replication from initiation (Thompson and McCarthy 1968, Kumar and Friedman 1972, Jawenski, Wang and Edelman 1976) to ligation of fragments (Francke and Hunter 1975, Tseng and Goulian 1975, Planck and Mueller 1977) and maintenance of the polymerization process is dependent on diffusible nondialysable cytoplasmic factors. The process is also dependent on ATP hydrolysis (Friedman

1974, Krokan, Bjorklid and Prydz 1975). Therefore, in addition to DNA polymerase, most eukaryotes need a number of enzyme activities for DNA replication. Many of these proteins are diffusible from the cytoplasm.

The Multiprotein "Replicase" Complex

In E. coli and its virus, T₄ phase, DNA is replicated by multiprotein complexes (Alberts 1975, Gefter 1975, Geider 1976). The specific chemical activity of many components within these complexes has been characterized. Some of these activities include polymerization, ligation, DNA dependent ATP hydrolysis, nucleolytic activity, and DNA helix destabilization. The discovery of these activities has led to an understanding of some of the mechanisms involved in the initiation, maintenance, and fidelity of prokaryotic DNA replication. A similar identification of factors involved in the replication of eukaryotic DNA is not immediately possible. Prokaryotic "replicase" systems have been developed by investigating DNA replication in bacterial mutants which are defective in DNA replication. Detection of missing or defective polypeptides is possible with these mutants. Once the polypeptide is identified, its biochemical activity may be determined. In this way, replicase enzyme complexes may be reconstructed in vitro. Eukaryotic mutant cells which are defective in DNA replication are not readily available. However, study of DNA replication in eukaryotes may be facilitated by the fact that the genome is compartmentalized in the

nucleus. Thus, diffusible cytoplasmic factors which stimulate DNA synthesis when added to isolated nuclei may be important in DNA replication. Some workers have proposed reconstituting S phase chromatin to identify components of the DNA replication complex (Wille personal communication). Other investigations have purified proteins which can be shown in vitro to exhibit activities found in DNA replicase complexes. Eukaryotic DNA polymerases, exonucleases, and ligases have been purified and characterized. In addition, nucleic acid unwinding activity has been found in certain polypeptide fractions isolated from eukaryotic cells. Polypeptides with this in vitro activity are interesting because they appear to be constitutive in the replicase systems of prokaryotes (Alberts 1975). Although these proteins are called unwinding proteins, there is no evidence concerning their actual in vivo activity. Unwinding proteins have been found to be important structural proteins for the efficiency and fidelity of prokaryotic replicase systems. The unwinding activity assay might therefore be used to identify important structural proteins in eukaryotic DNA replication.

Unwinding Proteins

Unwinding proteins bind preferentially to the single stranded form of DNA. The activity of these proteins may be based on this preferential binding (Alberts and Frey 1970). Von Hippel's group (Jensen and von Hippel 1976, Jensen, Kelly and von Hippel 1976)

has developed a theoretical model of nucleic acid helix destabilization by observing the action on nucleic acid helices by pancreatic ribonuclease, a biologically trivial unwinding protein, and Gene 32 protein of T₄ phage, a protein whose biological activity in DNA replication and recombination is based on its helix destabilizing activity. They define an unwinding protein as:

A protein which at equilibrium under the conditions of the experiment can achieve higher binding densities on the single stranded conformation than the double stranded conformation of a nucleic acid. Experimentally, this differential binding density brings about an equilibrium destabilization of the double helical conformation which is manifested by a shift of the the thermally induced helix coil transition of the nucleic acid to lower temperatures.

Several studies (Jensen and von Hippel 1976, Jensen et al. 1976, Herrick and Alberts 1976b) have explained the theoretical mechanisms of nucleic acid unwinding on the basis of the thermodynamic properties of the unwinding protein-DNA interaction. They propose an equilibrium destabilization in which several molecular transitions take place. This expression summarizes these transitions:



where 2D = native DNA, 2S = single stranded DNA, 2P = unwinding protein, 2PD = protein double stranded complex, 2PS = protein, K_1 = helix formation equilibrium constant without protein, K_2 = helix formation equilibrium constant with protein. Assumptions for these equations include noncooperativity of protein binding and

overlapping of homogeneous binding sites (i.e., nonspecific binding sites). Proteins also must bind to only one strand of DNA while DNA is in the double stranded conformation. If the protein binds to both strands of a nucleic acid double helix as does a histone or a polycation, the helix conformation will be stabilized (thus in the above equation the double helix is considered as "2D" rather than a unity). Theoretically, the degree and the temperature at which a helix will begin to unwind can be determined by the binding constants of the protein to the nucleic acid.

The formation of a single stranded nucleic acid-unwinding protein complex competes with the formation of a helix. If the free energy of the complex is greater than that of the helix, the nucleic acid will be in the single stranded conformation at equilibrium. This thermodynamic model of unwinding has been confirmed for noncooperatively binding proteins within experimental limits (Jensen and von Hippel 1976). Furthermore, the mechanism for unwinding has been suggested to begin from the middle of a nucleic acid helix rather than from the ends. The central idea of the model for the unwinding of DNA is that DNA free of protein is at equilibrium between single and double strandedness. This situation dictates that at any given time random sections of the DNA will be single stranded (actually it is not really random since G-C rich sections of DNA are more stable than A-T rich sections). There would be points of local DNA denaturation called "breaths". When an unwinding protein is present these "breaths" would become more

stable than the double stranded helix due to binding of the unwinding protein to the DNA in the single stranded "breaths". These protein-bound breaths might induce further unwinding of the double stranded helix by mechanical stress or more "breaths" may form randomly. Eventually at equilibrium due to the presence of the unwinding protein, the DNA would be mostly single stranded. This mechanism is stoichiometric. There is no catalysis of the reaction and no energy input from outside sources such as ATP. There has been a report, however, of a DNA dependent ATPase which unwinds DNA only in the presence of ATP (Abdel-Monem and Hoffman-Berling 1976).

Whether the above mechanism has any biological relevance in eukaryotes is uncertain. Eukaryotic DNA has complex tertiary and quaternary structure. It is wound around histone octamers called nucleosomes which are spaced from one another by DNA regions bound to histone H1 (for review see Kornberg 1977, for Physarum see Johnson et al. 1976, Vogt and Braun 1976, Staron et al. 1977). The unwinding protein model described above also uses an "unwinding protein" which has no significance in DNA replication (bovine pancreatic ribonuclease). Furthermore, these studies show that though the biological unwinding protein, the gene 32 protein (a gene necessary for DNA replication in T_4 phage), depresses the melting point of poly dAdT to greater degree than bovine pancreatic ribonuclease, but it does not unwind natural DNA. This is probably

because gene 32 protein binds cooperatively to DNA and must bind to significantly larger "breaths" than exist in natural DNA. (Cooperatively binding proteins require a separate thermodynamic model from noncooperatively binding proteins). Therefore gene 32 may not unwind DNA in vivo. Other proteins with DNA unwinding activity may not have a role in DNA regulation, and if they do, unwinding DNA may not be their activity.

Several functions have been suggested for proteins with unwinding activity. Alberts, the discoverer of gene 32 protein (Alberts and Frey 1970) has classified the various possible roles for unwinding proteins into three categories (Alberts 1974). The first possible role is the unwinding of DNA to permit DNA polymerase, which is more active on single stranded DNA *in vitro*, to work more efficiently. A second explanation is that the DNA unwinding proteins hold single stranded DNA at the replication fork rigid so that the DNA polymerase which synthesizes DNA in a 5' - 3' direction may make short DNA fragments in the antiparallel direction. The rigidity may also facilitate binding of DNA polymerase to DNA, ligation of the small nascent DNA fragments, and prevention of formation of hairpin loops which would reduce replication fidelity. A third possibility is that the unwinding protein simply protects nascent single stranded DNA from single strand specific deoxyribonucleases. Regardless of the real in vivo role of proteins with in vitro unwinding activity, they should correlate in some way with a DNA related biological function in order to be considered significant.

Unwinding activity has been discovered in several eukaryotic organisms using two basic assays. One assay is the filter binding assay (Hotta and Stern 1971). Single stranded DNA binds to cellulose filters while double stranded DNA does not. This assay simply reacts radioactively labeled with a protein that passes the mixture through a cellulose filter. The filter is then dried and counted for bound radioactive DNA. The other assay utilizes the hyperchromic shift that occurs when DNA denatures. U-V absorption and temperature are monitored in a protein DNA mixture. The temperature at which one-half of the DNA has denatured (T_m) is compared to the T_m of DNA without the protein. A significant depression of T_m is considered unwinding activity.

Using these two assays, unwinding activity has been identified in several eukaryotes. Unwinding proteins have been reported in lily cells (Hotta and Stern 1971, Mather and Hotta 1974), smut molds (Banks and Spanos 1975), rat prostate (Mainwaring, Rennie and Keen 1976), rat liver (Thomas and Patel 1976, Patel 1978), calf thymus (Herrick and Alberts 1976a and b), mouse cells (Otto and Baynes 1977), and NRK cells (Magun and Scott 1977). The intracellular location of these proteins has been found to be the nucleus (Thomas and Patel 1976) in some reports and in the cytoplasm (Herrick and Alberts 1976a and b) in other studies.

Three of the reports have correlated unwinding activity to biological events. Unwinding proteins occur in increased amounts in meiotic lily cells (Hotta and Stern 1971), transformation in

NRK cells (Magun and Scott 1977) and androgenic stimulation in rat prostate cells (Mainwaring, Rennie and Keen 1976). Other work (Herrick, Delius and Alberts 1976, Otto and Baynes 1976) has shown that unwinding proteins stimulated homologous DNA polymerase α but not DNA polymerase β or prokaryotic DNA polymerase. DNA polymerase α is considered to be active in DNA replication and DNA polymerase β active in repair (Weissbach 1977). One report (Yarrington, Moore and Spanos 1976) suggested that in the smut mold, Ustilago maydis, only one DNA polymerase exists, and that in conjunction with the DNA unwinding protein of the organism, the polymerase functions in replication, Without the unwinding protein, the polymerase is a repair enzyme. Other work indicates the DNA polymerase stimulation can be taken away by the experimental phosphorylation of the unwinding protein by cAMP dependent protein kinase (Otto and Baynes 1976). The work reported in this manuscript provides evidence that unwinding activity may be found in a phosphoprotein of the slime mold Physarum polycephalum. The protein has been previously reported to increase relative to other proteins just prior to and during S phase (Magun, in press). It is suggested that this protein might be worthy of further investigation as one of the diffusible components of the Physarum DNA replication complex.

MATERIALS AND METHODS

Culture of Physarum

Microplasmodial cultures of Physarum polycephalum (this colonial strain was a gift from Dr. Glen Kuehn) were grown continuously in sterile full nutrient media described by Daniel and Baldwin (1964). Cultures were grown in suspension in 2000 ml Bellco shaker flasks containing 250 ml of media (before autoclaving) in each flask.

Isotopic Labeling and Protein Determination

Microplasmodia were labeled continuously for at least 60 hr in media containing H^3 leucine (Amersham, 40-60 Ci per millimole) at a concentration of 1-2 μ Ci per milliliter. This labeling procedure allowed low specific activity protein (30-70 μ Ci per 1 μ g protein) to be obtained from the cultures. For this reason only one protein determination by the method of Lowry et al. (1951) was necessary to determine protein specific activity. Subsequent protein determinations were done by counting small aliquots of samples in a paraxylene based liquid scintillation cocktail (Amersham).

Preparation of Protein Extracts

Protein was prepared by the following method of Magun (1976). At the completion of the labeling period cultures were pooled, centrifuged at 250g for 30 sec and resuspended in several volumes of distilled water. This procedure was repeated once. Hereafter,

all extraction procedures were performed at 4°C. To the microplasmoidal pellet was added 1.5 volumes of ice cold buffer A (0.05 M NaCl, 0.01 M Tris-HCl pH 8.1, 0.1% polyoxyethyl-20-cetyl ether, 1 mM ethylene diamine tetra acetic acid). Phenyl methyl sulfonyl fluoride in isopropanol was added immediately to a concentration of 1 mM. Buffer and cells were homogenized in a glass dounce tube with 10 strokes of a "B" pestle. The homogenate was centrifuged at 20,000g for 15 min to remove most of the particulates then at 100,000g for 1 hr to obtain a soluble cell extract. The supernatant was made 2.0 M in NaCl by adding solid NaCl. Thirty percent polyethylene glycol in a M NaCl Buffer A was added to a final polyethylene glycol concentration of 10%. After 30 minutes, the solution was centrifuged at 7500g to remove the precipitate. The supernatant was dialysed for 40 hrs against four changes of 20 volumes of Buffer A. The dialysate was centrifuged for 15 min at 21,000g to remove the small precipitate. The supernatant was loaded onto DNA cellulose columns.

Preparation of DNA Cellulose Columns

Native and denatured DNA cellulose were prepared by the combined methods of Litman (1968) and Alberts and Herrick (1971). To prepare native DNA cellulose, 50 mg calf thymus DNA (Worthington Biochemicals, Freehold, N.Y.) were dissolved in 18 milliliters of Buffer B (0.01 M Tris HCl pH 7.4, 1 mM EDTA). Six grams cellex 410 (BioRad Laboratories, Richmond, Ca.) were washed extensively

with absolute ethanol. The washed cellulose was added to the DNA solution and the slurry was allowed to dry overnight. The next day the DNA cellulose was pulverized and resuspended in ethanol followed by a U-V irradiation. The dried native DNA cellulose was swollen for 2 hrs in Buffer B at 4°C, then washed free of "fines" by several low speed centrifugation followed by resuspension in Buffer B. DNA cellulose was then packed into a 2 x 5 cm column.

Denatured DNA was prepared by heating 1.3 mg per ml DNA in 0.05 M NaOH at 100°C for 15 min followed by rapid chilling by agitation in ice water. Three milliliters of denatured DNA solution was added per gram of ethanol washed cellex 410. The cellulose-DNA slurry was dried overnight and 3 more milliliters of DNA solution per gram of DNA-cellulose were added on the following day. The resulting slurry was again dried overnight and pulverized the next day. The dry powder was swelled 2 hr in Buffer B and washed and packed into a column in the same manner as native DNA cellulose.

DNA Cellulose Chromatography

One hundred to five hundred milligrams of protein in 300-500 mls of Buffer A were passed through the native and then denatured DNA cellulose columns arranged in tandem. Columns were then washed with 5-6 column volumes of Buffer A. Columns were disconnected and the native column was discarded. The denatured DNA cellulose column was washed with 3 column volumes 0.25 M NaCl in Buffer A. The column was subsequently eluted with 3 column

volumes 2.0 M NaCl in Buffer A. This fraction, containing tightly binding single strand specific DNA binding proteins was concentrated either by dialysis against solid sucrose or by letting dry Sephadex G-25 (Pharmacia, Piscataway, N.J.) absorb the solvent from the protein solution. After concentration to 2.5 ml, the protein solution was desalted into Buffer D (5 mM NaH_2PO_4 , 10% glycerol, pH 7.4) either by dialysis or by Sephadex G-25 columns (Pharmacia, Piscataway, N.J.).

Liquid Chromatography

Tightly binding single stranded DNA binding proteins were chromatographed on phosphocellulose (BioRad, Richmond, Ca.) and Sephadex G-150 SF (Pharmacia, Uppsala, Sweden) gel filtration columns. The phosphocellulose was eluted with a salt gradient from 0 to 2.0 M in NaCl. Fractions were collected and monitored with an Isco (Piscataway, N.J.) fraction collection and U-V monitor.

Polyacrylamide Gel Electrophoresis

Polypeptide profiles of column fractions were analyzed on polyacrylamide gels in the presence of sodium dodecyl sulfact (SDS) according to Laemmli (1970). The separation gel was 12.5 cm long and contained 8.75 percent acrylamide and 0.23 percent methylene bis-acrylamide. Molecular weight marker proteins were run with each tank of gels. Gels were stained with Coomassie Brilliant Blue and scanned with an Isco scanner at 580 nm.

Unwinding Assay

The spectrophotometric DNA unwinding assay was basically that described by Herrick and Alberts (1976b). The reaction mixture contained 3 μg poly d(AT) plus crude or purified protein extracts. Protein to nucleic acid ratio (wt/wt) varied from 5-20 to 1. The reaction mixture was begun at 0°C after equilibrium for one hr in a Beckman DU spectrophotometer. Absorbance was monitored at 260 nanometers. Temperature of the cuvette compartment was controlled by a Neslab circulating temperature bath fitted with a Neslab temperature programmer TP-2. The temperature was raised slowly over 3 hrs to 40°C then lowered over a similar period of time back to 0°C . Absorbance and temperature were monitored using a 2 channel strip recorder. Unwinding of the helix was considered to be the reversible increase in absorbance with increasing temperature. In the presence of added protein, the maximum temperature used was 40°C , since temperatures above that caused protein denaturation and turbidity. At low temperatures, it was sometimes (during high humidity) necessary to supply a steady stream of nitrogen to the cuvette chamber to prevent condensation.

RESULTS

The Unfractionated DNA-Binding Extract

Figure 1 shows the "melting" curve of poly d(AT) when it is allowed to react with a fraction of single stranded DNA binding proteins between the temperature of 0° - 40°C over a period of 3 hr (temperature increased almost linearly during this period). The protein fraction used will be called "2.0 M NaCl fraction" since the fraction was eluted from single stranded DNA cellulose by a buffer containing 2.0 M NaCl. The column had been previously washed with 0.25 M NaCl containing buffer (see Materials and Methods). Figure 1 also shows the renaturation of the nucleic acid over the same length of time and range of temperature. Renaturation is important for this study. Hyperchromicity can be observed when a double stranded nucleic acid is hydrolyzed. When the nucleic acid and protein mixture would not decrease in absorbance after a hyperchromic shift, the protein was considered to contain nucleases. Denaturation occurs over a small range of temperature in Figure 1. Hyperchromicity is maximal at 30° and the T_m of the denaturation is at 28°. This range of temperatures is close to the optimal temperature for the culture of Physarum. Since any protein which binds preferentially to the single stranded conformation of DNA will bring about some degree of helix destabilization over a wide range of temperature (Jensen and von Hippel 1976), for the purpose

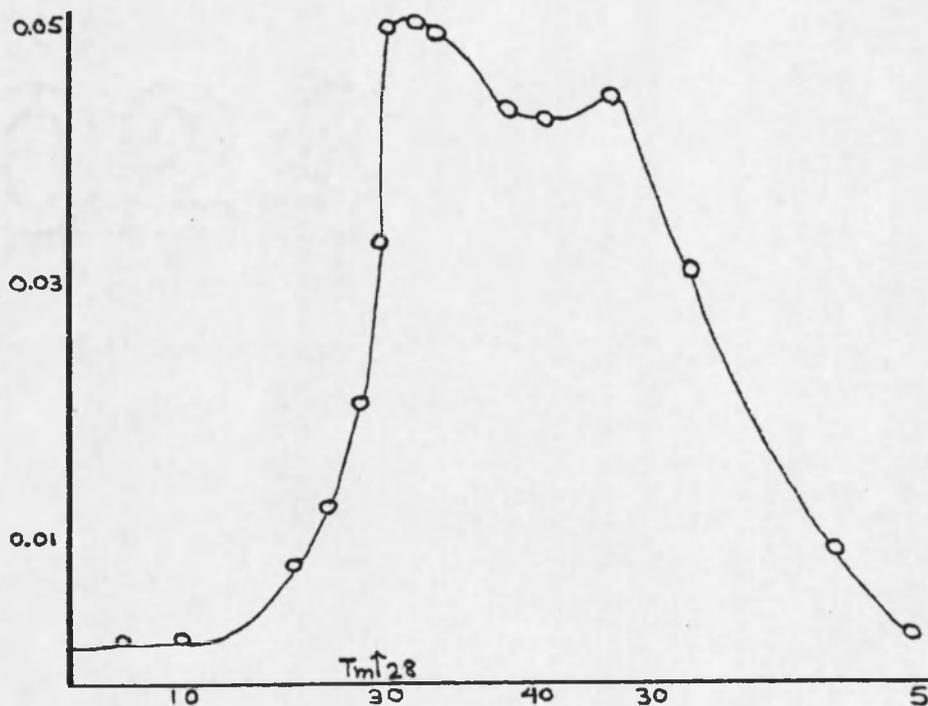


Figure 1: Nucleic Acid Helix Destabilization.

Nucleic acid unwinding was caused by the addition of 60 μg of protein which was eluted from a single stranded DNA cellulose column with a 2.0 M NaCl wash (see Materials and Methods). Reaction time was 6 hr. Reaction mixture contained 45 μl 5 mM MgCl_2 , 30 μl of 10% poly d(AT) solution, 120 μl of protein extract, and Buffer D up to 400 μl volume in the cuvette. The abscissa is temperature in degrees Celsius. The ordinate is ΔA_{260} units with an arbitrary baseline.

of this study we required hyperchromicity to occur within a small range of temperatures with a T_m near the physiologic range of Physarum in order to claim positive unwinding activity. Our unwinding curves are similar to those published for unwinding proteins which have been previously described (Herrick and Alberts 1976b).

In Figure 1 absorbance decreases at higher temperatures ($30^\circ - 40^\circ$). A similar phenomenon has been explained by Jensen and von Hippel (1976). They claim that as higher temperatures are reached, proteins will denature from their native configuration to a random coil. In this conformation, the protein may act as a polycation and bind to the phosphate backbone of the nucleic acid. Such binding would cause stabilization of the double stranded conformation of the nucleic acid and hyperchromicity would decrease. Such an explanation could be true in the case of the protein fraction used in the assay shown in Figure 1, since the fraction contains several polypeptides. Figure 2 shows densometer tracing of an SDS polyacrylamide gel of the polypeptides found in the 2.0 M NaCl fraction. This fraction contains six major polypeptides. To determine which polypeptide or polypeptides contributed to the activity shown in Figure 1, the 2.0 M NaCl fraction was fractionated on charge columns and a gel filtration column.

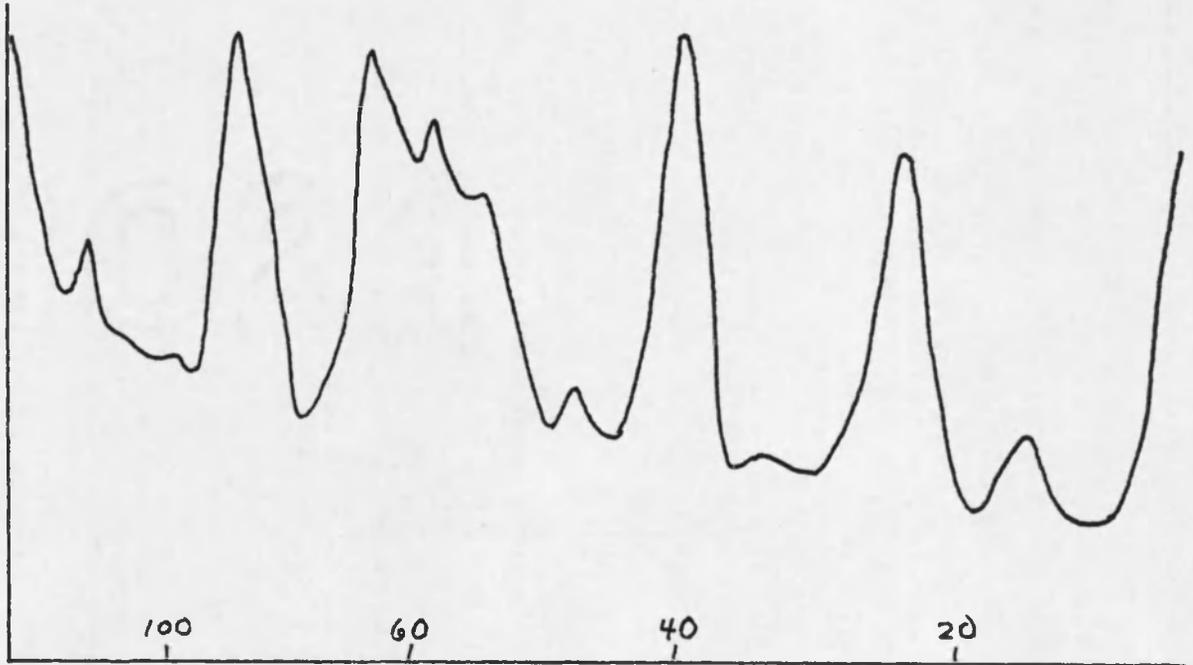


Figure 2. Densitometer Scan of a Coomassie Brilliant Blue Stained SDS Polyacrylamide Electrophoresis Gel of DNA Binding Proteins.

Polyacrylamide gel was loaded with 50 μ g of protein eluted from single stranded DNA cellulose with a 2.0 M NaCl wash. The abscissa is the length of the gel. The top of the gel is left. The bottom of the gel is right. The ordinate is relative A_{580} . Molecular weight makers are indicated in thousands of daltons. ^{64}UP is indicated by arrow.

Column Chromatography

Figure 3 shows the absorbance at 280 nanometers of an NaCl gradient (0-2.0 M) which was passed through a phosphocellulose column. The phosphocellulose column had been previously loaded with the 2.0 M NaCl fraction of Figure 2. As Figure 3 shows, there were no discrete peaks eluted. Therefore, arbitrary cuts were made in the gradient at the points shown in Figure 3. The fractions between these cuts were concentrated and called P₁, P₂, and P₃. The 2.0 M NaCl fraction which had been passed through the phosphocellulose column was loaded onto a DEAE cellulose column. This column was eluted in the same manner described above, but no detectable protein was found either on the column or in the buffer which passed through the DEAE column.

Figure 4 shows the polypeptides contained in fractions P₁, P₂, and P₃. P₁ and P₂ are very similar to the 2.0 M NaCl fraction. Both are reduced in the amount of a polypeptide of molecular weight 64,000 daltons. The two fractions are variable in amount of protein above 64,000 daltons. P₃ is highly enriched in polypeptides of low molecular weights. Almost all polypeptides above 35,000 daltons are absent. This fraction elutes in higher salt than the rest of the fraction and therefore may be considered more cationic. P₃ was the only fraction from the phosphocellulose column in which any purification of the 2.0 M NaCl fraction was achieved.

Further separations of the polypeptides by gel filtration chromatography were attempted. Figure 5 shows the polypeptide

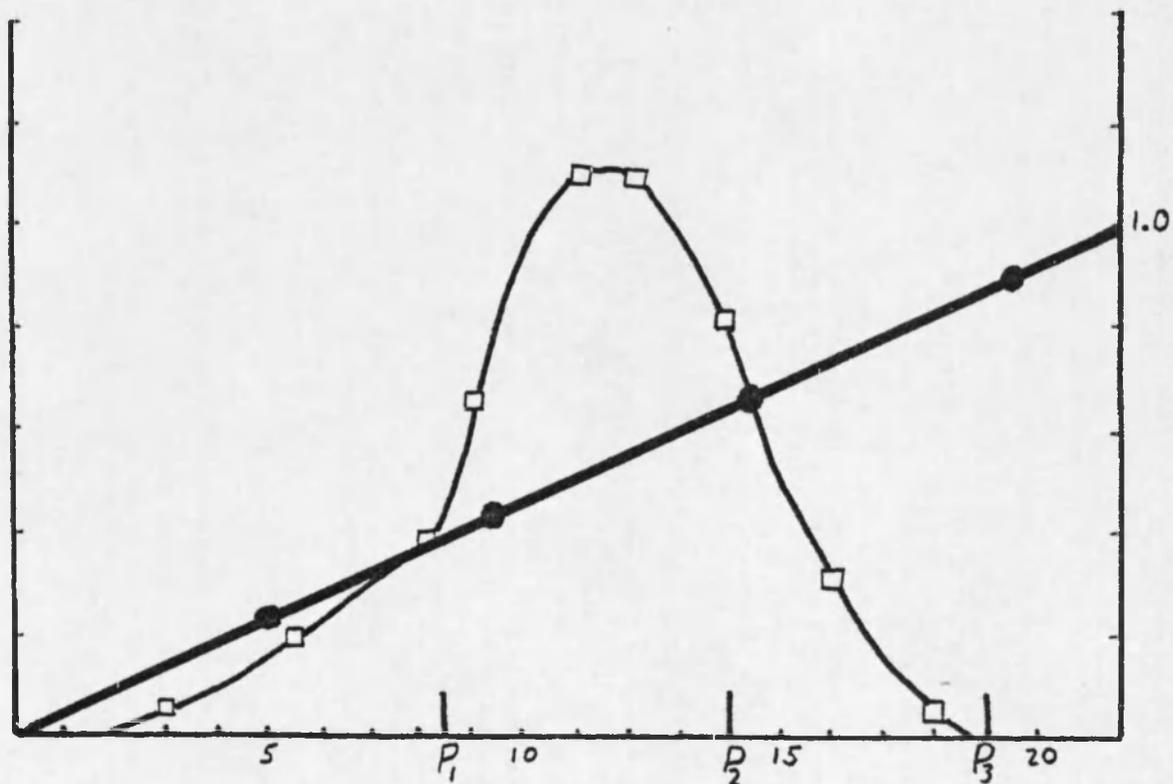


Figure 3: Relative A₂₈₀ of Fractions Collected from a Phosphocellulose column.

Closed circles (●—●) are molarity of NaCl in the elution buffer. Squares (□—□) represent relative A₂₈₀ of each fraction in the elution gradient. Fractions P₁, P₂, and P₃ were taken at points indicated. The abscissa is fraction number. The ordinate is relative A₂₈₀.

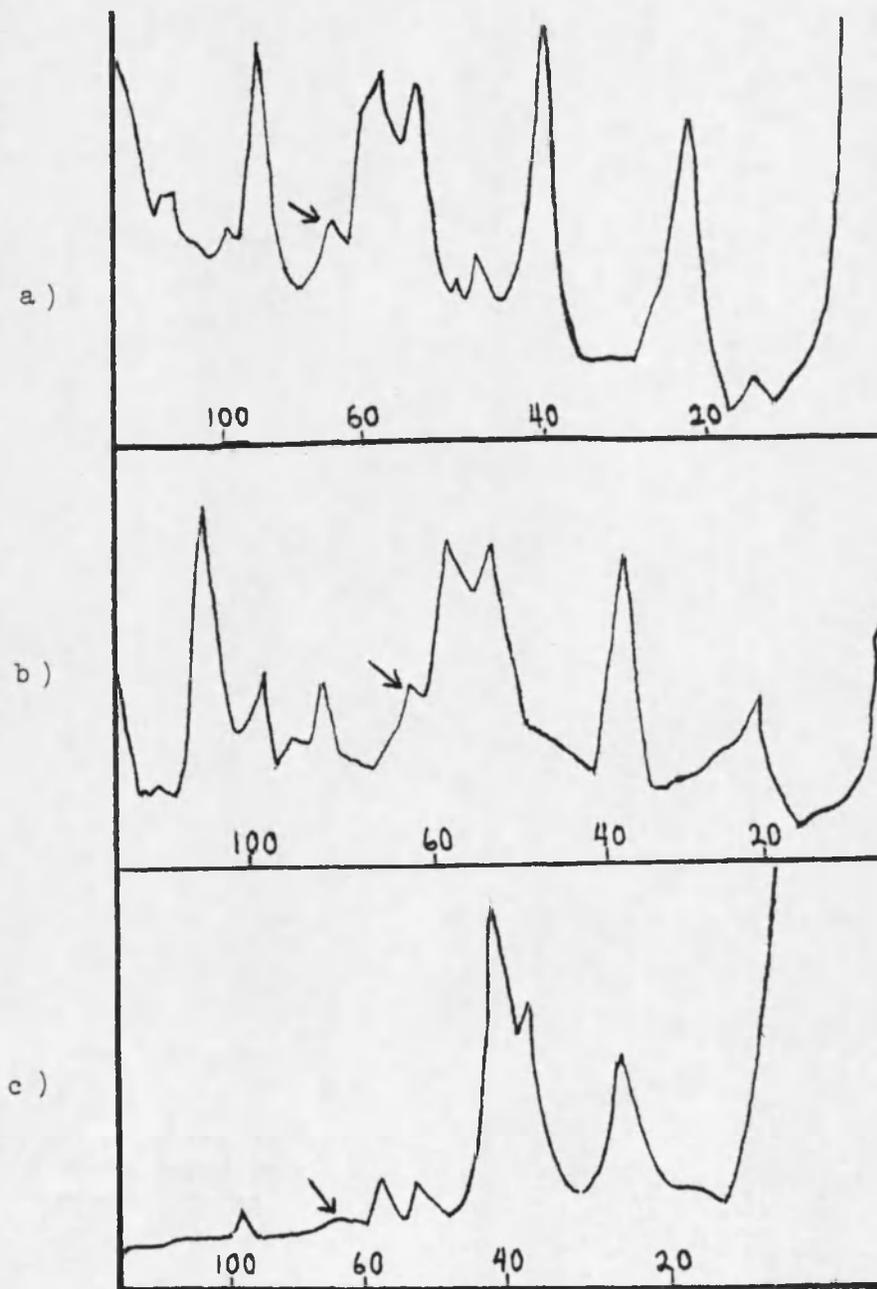


Figure 4: Densitometer Scans of Coomassie Stained "P" Fraction Gels.

Gels were loaded with from 35-50 μg of protein eluted from the phosphocellulose column of Figure 3. Fig. 4a is fraction P_1 . Fig. 4b is fraction P_2 . Fig. 4c is fraction P_3 . Gel scan is oriented as in Figure 2. The abscissa is the length of the gel. The ordinate is relative A_{580} . Molecular weights are indicated in thousands of daltons. 64UP is indicated by arrow.

profiles of fractions collected from the gel filtration column (Sephadex G-150 SF). Proteins eluted from the column were too dilute to be detected by any convenient method without substantial sample loss. Column fractions were pooled at arbitrary points, concentrated, assayed for unwinding activity, and analyzed on SDS polyacrylamide gels. Fractions were collected as described in Table 1. Fraction Fo was not assayed for unwinding activity because blue dextran interfered with the photometric assay. Apparently polypeptides of the 2.0 M NaCl fraction aggregated in high molecular weight complexes. Some polypeptides with variable molecular weight eluted with the blue dextran in fraction Fo (Fig. 5a). The largest fraction, FA (shown in Fig. 5b), was collected first after the blue dextran dye front. It contained essentially all the polypeptides which appeared in the unfractionated sample. However, a major 64,000 dalton polypeptide is reduced greatly in this fraction when compared to the 2.0 M NaCl fraction. This polypeptide is greatly enriched in the next sample FB (Fig. 5c), and by the standard of the polyacrylamide electrophoresis gel it is almost pure. The next sample to be collected from the column was FC (Fig. 5d). It is reduced in the 64,000 dalton protein and enriched in the two polypeptide impurities of fraction FB. Since the three polypeptides of FB and FC are separable by gel filtration from the other polypeptides of the 2.0 M NaCl fraction, they might not participate in the high molecular weight complex of polypeptides which apparently exist in Fo and FA. FD and FE had

Table I. Fractions Which Were Amalgamated*

Identification Fraction Numbers of Sample	
Fo	1 - 15
FA	16 - 65
FB	66 - 115
FC	116 - 165
FD	166 - 215
FE	216 - 265

* This table shows which of the one milliliter fractions from a gel filtration column (Sephadex G-150 SF) were amalgamated to form the fractions named and analysed in the text. Fraction contained the first detectable blue dextran.

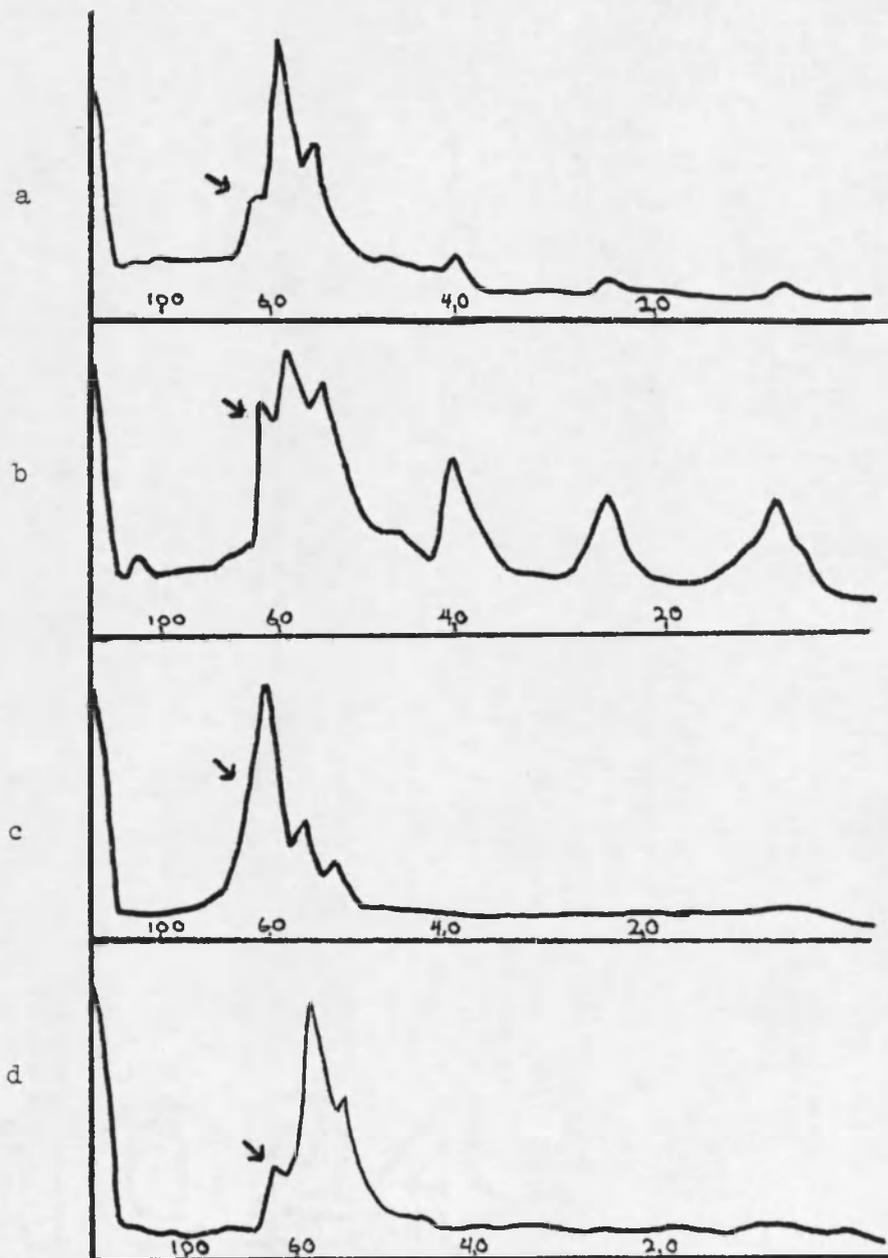


Figure 5: Scans of SDS Polyacrylamide Gels of Fractions Collected from a Gel Filtration Column.

Gels were oriented as in Figures 2 and 4. The abscissa is the length of the gel. The ordinate is relative A_{580} . Fig. 5a is fraction Fo. Fig. 5b is fraction FA. Fig. 5c is fraction FB. Fig. 5d is fraction FC. Molecular weight markers are shown in thousands of daltons. 64UP is indicated by arrow.

no detectable protein. Therefore, no SDS polyacrylamide electrophoresis gels or unwinding assays are shown for these fractions.

Unwinding Assays

The unwinding assay for P_3 is shown in Figure 6. P_1 and P_2 were not assayed since they really did not represent a particular subset of the proteins found in the 2.0 M NaCl fraction. The assay for P_3 is negative for unwinding activity since only minimal destabilization can be observed over a very wide range of temperature.

Of the fractions collected from the gel filtration column, only FB showed unwinding activity. This activity is shown in Figure 7 along with the negative assays of FA and FC. Since the 64,000 dalton protein is the major polypeptide in FB which showed unwinding activity, we conclude that it is a nucleic acid unwinding protein with an activity similar to the unwinding activity found in other eukaryotes. It is interesting to note that our "unwinding fraction" FB is impure. We considered further purification unnecessary for the purpose of this study since the fraction FC contained large amounts of the impurities of FB and very little of the 64,000 dalton protein. Fraction FC, as shown, contained no significant unwinding activity.

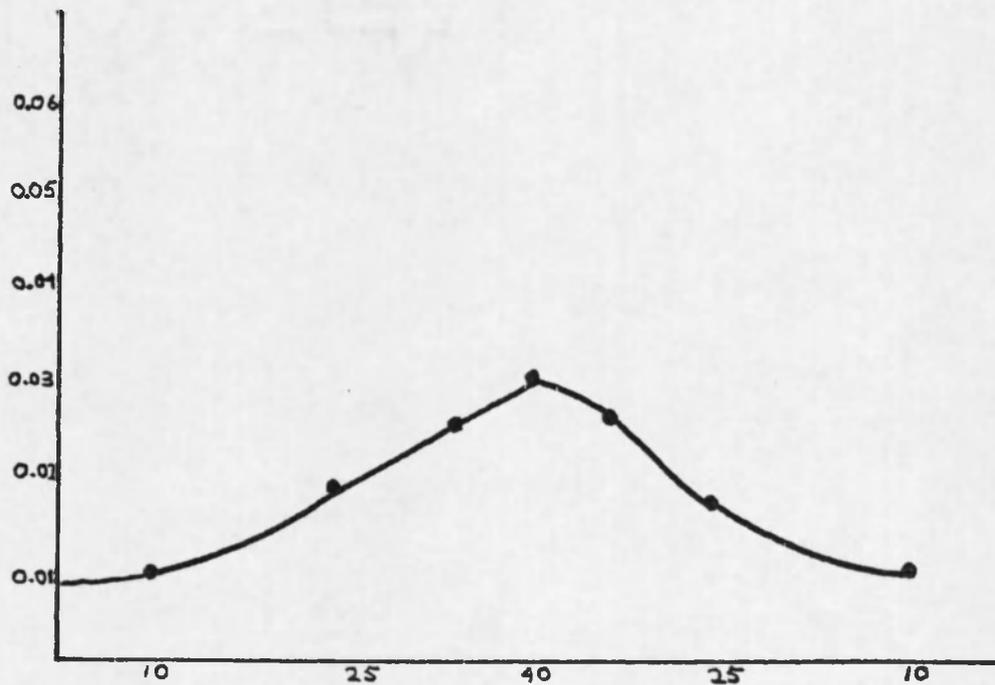


Figure 6: Unwinding Assay of Fraction P₃.

The reaction mixture was identical to the one used in Figure 1 except that 60 μ g of protein from fraction P₃ were added in 200 μ l of Buffer D. The abscissa is temperature in degrees centigrade. The ordinate is ΔA_{260} units with an arbitrary baseline.

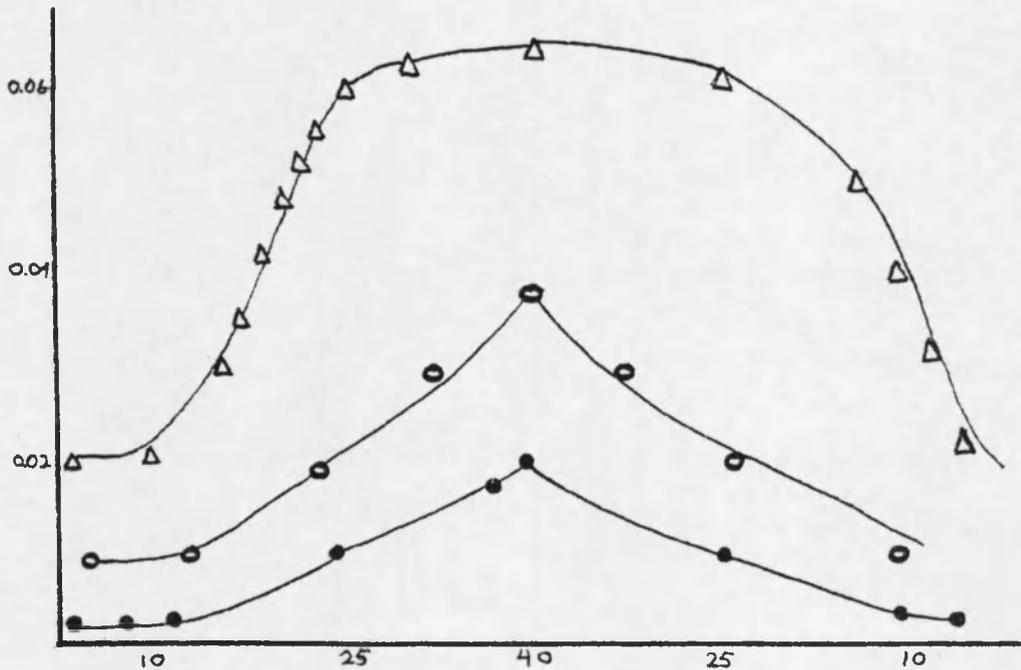


Figure 7: Unwinding Assays of Fractions from the Gel Filtration Column.

Electrophoreograms of these fractions are shown in Figure 5. Reaction is that used in Figure 1 except that the protein mixture use for the curved marked by triangles ($\Delta \rightarrow \Delta$) was 25 μg of protein from fraction FB in 150 μl of Buffer D. Open circles ($\circ \rightarrow \circ$) represent 35 μg from fraction FC in 200 μl of Buffer D. Closed circles ($\bullet \rightarrow \bullet$) represent 40 μg of protein from fraction FA in 200 μl of Buffer D. The abscissa is temperature in degrees centigrade. The ordinate is A_{260} units with an arbitrary baseline.

DISCUSSION

Column fractions from the gel filtration column were assayed for unwinding activity before the polypeptide compositions of the fractions were known. The fact that unwinding activity was found in fraction FB was unexpected. This fraction was of relatively high (64,000 daltons) molecular weight since it was the second fraction collected after the blue dextran dye front from a gel filtration column. Unwinding protein monomers previously described have been relatively low (22,000 - 52,000 daltons) in molecular weight (Banks and Spanos 1975, Herrick and Alberts 1976a, Patel 1978). Equally unexpected was the fact that the low molecular weight polypeptides under non-denaturing conditions exist as a high molecular weight complex in fraction FA. Polypeptides found in fractions FB and FC are also in fraction FA and FO. They may be constituents (albeit a minor constituent in the case of the 64,000 daltons protein) of the high molecular weight complex formed by the smaller polypeptides of the fraction. Alternatively they may form complexes with themselves. The polypeptide complex with molecular weight from about 60,000 - 50,000 daltons (Fig. 5d) is a major part of every fraction analyzed except P₃ and FB. This complex can be resolved into five polypeptides under optimal conditions for SDS polyacrylamide gel resolution (personal observation). Why these polypeptides are so ubiquitous is unknown.

They have no significant unwinding activity (see results for unwinding curve of fraction FC), and we do not suggest any function for them. We cannot assign any function to lower molecular weight polypeptides found in fraction P₃ which has no significant unwinding activity. We do suggest, however, that the 64,000 daltons unwinding protein of fraction FB might be involved in DNA replication. For the purpose of further discussion about this protein, it will be called 64UP.

Biological Significance

Unwinding activity alone cannot justify the claim that the 64UP protein functions in Physarum DNA replication. A review of Magun's previous work (in press) added further evidence to suggest this polypeptide might participate in DNA replication. Magun characterized the polypeptide polyacrylamide gel electrophoresis profiles of both double and single stranded DNA binding phosphoproteins during the cell cycle of Physarum. He collected soluble protein fractions from one time point during mitosis, one during S phase, and one during G2 phase. He found that a phosphoprotein with a molecular weight of around 64,000 daltons increased relative to other polypeptides which co-electrophoresed with the protein in samples collected during mitosis and S phase (see Fig. 8). This phosphoprotein was eluted from single stranded DNA with a buffer which was 0.6 M in NaCl. Since our 64UP polypeptide was of similar molecular weight and was contained in a similar fraction, we

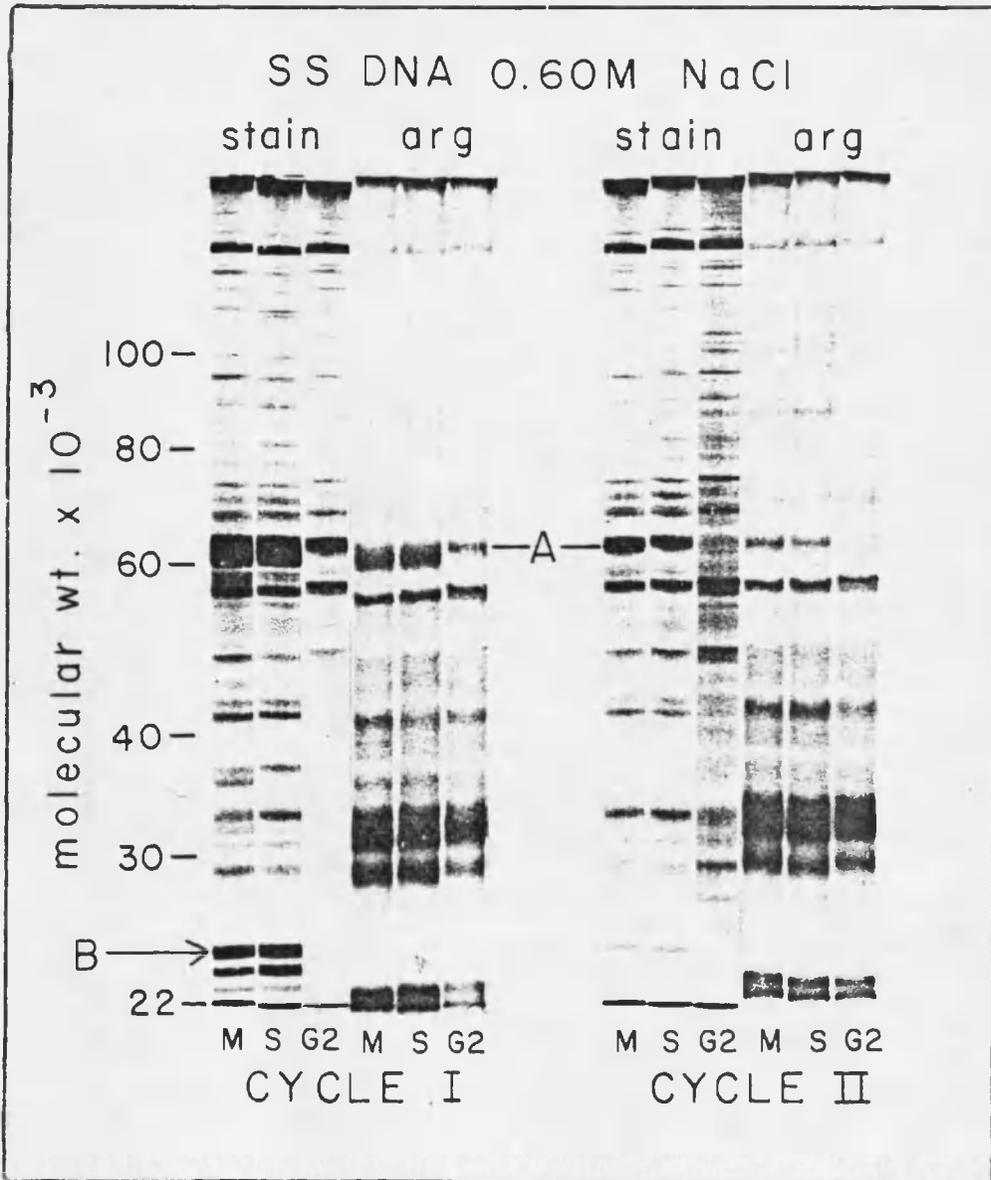


Figure 8: SDS Polyacrylamide Electrophoresis Gels of Magun (in press).

Proteins on these gels were eluted from single stranded DNA cellulose with a 0.6 M NaCl wash. 64UP may be "A". "Stain" gels are autoradiograms of ^{32}P labeled proteins on the same gels.

repeated Magun's experiment in order to confirm his results. We did find the same increase of the polypeptide around 64,000 daltons during mitosis and S phase. This 64,000 daltons polypeptide which Magun characterized migrates with the same apparent molecular weight as our 64UP. It is also in the same position on the electrophoresis gel relative to other polypeptides in the fraction (compare Figs. 2 and 8). We therefore tentatively conclude that the 64UP increases during mitosis and S phase.

Since there is no significant G_1 in Physarum, the increased levels of the 64UP during mitosis would be consistent with the hypothesis that this protein is important in Physarum DNA replication. Proteins required for the onset and/or maintenance of DNA synthesis would logically be synthesized during late G_2 and mitosis as well as S phase. Since our fractionation procedure utilized only soluble proteins most of the polypeptides are probably cytoplasmic in origin. If we assume the action of the unwinding protein is in the nucleus, we therefore would be observing newly synthesized proteins, or proteins which are easily solubilized from the nucleoplasm.

Possible Future Experiments

We have no evidence concerning the intracellular location of any proteins we have studied. Most reports of previously described unwinding proteins have studied soluble presumably cytoplasmic proteins. Only one report has appeared in which nuclear

proteins have been shown to destabilize nucleic acid helices (Thomas and Patel 1976). Physarum might provide an attractive model in which to immunocytochemically determine whether or not an unwinding protein migrates to the nucleus during S phase.

Further in vitro studies are necessary in order to ascertain the significance of the 64UP protein. First, the protein must be purified to electrophoretic homogeneity. Then more direct evidence that the protein fluctuates during the cell cycle could be obtained. Labeled 64UP protein might be coelectrophoresed with unlabeled 2.0 M NaCl fractions extracted from synchronous cells. It must be proven that this protein is not a nuclease. In our experiments, we considered the fact that nucleic acid hyperchromicity was reversible as evidence against nucleolytic activity. However, such evidence does not rule out the possibility that the protein is an RNase. The effect of the protein on both endogenous and exogenous DNA polymerases should be studied. Magun's work suggests that the protein is a phosphoprotein. The effect of dephosphorylation and rephosphorylation on the protein's activity could be determined. Physical DNA binding characteristics should be studied further. RNA binding should be reviewed also. The protein might be added to cell free DNA synthesizing systems to study the effect of the quantity or quality of the DNA produced.

The above studies are needed in order to further relate the 64UP to DNA synthesis. We have indirectly shown that this protein which can unwind DNA-like helices increases coincidentally

with DNA synthesis. Helix destabilizing activity has never been temporally related to DNA replication in normal cycling cells. However, the protein might be unrelated to DNA replication. The protein might function in RNA synthesis or processing since RNA has a peak transcription period which occurs during S phase in Physarum. In fact, protein synthesis in general increases during S phase. It should be noted, therefore, that Magun observed an increase in this protein relative to the levels of other proteins, and its increase during S is not simply a manifestation of an increase in overall protein synthesis. The fact remains, however, that the relative increase in 64UP protein during S phase might be related to some other metabolic process in Physarum than DNA replication.

If further characterization were to indicate that the protein did function in DNA replication, this work could provide a useful insight into eukaryotic DNA replication. Magun's work (in press) suggests that the protein constitutes 0.45 percent of the total soluble cellular proteins during S and mitosis phases. With such high levels of the protein present, it is more likely that the protein has a structural rather than catalytic role. Very little is known about the eukaryotic DNA replication complex, and the identification of one of its structural components might help in the understanding of the physical mechanisms involved in the replication process.

ABBREVIATIONS USED

M	Mitosis
DNA	deoxyribonucleic acid
AMP	adenosine monophosphate
RNA	ribonucleic acid
cGMP	cyclic guanosine monophosphate
H ³	tritium
BUdR	bromo-deoxyuridine
TTP	thymidine triphosphate
ATP	adenosine triphosphate
A-T	adenylate-thymidylate
dAdT	synthetic DNA, a polymer adenylate and thymidylate
μCi	micro Curie
Tris	tris hydroxymethyl amino ethane
NaCl	sodium chloride
EDTA	ethylene diamine tetra acetic acid
U-V	ultraviolet
min	minute
DEAE	diethyl amino ethane

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