Purification and Initial Characterization of the 71-Kilodalton Rat Heat-Shock Protein and Its Cognate as Fatty Acid Binding Proteins†

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Received September 16, 1985; Revised Manuscript Received January 15, 1986

ABSTRACT: The major rat heat-shock (stress) protein and its cognate were purified to electrophoretic homogeneity from livers of heat-shocked rats. Both proteins exhibited similar behavior on a variety of column chromatography matrices but were separable by preparative isoelectric focusing under nondenaturing conditions by virtue of a 0.2 pH unit difference in isoelectric point. Both purified proteins had similar physical properties, suggesting the possibility that they may have similar biological functions as well. Both proteins were homodimers under nondissociative conditions (M_r 150 000) with isoelectric points of 5.0 (cognate) and 5.2 (major stress protein). After denaturation, both proteins had an increase in isoelectric point of 0.6 pH unit, and the resulting polypeptide chains had apparent molecular weights of 73 000 (cognate) and 71 000 (major stress protein). Similarities in the electrophoretic properties of these two proteins and serum albumin, which also undergoes a large basic shift in isoelectric point due to loss of fatty acids and conformational changes accompanying denaturation, prompted us to search for lipids associated with the purified 71-kilodalton stress protein and its cognate. Thin-layer chromatography of chloroform/methanol extracts of these two proteins revealed nonesterified fatty acids bound to both proteins. Palmitic acid, stearic acid, and a small amount of myristic acid were identified by gas chromatography/mass spectroscopy. Both proteins contained approximately four molecules of fatty acid per dimer with palmitate and stearate present in a one to one molar ratio. Possible roles of the major stress protein and its cognate as fatty acid associated proteins in cellular responses to stress are discussed.

The eucaryotic heat-shock response was first observed as a change in the pattern of puffs in *Drosophila* polytene chromatin at elevated temperature (Ritossa, 1962), and the heat-shock proteins were discovered 12 years later (Tissieres et al., 1974). Because a variety of potentially injurious agents besides heat stimulate cells in culture and in stressed animals to synthesize heat-shock proteins, these proteins also have been called stress proteins (Hightower & White, 1981). The most abundant stress protein is encoded by a family of evolutionarily conserved genes which include both inducible and constitutively expressed members known as cognate genes (Ingolia & Craig, 1982; Ellwood & Craig, 1984; Lowe & Moran, 1984). In rats, the major stress protein and its constitutively produced stress cognate protein have apparent molecular weights of 71 000 (sp71)[1] and 73 000 (scp73), respectively, under denaturing conditions (Hightower & White, 1981). We have designated scp73 as a cognate protein based on the findings that it is synthesized to high levels in nonstressed cells, that it has a peptide map similar to but not identical with sp71, and that it appears to be a primary translation product (Hightower & White, 1981). These proteins are methylated in avian and mammalian cells (Wang et al., 1981, 1982), but other post-translational modifications such as phosphorylation and glycosylation have not been found on the mammalian proteins (Welch et al., 1983).

The most abundant stress protein and its cognate have been purified from HeLa cells, but the physical and chemical properties of the two proteins were so similar that they could not be separated from each other (Welch & Feramisco, 1982, 1985). Both proteins bound to ATP–agarose columns, suggesting that they may contain nucleotide or nucleic acid binding sites. The native molecular weight of both of the purified proteins was 73 800 based on ultracentrifugation, suggesting that they are monomers. In contrast, the major chicken stress protein [M_r 71 000 determined by SDS–PAGE (Hightower, 1980)] had an estimated molecular weight of 165 000 on gel filtration columns, suggesting that it is a dimer (Kelley & Schlesinger, 1982).

It is generally thought that stress proteins are induced in response to damage or metabolic perturbations caused by the interactions of stressors with cells and that the induced proteins have homeostatic functions (Lewis et al., 1975). The induction of stress proteins has been correlated with the acquisition of thermotolerance in a variety of organisms [e.g., see Li & Werb (1982)]; however, results of a recent molecular genetic study in yeast indicate that the major inducible stress protein is required for optimal growth at elevated temperatures but not for acquired thermotolerance (Craig & Jacobsen, 1984). Efforts to localize the major stress protein in cells have implicated a variety of nuclear and cytoplasmic structures in interactions with this protein including nucleoli (Pelham, 1984; Welch & Feramisco, 1984, 1985), chromatin (Velazquez et al., 1980), nuclear matrix (Levinger & Varshavsky, 1981; Sinibaldi & Morris, 1981), ribonucleoproteins and RNA (DiDomenico et al., 1982; Koetzel & Bautz, 1983; Velazquez

† This work was supported by National Science Foundation Grants PCM 8118285 and DCB 8419937. We benefited from the use of a Cell Culture Facility supported by The University of Connecticut Research Foundation.

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[1] Abbreviations: sp71, 71-kilodalton stress protein; scp73, 73-kilodalton stress cognate protein; IF, isoelectric focusing; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; nefa, nonesterified fatty acids(s); fames, fatty acyl methyl ester(s); ATP, adenosine 5′-triphosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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& Lindquist, 1984), cytoskeletal elements (Wang et al., 1981), and the plasma membrane (Velazquez et al., 1980; Hughes & August, 1982; LaThangue, 1984). Cell fractionation studies by numerous investigators [e.g., see Mitchell & Lipps (1975) and Currie & White (1983)] support both a nuclear and cytoplasmic distribution for the major inducible stress protein. Using molecular genetic approaches, Munro and Pelham (1984) recently obtained evidence for two functional domains on *Drosophila* sp70, an N-terminal domain necessary for nucleolar localization during stress and a C-terminal domain also involved in nuclear accumulation.

Our strategy for seeking clues to the function of the major stress protein and its cognate has been to purify these proteins from the livers of heat-shocked rats and to begin biochemical characterization of these proteins. Comparison of sp71 with scp73 could yield clues to why cells need two forms of these proteins, the expression of each apparently under different control. Here, we report the separation and purification of sp71 and scp73 along with the unexpected finding that both proteins contained nonesterified, saturated fatty acids of long chain length. Several viral and cellular proteins containing covalently bound myristic acid and palmitic acid have been described recently (Henderson et al., 1983; Carr et al., 1982), and several small (M_r 15 000) intracellular proteins such as Z protein containing noncovalently bound fatty acids have been discovered in mammalian cells (Mishkin et al., 1972; Ockner et al., 1972). The possible existence of higher molecular weight intracellular fatty acid binding proteins has been suggested (Brandes & Arad, 1983), and sp71/scp73 may be specific examples of this class of proteins.

Experimental Procedures

Analytical Polyacrylamide Gel Electrophoresis. Fractions collected during column chromatography and the final purification products were assayed for sp71 and scp73 by using SDS–PAGE on 9% polyacrylamide slab gels after the methods of Laemmli (1970) and two-dimensional polyacrylamide gel electrophoresis. The latter procedure involved IF in the first dimension and SDS–PAGE in the second. For IF under denaturing conditions, the methods of O'Farrell (1975) were used. For IF under nondissociative conditions, 9.5 M urea, 2% (w/v) Nonidet P-40 detergent, and 5% (v/v) 2-mercaptoethanol were omitted from the sample buffer and the IF gels whereas the concentration of acrylamide in the gels was increased from 5% to 7.5%. Proteins were visualized by staining with either Coomassie brilliant blue (Hightower, 1980) or silver (Merril et al., 1982).

Purification of Sp71 and Scp73. Male CD rats 6–8 weeks old (ca. 200–225 g) were obtained from Charles River Laboratories. The animals were anesthetized with sodium pentabarbitol and their internal body temperatures raised to 41.5 °C for 15 min by placing them between two heating pads. The animals were allowed to recover at room temperature for 24 h, then sacrificed, and perfused with phosphate-buffered saline. Livers were excised, placed in a buffer containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 0.5 mM EDTA, and aprotinin (10 μg/mL), and homogenized with a Polytron tissue grinder. The homogenate was subjected to centrifugation at 16300*g* for 30 min at 4 °C and the resulting supernatant subjected to ultracentrifugation at 25000*g* for 4 h at 4 °C. The supernatant (S-100 fraction) was adjusted to 20% saturation in ammonium sulfate and loaded onto a phenyl-Sepharose column which had been equilibrated with 10 mM NaCl/10 mM Tris-HCl (pH 7.4)/0.3 mM phenylmethanesulfonyl fluoride (buffer A) and a 20% saturated solution of ammonium sulfate. This and all subsequent purification steps were carried out at 4 °C. The column was developed with a linear gradient from 20% to 0% ammonium sulfate in buffer A, and fractions were collected. Column fractions were analyzed by one-dimensional and two-dimensional PAGE as described above. Scp73/sp71-enriched fractions were pooled, dialyzed against buffer A, and concentrated by dehydration using polyethylene glycol. The sample was then loaded onto a Bio-Gel P-300 gel filtration column equilibrated in buffer A. Fractions were collected and analyzed as described previously. Scp73/sp71-enriched fractions were pooled, loaded onto a hydroxylapatite column equilibrated in buffer A, and eluted with a linear phosphate gradient ranging from 0 to 0.3 M sodium phosphate (pH 7.4) in buffer A. Fractions were analyzed by SDS–PAGE, and those enriched for scp73 and sp71 were pooled and dialyzed against buffer A. Preparative IF was used to separate sp71 from scp73. A 5% polyacrylamide IF bed containing 1% pH 4–6 ampholines and 1% pH 3.5–10 ampholines was prepared and allowed to polymerize under nitrogen gas. The bed was prerun for 30 min at 500 V and 90 min at 1000 V. A 2-cm-wide trough was cut in the bed near the region of pH 8.0; the sample containing scp73 and sp71 was applied and subjected to electrophoresis for 5 h at 1000 V. The pH gradient of the bed was determined by a surface electrode. To determine the location of scp73 and sp71, a thin slice of the bed was excised and subjected to SDS–PAGE on a 9% polyacrylamide gel for 3 h at 15 mA. The gel was stained with Coomassie brilliant blue, and the distances between the bands containing scp73 and sp71 and the anode were measured. The sections of the preparative bed containing scp73 and sp71 were excised by using this distance. The proteins were electroeluted from acrylamide slices, dialyzed against buffer A, lyophilized, and stored at −70 °C.

Lipid Extraction and Thin-Layer Chromatography. Sp71 and scp73 were rehydrated in 0.3 mL of phosphate-buffered saline and extracted by the method of Folch (Folch et al., 1957). Material extracted by chloroform/methanol was evaporated to dryness under nitrogen, dissolved in chloroform, and applied to silica gel H (E. Merck, Inc.) thin-layer chromatography plates containing fluorescent indicator. The plates were subjected to chromatography in a polar solvent system consisting of 30 parts chloroform/40 parts acetone/10 parts methanol/10 parts acetic acid/5 parts water, dried under nitrogen, and then placed in a nonpolar solvent system consisting of 60 parts hexanes/40 parts ethyl ether/1 part acetic acid. Chloroform-soluble components were visualized under ultraviolet light. Fluorescent spots which comigrated with nefa standards were scraped from thin-layer plates, extracted with chloroform, and evaporated under nitrogen.

Methyl Esterification and Gas Chromatography/Mass Spectroscopy. Nefa extracted from sp71 and scp73 were methyl esterified by using a boron trichloride method (Godchaux & Leadbetter, 1984). The resulting fames were dissolved in chloroform and analyzed by gas chromatography/mass spectroscopy using a Hewlett-Packard 5985 GC/MS system. Standard mixtures of saturated and unsaturated fames were purchased from Supelco Inc.

Peptide Mapping. Cultured rat embryo cells were prepared, heat shocked, and labeled with [^{35}S]methionine as previously described (Hightower & White, 1981). Cells were scraped from tissue culture plates into buffer A and lysed by freeze–thawing. The extract was subjected to centrifugation for 5 min in a Brinkmann microcentrifuge to remove cellular debris, and the supernatant was analyzed by IF under either nondenaturing or denaturing conditions. Second-dimension SDS–

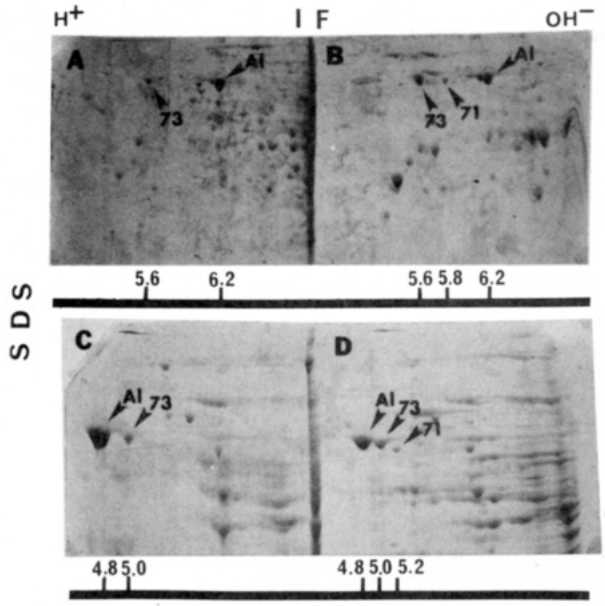

FIGURE 1: Two-dimensional polyacrylamide gels of proteins from 100000*g* supernatants obtained from homogenates of control (panels A and C) and heat-shocked (panels B and D) rat livers. IF was carried out under either denaturing (panels A and B) or nondenaturing (panels C and D) conditions in the first dimension, and all samples were analyzed by SDS–PAGE in the second dimension as described under Experimental Procedures. Proteins were stained with Coomassie brilliant blue. Approximate pH scales drawn below each panel were determined with a surface electrode. Approximately 100 μg of protein was loaded on each gel.

PAGE and partial digest peptide mapping (Cleveland et al., 1977) were carried out as previously described (Hightower & White, 1981), except that papain and *Staphylococcus aureus* V8 proteases were used to generate peptide fragments.

Results

Unusual Properties of Sp71 and Scp73 Revealed by PAGE. Rat liver was chosen as a source of scp73 and sp71 because this organ responds to heat shock by producing a single major isoelectric form of each protein and because the behavior of rat stress proteins on two-dimensional gels is well characterized (Hightower & White, 1981; Currie & White, 1983). As shown in Figure 1, the 100000*g* supernatant (S-100 fraction) obtained from control liver homogenate and analyzed by two-dimensional PAGE under denaturing conditions (panel A) contained substantial amounts of scp73 (p*I* = 5.6) as well as residual albumin (p*I* = 6.0–6.2) not removed by perfusion. S-100 fractions of heat-shocked liver analyzed under the same conditions (panel B) contained sp71 (p*I* = 5.8) as well. When dissociating agents were omitted from the IF dimension (panel C, control; panel D, heat shock), many cytosolic proteins produced streaks in the gels. This heterogeneity was probably due to incomplete solubilization or aggregation of proteins in the absence of detergent and urea in the IF dimension. Streaking was not due to failure of IF to reach equilibrium since albumin moved from the basic to the acidic end of the focusing gel to achieve its expected p*I* of 4.8. Scp73 and sp71 along with albumin focused well under both denaturing and nondenaturing conditions. The patterns of proteins in S-100 fractions from control and heat-shocked livers contained many differences besides the induction of the major stress proteins when analyzed under either denaturing or nondenaturing conditions (see Figure 3 also). In contrast, crude homogenates of control and heat-shocked livers had very similar protein patterns except for the accumulation of the major stress proteins (data not shown), suggesting that there are major

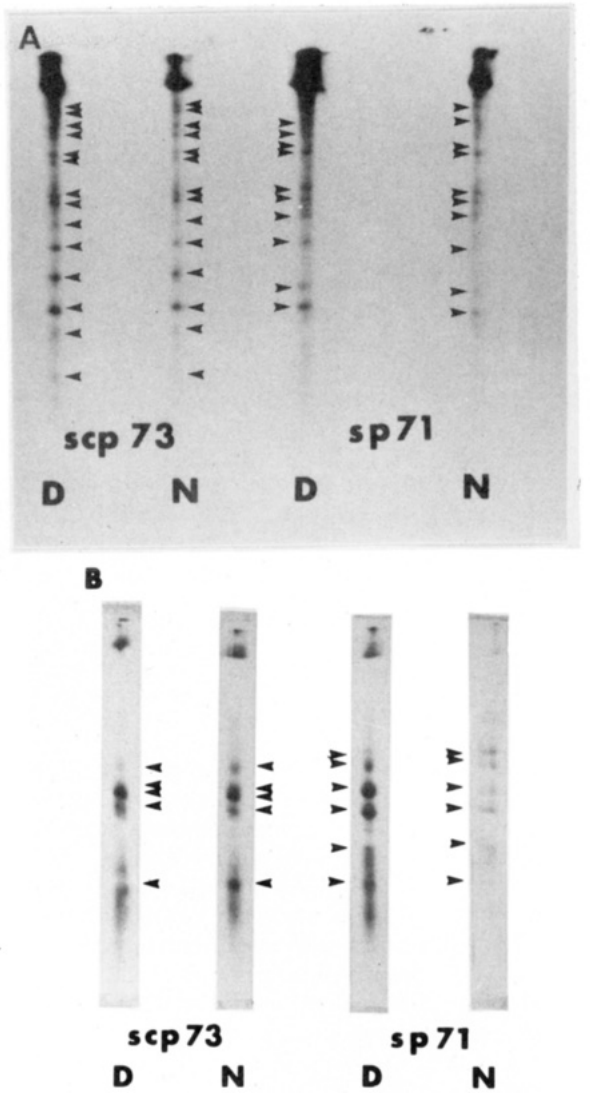

FIGURE 2: Partial digest peptide maps of scp73 and sp71 excised from two-dimensional polyacrylamide gels. IF was carried out under either denaturing (D) or nondenaturing (N) conditions as described under Experimental Procedures. [^{35}S]Methionine-labeled proteins extracted from heat-shocked cultures of rat embryo cells were used. Fluorograms of the radioactive peptides generated by 50 μg of papain (panel A) and 1.25 μg of *S. aureus* V8 protease (panel B) digestion are shown. Major fragments were marked with darts.

differences in the distribution of proteins between soluble and particulate fractions from control and heat-shocked rat liver. The large difference in the p*I* of albumin under denaturing and nondenaturing conditions is well documented (Basu et al., 1978). Albumin with anionic forms of fatty acids noncovalently bound focuses at pH 4.8, whereas rat albumin stripped of fatty acids by dissociating agents such as Nonidet P-40 detergent and urea focuses at pH 6.0–6.2. Liver scp73 and sp71 also had large differences in p*I*'s under conditions of nondenaturing and denaturing IF. When exposed to dissociating conditions, the p*I* of scp73 changed from 5.0 to 5.6, and the p*I* of sp71 changed from 5.2 to 5.8. The major stress protein and its cognate from cultured chicken and rat embryo cells and from rat heart and brain tissue heat shocked in vivo exhibited similar behavior (data not shown).

To confirm that the proteins focusing at pH 5.2 and 5.0 under nondenaturing conditions were the major stress protein and its cognate, partial digest peptide maps were prepared and compared to those of sp71 and scp73 separated under denaturing conditions (Figure 2). The protein which focused at pH 5.0 in nondissociative IF gels (lanes marked N) had a

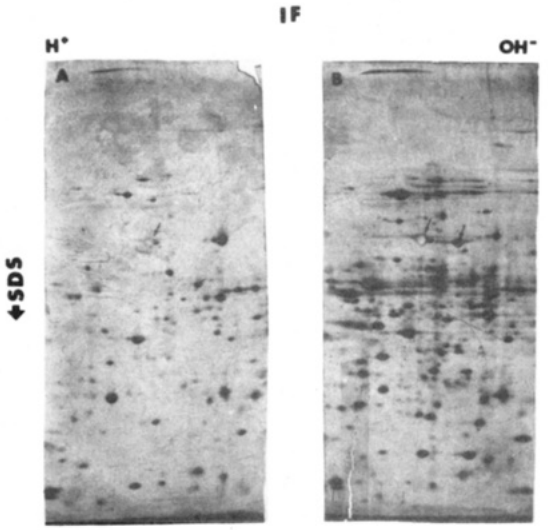

FIGURE 3: Two-dimensional polyacrylamide gels of proteins from S-100 fractions obtained from homogenates of control (panel A) and heat-shocked (panel B) rat livers visualized with silver stain. Aliquots of high-speed supernatant containing 10 μg (panel A) and 30 μg (panel B) of protein were diluted into buffer and subjected to IF under denaturing conditions in the first dimension and SDS–PAGE in the second dimension. The positions of scp73 and sp71 are marked with arrows.

peptide map identical with that of the protein (scp73) focusing at pH 5.6 in dissociative gels (lanes marked D). Identical maps were also obtained for the protein focusing at pH 5.2 on a nondissociative IF gel and the protein focusing at pH 5.8 on a dissociative IF gel (sp71). As reported previously (Hightower & White, 1981), sp71 and scp73 had similar but not identical peptide maps. In addition to peptide mapping, purified sp71 and scp73 were identified as members of the 70-kilodalton heat-shock protein family by immunoblot analysis (data not shown) of one- and two-dimensional polyacrylamide gels using a monoclonal antibody (7.10, orginally raised against the *Drosophila* 70-kilodalton heat-shock protein) provided by Dr. Susan Lindquist (Velazquez et al., 1983). The purified proteins had the same changes in p*I* on denaturing and nondenaturing IF gels as shown in Figure 1.

Sp71 and scp73 exhibited unusual silver staining properties. When homogenates from both control and heat-shocked rat livers were separated by two-dimensional PAGE and the resulting gels were silver stained, most proteins stained shades of grey or brown as expected (Merrill et al., 1982). In contrast, gel regions containing scp73 and sp71 excluded the silver stain and appeared as large colorless spots usually surrounded by a halo of silver (Figure 3). Only three other proteins detectable on gels of the liver homogenates exhibited similar behavior. The amount of liver protein loaded on two-dimensional gels was varied to determine whether or not the staining properties of sp71 and scp73 were concentration dependent. The intensity of silver staining of these proteins varied inversely with the amount of protein on the gels except at very low protein concentrations where the staining intensity decreased as the amount of protein was reduced. When gels containing amounts of scp73 and sp71 which ordinarily would result in exclusion of silver stain were first stained with Coomassie blue, destained, and then counterstained with silver, scp73 and sp71 became stainable with silver (data not shown).

Purification of the Major Heat-Shock Protein and Its Cognate. Scp73 and sp71 were purified to electrophoretic homogeneity from homogenates of heat-shocked rat livers. A purification protocol was developed (Table I) that maintained predominantly single major isoelectric forms of scp73 and sp71

Table I: Summary of Recoveries of Sp71 and Scp73 after Each Major Step in the Purification

purification step	total protein[a] (mg)	total scp73[b] (mg)	total sp71[b] (mg)	yield (%) scp-73	yield (%) sp-71
(1) cytosol (S-100)	480	10	8	100	100
(2) phenyl-Sepharose	90	6	4.2	60	52
(3) Bio-Gel P-300	32.5	4.5	3.1	45	40
(4) hydroxylapatite	7.0	2.5	1.5	25	18
(5) isoelectric focusing	0.75	0.5	0.25	5	3

[a] Total protein was measured with a dye binding assay (Bradford, 1976) using bovine serum albumin as a standard. [b] Total scp73 and sp71 were estimated from scans of Coomassie blue stained gels using known amounts of purified sp71 and scp73 as standards.

which focused at pH 5.0 and 5.2, respectively, under nondenaturing conditions. Preservation of this difference in p*I* was crucial for the separation of sp71 from scp73 because these proteins copurified in all other steps in our protocol. Our initial efforts to purify these proteins as well as published work (Welch & Feramisco, 1982) indicated that sp71 and scp73 eluted from a variety of column chromatography matrices in very broad bands. Several unusual properties of sp71 and scp73 including albumin-like shifts in p*I* following denaturation and exclusion of silver stain suggested that these proteins might have considerable hydrophobic character; therefore, phenyl-Sepharose, a hydrophobic interaction matrix, was tried and found to be very useful. Scp73 and sp71 exhibited relatively homogeneous behavior and were recovered in excellent yields after phenyl-Sepharose chromatography. These proteins were further purified by using gel filtration on Bio-Gel P-300 columns which also allowed molecular weight estimates under nondissociating conditions. Both scp73 and sp71 had apparent molecular weights of approximately 150000, indicating that both were dimers. Hydroxylapatite chromatography was used to remove most of the remaining major contaminating proteins from sp71/scp73. Preparative IF under nondenaturing conditions was used as a final step to separate scp73, which focused at p*I* = 5.0, from sp71, which focused at p*I* = 5.2. The purification was monitored by one-dimensional SDS–PAGE, and gel analysis of representative aliquots taken at various stages during a typical purification is shown in Figure 4. Densitometer scans of Coomassie blue stained one-dimensional gels containing purified sp71 and purified scp73 have been published previously (Hightower et al., 1985). The final purity of sp71 and scp73 was determined by using silver staining of one-dimensional gels (Figure 4B) loaded with sufficient amounts of purified proteins (0.2 μg per lane) to be detectable by Coomassie blue stain. Silver stain is approximately 100-fold more sensitive than Coomassie blue stain, allowing detection of contaminants at the level of approximately 1% of loaded protein. As described above for Figure 3, both sp71 and scp73 stained very poorly with silver and were vastly underrepresented; however, only trace amounts of contaminating proteins were detectable. The purities of sp71 and scp73 were estimated to be at least 95%. The most significant contaminants (marked by arrows in Figure 4B) appeared to be degradation products of sp71 and scp73 since these smaller polypeptides bound monoclonal antibody 7.10 on immunoblots (data not shown). Some degradation of sp71 and scp73 occurred during purification despite the inclusion of aprotinin in sample buffers and phenylmethanesulfonyl fluoride in column buffers and execution of all purification steps in the cold.

Final purity and charge heterogeneity of scp73 and sp71 were also assessed by two-dimensional PAGE (Figure 5).

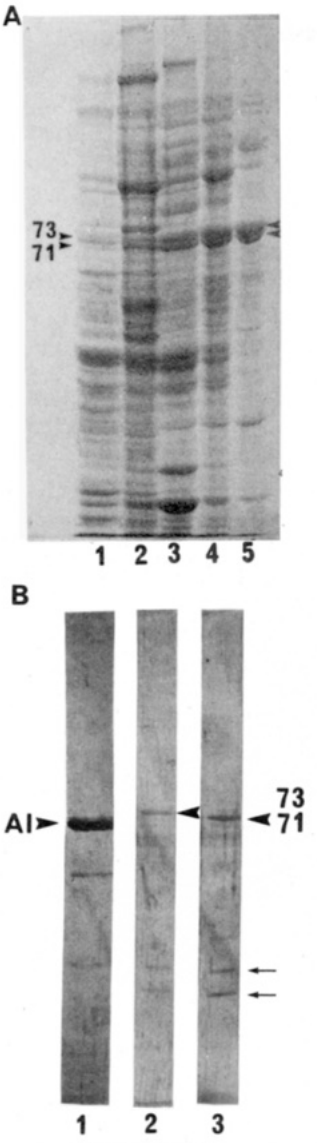

FIGURE 4: One-dimensional SDS–PAGE of samples taken after major steps of the purification protocol. Proteins were visualized by Coomassie blue staining in panel A: channel 1, S-100 fraction from control rat liver; channel 2, S-100 fraction from heat-shocked rat liver; channel 3, pool of sp71/scp73-enriched fractions after phenyl-Sepharose chromatography; channel 4, sp71/scp73-enriched pool after Bio-Gel P-300 chromatography; channel 5, sp71/scp73-enriched pool after hydroxylapatite chromatography. Following the final IF step, proteins were visualized by silver staining in panel B: channel 1, purified bovine serum albumin (A1); channel 2, purified scp73; channel 3, purified sp71 (0.2 μg of protein per channel). Major contaminants were marked with arrows.

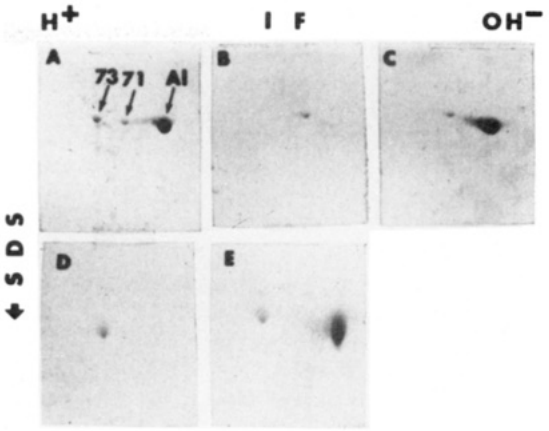

FIGURE 5: Analysis of purified scp73 (panel D) and sp71 (panel B) by two-dimensional PAGE using denaturing IF. Five micrograms of purified serum albumin was added to 1 μg of purified sp71 (panel C), to 1 μg of purified scp73 (panel E), and to a mixture (panel A) of scp73 and sp71 for reference. All gels were stained with Coomassie blue.

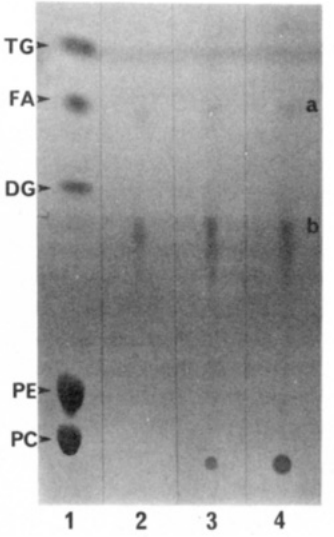

FIGURE 6: Thin-layer chromatogram of chloroform/methanol-extracted material from 100 μg of purified rat serum albumin (lane 2), 100 μg of purified scp73 (lane 3), and 100 μg of purified sp71 (lane 4) separated on a silica gel 60 thin-layer plate developed with a polar solvent system followed by a nonpolar solvent system as described under Experimental Procedures. Lipids were visualized with iodine vapors. Standards (lane 1) were phosphatidylcholine (PC), phosphatidylethanolamine (PE), diglycerides (DG), fatty acids (FA), and triglycerides (TG). Spot a, fatty acids; spot b, unidentified yellow pigmented material. The discontinuity just above spot b was the polar solvent front.

Purified rat serum albumin was added to purified sp71 (panel C), to purified scp73 (panel E), and to a mixture of sp71 and scp73 (panel A) to serve as a marker protein. Purified preparations of sp71 (panel B) and scp73 (panel D) contained only a single protein detectable by staining with Coomassie brilliant blue.

Lipid Analysis and Identification of Fatty Acids. The similarities in the electrophoretic behavior of sp71, scp73, and albumin under denaturing and nondenaturing conditions raised the possibility that, like albumin, the major stress protein and its cognate might contain noncovalently bound fatty acids. To test this possibility, 100 μg of purified scp73, sp71, and rat serum albumin was extracted with chloroform/methanol. The organic phase was evaporated and the residue dissolved in chloroform for analysis by thin-layer chromatography using both polar and nonpolar solvent systems (Figure 6). The patterns of neutral lipids extracted from scp73 (lane 3) and sp71 (lane 4) were virtually identical and consisted of one major species (spot a). The pattern of neutral lipids extracted from scp73 from normal livers was indistinguishable from that obtained from heat-shocked livers (data not shown). Spot a comigrated with a palmitic acid standard and with fatty acids extracted from serum albumin. No polar lipids were found associated with either scp73 or sp71, although a yellow pigment (spot b) which migrated with the polar solvent front routinely copurified with sp71 and scp73. The intensities of the iodine-stained fatty acid spots isolated from scp73 and sp71 were nearly equivalent to that extracted from the same amount of albumin (lane 2).

To identify the fatty acids noncovalently associated with scp73 and sp71, spot a was removed from a thin-layer plate and methyl-esterified. The resulting fames were separated by

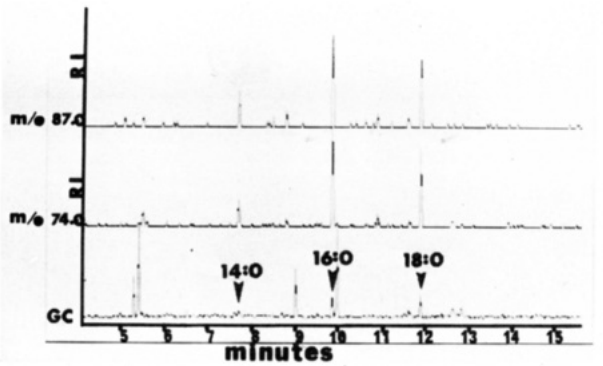

FIGURE 7: Analysis by gas chromatography/mass spectroscopy of fatty acids extracted from scp73. Spot a was removed from a preparative thin-layer chromatography plate (silica gel H with fluorescent indicator) and eluted into chloroform. Fatty acids were converted into fames by methyl esterification and separated on an OV101 gas chromatography column programmed to increase 10 °C/min from 125 to 250 °C [gas chromatogram (GC), lowest scan]. The retention times of standard fames of myristic (14:0), palmitic (16:0), and stearic (18:0) acids are marked. Peaks were analyzed by mass spectroscopy (Hewlett-Packard 5985 GC–MS system), and scans of the relative intensities (RI), i.e., frequencies, of molecular fragments with mass numbers (m/e) 74.0 and 87.0 are shown. Other major GC peaks were contaminating silicates and phthalates.

gas chromatography (lower scan, Figure 7). Material which comigrated with fames standards for palmitic (16:0), stearic (18:0), and myristic (14:0) acids was detected in spot a from both sp71 and scp73. Mass spectra were obtained, and scans at the mass numbers of the most frequently occurring fragments of saturated straight chain fames are shown in Figure 7, top and middle scans. The complete mass spectra of the fames contained signals for the two characteristic major peaks with mass numbers of 74.0 and 87.0 and for molecular ions with mass numbers of 252 for myristic acid, 270 for palmitic acid (Figure 8A), and 298 for stearic acid (Figure 8B).

The amount of fatty acid extracted from known amounts of purified stress proteins was estimated by comparing the intensity of iodine staining of the fatty acid containing spots on thin-layer plates to that of known amounts of palmitic acid run on the same plate. An approximate molar ratio of two molecules of nefa per molecule of scp73 and sp71 was calculated. The molar ratios of palmitic and stearic acids were estimated by measuring peak areas from gas chromatograms such as the one shown in Figure 7. The one to one ratio of these fatty acids was also apparent from the relative frequencies of the major molecular fragments of the corresponding fames analyzed by mass spectroscopy (Figure 7). Palmitic and stearic acids were present in a one to one molar ratio in lipid extracts from both sp71 and scp73. The physical properties of these two proteins are summarized in Table II.

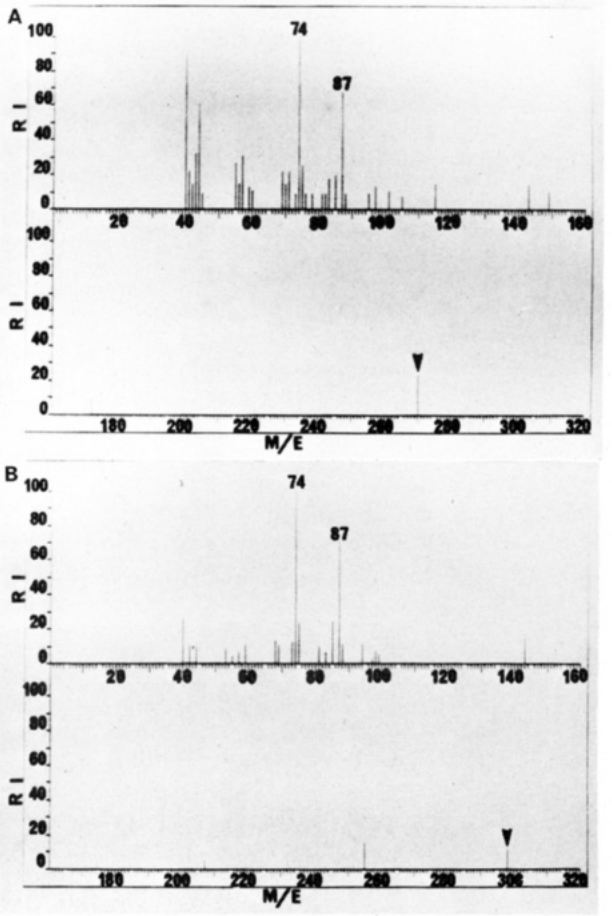

FIGURE 8: Mass spectra of fames of palmitic (panel A) and stearic (panel B) acids extracted from sp71. Relative intensities (RI) of molecular fragments were plotted vs. mass number (M/E). The major fragments characteristic of fames are marked with their corresponding mass number, and the positions of the molecular ions are marked with arrowheads.

Table II: Physical Properties of Sp71 and Scp73

	scp73	sp71
(1) p*I* under nondenaturing condn[a]	5.0	5.2
(2) p*I* under denaturing condn[b]	5.6	5.8
(3) mol wt under nondenaturing condn[c]	150000	150000
(4) mol wt under denaturing condn[d]	73000	71000
(5) fatty acid molecules per dimer[e]	4	4

[a] Two-dimensional IF/SDS–PAGE without Nonidet P-40 detergent and urea. p*I* determined by measuring the pH gradient after the IF step. [b] Two-dimensional IF/SDS–PAGE with Nonidet P-40 and urea by the methods of O'Farrell (1975). [c] Measured on a Bio-Gel P-300 chromatography column calibrated with a mixture of thyroglobulin, ovalbumin, myoglobin, γ-globulin, and vitamin B_{12} (Bio-Rad gel filtration standard). [d] Measured by SDS–PAGE (Hightower & White, 1981). [e] Measured with thin-layer chromatography using palmitic acid as a calibration standard.

Discussion

The purification protocol described herein was effective in purifying both sp71 and scp73 to electrophoretic homogeneity. As a result, we were able to begin comparison of the biochemical properties of the major heat-shock protein and its cognate. None of the chromatography matrices effected a significant separation of these proteins, indicating further similarities in their physical and chemical properties in addition to similar molecular weights and peptide maps. Lipid analyses showed that both sp71 and scp73 were associated with palmitic acid and stearic acid in a one to one ratio and with a stoichiometry of approximately four nefa per dimer. These similarities encourage the hypothesis that sp71 and scp73 may have similar biological functions. Our native molecular weight estimates for rat sp71 and scp73 are different than published molecular weights of the analogous purified human stress proteins, sp72 and sp73 (Welch & Feramisco, 1982), but agree with estimates of the molecular weight of the major chicken stress protein (Kelley & Schlesinger, 1982).

Our main purpose for describing the staining properties of sp71 and scp73 was to show that silver staining by Merril's procedure does not provide reliable quantitative data on these proteins except when very low amounts of protein are used. However, the interactions of sp71 and scp73 with the Merril silver stain may also be a reflection of hydrophobic properties of these proteins. The Coomassie blue dye molecule has substantial hydrophobic character (Tal et al., 1985) whereas

the silver stain reaction is thought to involve hydrophilic amino acid residues (Wray et al., 1981). If sp71 and scp73 underwent concentration-dependent aggregation in polyacrylamide gels involving hydrophobic interactions, the silver stain might react with the surface of the aggregated protein but not penetrate, creating the silver halo effect observed in our study. Coomassie stain by virtue of its hydrophobic properties might be able to penetrate such an aggregate, and once penetrated by dye molecules, the proteins may be more accessible to silver stain.

The major clue that sp71 and scp73 might contain fatty acids was the similarity in electrophoretic behavior of these proteins upon denaturation to the known properties of serum albumin. The large increase in p*I* of albumin treated with Nonidet P-40 and urea must reflect the effect of conformational changes in albumin upon denaturation in addition to loss of fatty acids. Since the calculated change of p*I* for loss of a single anionic charge is 0.07 pH unit (Spencer & King, 1971) and an albumin molecule carries on average 1.3 fatty acids (Peters, 1975), loss of fatty acids actually accounts for only a small fraction of the 1 pH unit change in the p*I* of albumin upon denaturation. However, loss of an estimated four molecules of fatty acid per dimer of sp71 and scp73 can account for about half of their 0.5 pH unit increase in p*I* after denaturation. Whether such changes in p*I* are characteristic of fatty acid binding proteins or merely coincidental in this case is not clear at present; however, the similarity to albumin prompted us to analyze purified sp71 and scp73 for noncovalently bound lipids.

We have shown here that sp71 and scp73 were capable of associating with fatty acids. The question now is whether or not fatty acid association has significance for the function(s) of these proteins. Several facts argue against this association arising from nonspecific interactions between hydrophobic ligands and hydrophobic regions of these proteins. First, there is selectivity in the lipids bound to sp71 and scp73, and the fatty acids bound to these proteins do not simply reflect either qualitatively or quantitatively the distribution of free fatty acids in rat liver. No phospholipids or other polar lipids were detected in lipid extracts of these purified proteins nor were unsaturated fatty acids detected despite the fact that free (noncovalently associated) linoleic (16:1) and oleic (18:1) acids are twice as abundant as free stearic acid in rat liver (Upreti et al., 1983). Palmitic and stearic acids were found in a one to one ratio in sp71 and scp73 whereas the ratio of free palmitic acid to free stearic acid in liver is approximately three to one. Scp73 isolated from rat brain, an organ with a dramatically different fatty acid composition than liver, contains the same nefa in the same proportions as scp73 isolated from rat liver, indicating again that the fatty acids associated with this protein are not simply a nonselective sampling of the free fatty acids in a tissue (Guidon & Hightower, 1986). Second, the stoichiometry of fatty acid binding to these proteins is approximately as high as fatty acid binding to albumin, the major fatty acid carrier in the blood. If we assume that a major component of the change in p*I* of sp71 and scp73 upon denaturation is due to loss of nefa, then the data in Figure 1 indicate that the vast majority of these proteins in the S-100 fraction of a rat liver homogenate contain nefa since essentially all detectable sp71 and scp73 underwent the shift in p*I*. Currie and White (1983) reported the greatest enrichment for sp71 and scp73 in the supernatant fraction from heat-shocked rat brain and liver, suggesting that our starting material for purification contained the major portion of the major stress protein and its cognate. And finally, initial studies of fatty acid binding to scp73 involving the addition of [^{3}H]palmitate to purified scp73 in vitro showed that this protein does not bind additional fatty acids under conditions which allow facile binding/exchange of fatty acids on albumin (Guidon & Hightower, 1986), indicating that the fatty acids are tightly bound rather than adventitiously associated.

We are considering several possible roles for the palmitic and stearic acids noncovalently associated with sp71 and scp73. These fatty acids may have structural roles. For example, sp71 and scp73 may assume a conformation when associated with fatty acids that affects either the function, the solubility, or the cellular location of these proteins. Recently, the importance of amino-terminal myristylation for functional integrity of the pp60^{src} kinase was demonstrated (Cross et al., 1984). Although the fatty acids which we identified were not covalently attached to sp71 and scp73, they could conceivably bring about changes in the tertiary structure of these proteins as has been demonstrated for noncovalent binding of fatty acids to albumin [reviewed in Peters (1975)].

As structural components, fatty acids might associate with sp71 and scp73 during or shortly after synthesis and could participate in protein folding, becoming integral parts of the mature proteins. The stability and solubility of serum albumin increase when albumin associates with fatty acids (Peters, 1975), and fatty acids might have similar effects on sp71/scp73. Alternatively, fatty acids may associate with sp71 and scp73 as regulators of stress protein function following tissue stress. Recently, several nonlysosomal proteases isolated from mammalian muscle have been activated in vitro by SDS and fatty acids (Dahlmann et al., 1985), providing interesting models for enzyme activation by fatty acids. It may be more than coincidental that members of the 70-kilodalton family of stress proteins have been reported to undergo self-degradation after denaturation with SDS (Mitchell et al., 1985). Mitchell and co-workers suggested that the major stress protein might interact with SDS molecules in order to assume a proteolytically active conformation and that fatty acids might provide this function in vivo.

Since sp71 and scp73 contained mainly saturated fatty acids of long chain length, it is not likely that these proteins serve as general intracellular carriers of fatty acids. Such a role has been proposed for the intracellular fatty acid binding protein which carries about 60% of the cytosolic long chain fatty acids of rat liver (Ockner et al., 1982) and reportedly binds more unsaturated than saturated fatty acids [reviewed in Glatz & Veerkamp (1985)]. If scp73 and sp71 do have roles in fatty acid metabolism in normal and stressed cells, respectively, they are more likely to specifically involve the metabolism of palmitate and stearate. However, it is interesting to note that sterol carrier protein (i.e., fatty acid binding protein) highly purified from rat liver contains primarily palmitic and stearic acids in a 1:1 ratio (Dempsey et al., 1981). One interesting possibility is that sp71 and scp73 may be involved in membrane remodeling. In heat-stressed animal cells, this might involve structural alterations in membranes to counter the effects of heat on membrane lipid properties and/or membrane protein function [reviewed in Lepock (1982)]. Although recent studies indicate that bulk changes in membrane lipid fluidity (Lepock et al., 1981) and gross changes in membrane lipid composition (Gonzalez-Mendex et al., 1982) do not correlate with cellular responses to hyperthermia and thermotolerance, these studies do not rule out more selective and localized changes in membrane structure that could involve saturated fatty acids. We (Hightower et al., 1985) and others (DiDomenico et al., 1982; Minton et al., 1982; Pelham, 1984) have suggested that stress proteins may protect or repair labile structures in cells. Such

stress-sensitive structures might include decondensed regions of chromatin, nucleolar and cytoplasmic ribonucleoproteins, and regions of cytoskeletal and membrane biosynthesis. This hypothesis could explain why sp71 and scp73 appear to be associated with such a variety of intracellular components. It is possible that saturated fatty acids delivered to a variety of cellular locations by sp71 and scp73 protect sensitive molecules from oxidative damage, for example, by creating localized hydrophobic environments capable of excluding hydrophilic free radicals and peroxides. There is suggestive evidence already of links between oxidative stress and induction of stress proteins (Lee et al., 1983; Sciandra, 1984; Whelan & Hightower, 1985). These are of course only speculative ideas at this point; however, they serve to illustrate some of the new directions for which the finding that sp71 and scp73 bind fatty acids now offers a tangible reason to pursue.

ACKNOWLEDGMENTS

We thank Richard Crain and Walter Godchaux, III, for many helpful discussions about lipidology, Fred White for sharing his insights about stress proteins, and Michelle Mailloux for assistance in preparing the manuscript.

Registry No. Myristic acid, 544-63-8; palmitic acid, 57-10-3; stearic acid, 57-11-4.

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Purification and Characterization of a Deoxyribonucleic Acid Dependent Adenosinetriphosphatase from Mouse FM3A Cells: Effects of Ribonucleoside Triphosphates on the Interaction of the Enzyme with Single-Stranded DNA†

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Received August 9, 1985; Revised Manuscript Received January 17, 1986

ABSTRACT: There are at least four forms of DNA-dependent ATPase in mouse FM3A cells [Tawaragi, Y., Enomoto, T., Watanabe, Y., Hanaoka, F., & Yamada, M. (1984) *Biochemistry 23*, 529–533]. One of these, ATPase B, has been purified and characterized in detail. During the purification of the enzyme, we encountered the difficulties that the enzyme could not be recovered well from the single-stranded DNA–cellulose column and that the enzyme activity was distributed very broadly. The problems were resolved by the addition of ATP in the elution buffer. The ATPase has a sedimentation coefficient of 5.5 S in both high salt and low salt. The enzyme hydrolyzes rNTPs and dATP, but ATP and dATP are preferred substrates. Adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S), 5′-adenylyl methylenediphosphate (AMP-PCP), and 5′-adenylyl imidodiphosphate (AMP-PNP) inhibit the enzyme activity. The enzyme is insensitive to ouabain, oligomycin, novobiocin, and ethidium bromide. A divalent cation ($Mg^{2+} \simeq Mn^{2+} > Ca^{2+}$) as well as a nucleic acid cofactor is required for activity. Poly(dT), single-stranded circular DNA, and heat-denatured DNA were very effective. Native DNA was little effective with an efficiency of 29% of that obtained with heat-denatured DNA. In addition, the enzyme showed almost no activity with poly(dA)·poly(dT) although it showed very high activity with the noncomplementary combination of poly(dT) and poly(dC), suggesting that ATPase B requires single-stranded DNA for activity. ATP altered the affinity of ATPase B for single-stranded DNA. The interaction of the enzyme with DNA was studied by Sephadex G-200 gel filtration assay. In the presence of 0.15 M KCl, almost all of the enzyme applied to the column was coeluted with single-stranded circular *fd* DNA, while in the presence of 1 mM ATP under the same salt condition the amount of the enzyme coeluted with DNA decreased greatly. The effect of dissociation of the enzyme from the single-stranded DNA was not observed with other ribonucleoside triphosphates, CTP, GTP, and UTP.

It has been shown that ATP is required for DNA replication in eukaryotic cells as well as prokaryotes. We have demonstrated an absolute requirement for high levels of ATP for DNA synthesis in isolated nuclei, especially for the synthesis of Okazaki fragments (Enomoto et al., 1981, 1983a). However, the molecular basis of the requirement for high levels of ATP remains to be resolved. In prokaryotic systems, the role of ATP in DNA replication has been related to several proteins that have DNA-dependent ATPase activity by a combination of genetic and biochemical approaches. These proteins include the *rep* protein, which catalyzes the separation of DNA strands, the *Escherichia coli dnaB* gene product, which acts as a "mobile promoter" enabling the primase to synthesize initiator RNAs, and *E. coli* DNA gyrase, which introduces superhelical turns into closed circular DNA. Therefore, the requirement for ATP for DNA replication in eukaryotic cells could be explained by the participation of analogous proteins in the DNA replication, which perform their functions concomitantly hydrolyzing ATP in a DNA-dependent manner.

During this decade, eukaryotic DNA-dependent ATPases have been isolated from mouse myeloma (Hachmann & Lezius, 1976), bovine lymphocyte (Otto, 1977), lily (Hotta & Stern, 1978), human EUE cells (Cobianchi et al., 1979), calf thymus (Assairi & Johnston, 1979), human KB cells (Boxer & Korn, 1980; DeJong et al., 1981), yeast (Plevani et al., 1980), mouse FM3A cells (Hyodo & Suzuki, 1981), rat liver mitochondria (Yaginuma & Koike, 1981), rat hepatoma (Thomas & Meyer, 1982), and monkey CV-1 cells (Brewer et al., 1983). We have also isolated three forms of DNA-dependent ATPase from calf thymus (Watanabe et al., 1981). The lily enzyme has been shown to have helicase activity, and a limited degree of stimulation of DNA polym-

†This work was supported in part by a Grant-in-Aid for Scientific Research and a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.
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0006-2960/86/0425-3239$01.50/0