Disassembly and Characterization of the Nuclear Pore Complex–Lamina Fraction from Bovine Liver Nuclei†

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ABSTRACT: The nuclear pore complex–lamina (PCL), composed of nuclear pore structures attached to fibrous lamina, was isolated from bovine liver nuclei. We found that the highly aggregated PCL was disrupted and 75% of the constituent polypeptides could be solubilized by extraction for 1 h with 2% deoxycholate (DOC) and 3% 2-mercaptoethanol. While some differential solubilization was observed at lower detergent concentrations, all PCL proteins were solubilized equally at 2% DOC. The reducing agent was necessary to achieve maximum dispersal of the PCL and to prevent aggregation of the solubilized proteins. No tightly bound phospholipid or Triton X-100 could be detected in these preparations. Rapid removal of DOC, by dialysis or gel filtration, resulted in aggregation and precipitation of the PCL proteins, but the detergent could be removed by centrifugation through sucrose gradients. The sedimentation profiles indicated that the three major polypeptides, lamins A, B, and C, each sedimented as a single peak with a shoulder of more rapidly sedimenting material, possibly higher oligomeric forms. The sedimentation coefficient of lamins B and C, in the presence and absence of detergent, was 4.5 S. In the presence of DOC, lamin A had a sedimentation coefficient of 5.6 S, but this value was decreased to 4.1 S, when DOC was omitted from the gradient. These studies suggested that lamins B and C do not interact with or bind DOC, while lamin A may bind appreciable amounts of the detergent. The Stokes radii of lamins A, B, and C were found by gel filtration to be 75, 75, and 70 Å, respectively. The molecular weights and frictional ratios estimated from the sedimentation and gel filtration data indicated that the lamins are dimeric, rod-shaped molecules.

The nuclear pores are the most conspicuous morphological feature of the eukaryotic nuclear membrane. They are large structures, 850–1200 Å in diameter, and so numerous that they can cover up to 35% of the surface area of the nucleus (Feldherr, 1972). Electron microscopy (Franke, 1974; Maul, 1977) has shown that the nuclear pore has an elaborate structure that spans the double membrane of the envelope and is anchored to a fibrillar network, the lamina, on the nucleoplasmic side. Recently, a three-dimensional map to a resolution of 90 Å has been determined from electron micrographs by using Fourier averaging methods (Unwin & Milligan, 1982). There is considerable evidence (Franke & Scheer, 1970; Wise et al., 1972; Stuart et al., 1977; Agutter et al., 1976) indicating that the nuclear pore complex is actively involved in the translocation of mRNA and other cytoplasmic exchange processes. Despite the important function postulated for the nuclear pore complex, very little is known of its structure components.

Aaronson & Blobel (1975) succeeded in obtaining a preparation from rat liver nuclear envelopes, which consisted of nuclear pore complexes firmly attached to a filamentous structure, presumably the lamina. This preparation, the pore complex–lamina (PCL),[1] consisted almost exclusively of protein. Three polypeptides, in the 60K–70K molecular weight range, dominated the $NaDodSO_4$–polyacrylamide gel electrophoresis pattern.

Gerace et al. (1975, 1980) later presented evidence, obtained by immunolocalization in the electron microscope, that these polypeptides were constituents of the lamina and not the pore complex itself. These proteins were therefore designated lamins A, B, and C. The lamins have been found to be characteristic of the nuclear envelope of a variety of organisms. Recently Stick & Krohne (1982) also using immunolocalization found that antiserum prepared against the undenatured proteins reacted strongly with both nuclear pores and lamina. Preparations obtained from *Xenopus* (Krohne et al., 1978a,b, 1981) and the clam, *Spisula solidissima* (Maul & Avdalovic, 1980) oocytes, which have nuclei highly enriched in pore complexes and minimal amounts of lamina, consisted of a single M_r 67 000 polypeptide in this molecular weight range. These same three polypeptides are also prominent in the rat liver nuclear matrix (Berezney & Coffey, 1976, 1977), a skeletal structure remaining after extensive extraction of the nuclei, that may represent the site of DNA replication (Pardoll et al., 1980). More recently, it has been suggested (Jackson & Patel, 1982) that the nuclear substructures may be involved in mRNA processing and splicing. Thus, the structural and functional role of these proteins remains controversial.

There have been several interesting biochemical studies (Shelton & Cochran, 1978; Lam & Kasper, 1979a,b; Shelton et al., 1980a; Richardson & Maddy, 1980a,b) of the nuclear envelope proteins, but further work has been hampered by the extreme insolubility of the pore complex–lamina. Although these structures are believed to undergo reversible depolymerization during mitosis (Gerace et al., 1975), it has heretofore not been possible to disassemble the PCL except by using strong denaturants. We report here the solubilization of the pore complex–lamina under relatively mild conditions and the preliminary characterization of the major constituent proteins. Preliminary accounts of these studies have been presented (Havre & Evans, 1981, 1982).

Materials and Methods

The method of Kay & Johnston (1977) was used for the isolation of nuclei from 100 g of bovine liver obtained immediately following slaughter. Nuclear envelopes were pre-

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[1] Abbreviations: PCL, pore complex–lamina; $NaDodSO_4$, sodium dodecyl sulfate; DOC, sodium deoxycholate; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; cmc, critical micelle concentration.

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pared as described by these authors except that the nuclei were suspended in a buffer consisting of 10% sucrose, 0.1 mM $MgCl_2$, and 10 mM triethanolamine, pH 8.5, rather than the Tris buffer used previously and the first of the two DNase incubations was carried out for 45 min instead of 15 min. The procedure for the isolation of the pore complex–lamina (PCL) was essentially that described by Dwyer & Blobel (1976). For this procedure, the nuclear envelopes were suspended in 0.17 mL of 0.1% Triton X-100/g of liver processed. The purified PCL was then washed with 10 mL of 0.1% Triton X-100 in the suspension buffer described above (except adjusted to pH 7.5).

The DOC extract was prepared by suspending the PCL from 100 g of bovine liver (about 1.5 mg) in 4.0 mL of the suspension buffer given above (pH 8.5) with a Potter-Elvehjem tissue grinder with a Teflon pestle to disperse the pellet. An equal volume of 4% sodium deoxycholate in water was added, and the suspension was made 3% in 2-mercaptoethanol. Following incubation for 1 h at 22 °C, the extract was centrifuged at 40 000 rpm in a Beckman L5-65 ultracentrifuge with a Ti 65 rotor (100000*g*) at 20 °C. The supernatant is referred to as the DOC extract. Phenylmethanesulfonyl fluoride in 2-propranol (10 mg/mL) was added to a final concentration of 2 mM. The DOC extract was stored at −20 °C.

Protein was determined by Goldin's modification (1977) of the Lowry procedure in which $NaDodSO_4$ was added to the reagent. DNA (Ceriotti, 1952) and RNA (Munro & Fleck, 1966) were analyzed after separation by the Schmidt & Thannhauser (1945) procedure as modified by Munro & Fleck (1966). Phospholipid was extracted (Folch et al., 1956) and determined by the Ames method (1966). The chemical composition of the nuclei and nuclear envelopes measured when these procedures were used was very similar to that reported by Kay & Johnston (1977) for rat liver.

$NaDodSO_4$–polyacrylamide gel electrophoresis was carried out on 1.5 mm thick slabs with the buffer system described by Laemmli (1970). A 3.75% spacer gel and 12% resolving gel were used routinely. The gels, stained with Coomassie brilliant blue R, were scanned with a Zeineh soft laser scanning densitometer. The gain was set to full scale on the darkest band on the gel, and all lanes were then scanned at these settings. Gels containing known amounts of standard proteins were also scanned and used to construct standard curves that were linear to 10 μg. Samples were concentrated for electrophoresis by coprecipitating with 1.25 mg of dextran (Sigma Chemical Co.; M_r 80 000) by the addition of 2 volumes of absolute ethanol. After standing overnight at −20 °C, the precipitate was collected by low-speed centrifugation, dissolved in 3% $NaDodSO_4$, 5% 2-mercaptoethanol, 0.063 M Tris-HCl, pH 6.8, 1 mM EDTA, 0.003% bromphenol blue, and 20% glycerol and heated for 3 min at 100 °C (Richardson & Maddy, 1980a,b).

Two-dimensional gel electrophoresis was carried out in our laboratory by Terrell Van Aken. Isoelectric focusing was essentially as described by O'Farrell (1975) with 2.5 mm × 135 mm cylindrical polyacrylamide gels (4%) in urea. Focusing was at a constant voltage of 400 V for 6 h. The gels were then either stained with Coomassie blue or used for two-dimensional electrophoresis. The pH gradient was measured by using standard proteins of known isoelectric point (Sigma, kit) and by measuring the pH of gel slices extracted into CO_2-free water.

Sucrose gradient centrifugation was carried out with 10–30% sucrose (w/v) in 0.05 M Tris-HCl, pH 7.5, 0.10 M NaCl, and, when indicated, deoxycholate ranging from 0 to 2%. Centrifugation was typically at 50 000 rpm (234000*g*) at 20 °C in a Beckman L565 ultracentrifuge with an SW 50.1 rotor. The positions of the individual proteins in the gradient were determined by analytical $NaDodSO_4$ gel electrophoresis and where necessary by scanning densitometry of stained gels. The sucrose concentration was measured by refractometry. The sedimentation coefficients were calculated by using the method of McEwen (1967).

The Stokes radius was determined by gel filtration (Ackers, 1964) on a Bio-Gel A-5m column, 10.0 cm × 1.8 cm^2, equilibrated with 1% deoxycholate, 0.01 M Tris-HCl, pH 8.5, and 1 mM EDTA. The column was eluted at a flow rate of 1.33 mL/h. The column effluent was collected, and the protein was precipitated by the ethanol–dextran method described above. The fractions were analyzed by $NaDodSO_4$ gel electrophoresis, and the amount of each lamin was determined by scanning the stained gel. The column was calibrated with five proteins of known molecular dimensions (Ackers, 1964; Sober & Harte, 1968), and the Stokes radius was determined from this standard curve. The molecular weight and frictional ratios of the particles were estimated as described by Siegel & Monty (1966).

To measure residual Triton X-100 bound to the extracted proteins, nuclear envelopes (2 mL, 1 mg/mL) were extracted with 40 mg of [H^3]Triton X-100 (2.5 μCi/mg). The remaining steps in the preparation of the pore complex–lamina and its extraction with DOC–mercaptoethanol were the same as described above. Isotopically labeled samples were counted in 10 mL of Aquasol (New England Nuclear) in a Packard Tricarb 400-C liquid scintillation counter. The partial specific volumes of the proteins in the absence of detergent were assumed to be 0.73 (Cantor & Schimmel, 1977). The expression given by Smigel & Fleischer (1977) was used to estimate the effect of bound detergent on the partial specific volume of the particle. The partial specific volumes of the detergents used in these calculations were 0.908 cm^3/g for Triton X-100 and 0.778 cm^3/g for deoxycholate (Makino et al., 1973).

Results

As expected, the Dwyer & Blobel (1976) procedure devised for the isolation of pore complex–lamina from rat liver worked equally well for bovine liver. The resulting preparation was insoluble, fibrous material consisting almost exclusively of protein. Three major polypeptides, representing 43% of the total protein, were found on $NaDodSO_4$ gels (Figure 2, lane NPL). The molecular weights of these polypeptides were 78 000, 73 000, and 68 000 corresponding to the values obtained previously (Krohne et al., 1978a,b; Lam & Kasper, 1979b) for lamins A, B, and C, respectively. The isoelectric points were 6.5 for lamin A, 5.7 for lamin B, and 6.5 for lamin C. Moreover, the pattern obtained by two-dimension gel electrophoresis (data not shown) was very similar to that obtained from rat liver nuclear envelope preparations (Shelton & Egle, 1979). The relative amounts of the lamins was quite constant from preparation to preparation. Typical values were 16% lamin A, 41% lamin B, and 43% lamin C. The remaining protein of the PCL was distributed among numerous other polypeptides that were present in much smaller amounts than the lamins.

The pore complex–lamina was extracted with a variety of agents, alone and in combination, in an attempt to solubilize the constituent proteins. High and low ionic strength buffers, metal ions, detergents, chelating agents, and reducing agents were all used in these experiments. For the most part, only denaturants such as urea and $NaDodSO_4$ produced significant

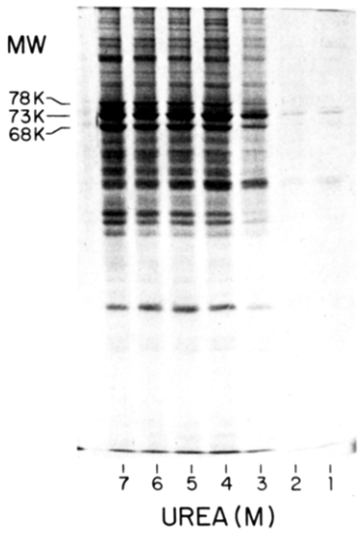

FIGURE 1: Urea extraction of the pore complex–lamina fraction. Pore complex–lamina fraction (0.6 mL, 0.27 mg/mL) was incubated in 10 mM Tris-HCl, pH 7.6, and the indicated concentrations of urea for 20 min at room temperature. The extract was centrifuged for 5 min at 3000*g*. The supernatants from the low-speed spin were then centrifuged for 1 h at 100000*g*. The high-speed supernatant was concentrated and analyzed (data shown in figure) along with the pellets from each centrifugation step (data not shown) by $NaDodSO_4$ gel electrophoresis as described under Materials and Methods. At high urea concentrations, where all of the material was solubilized, 12 μg of protein was applied to the gel. The molecular weights (MW) of the major polypeptides are also shown.

solubilization. The effect of increasing urea concentration is shown in Figure 1. Virtually complete solubilization of the PCL could be achieved with 4 M urea. However, we found that low concentrations of sodium deoxycholate (DOC), in the presence of 2-mercaptoethanol, were remarkably effective in dissociating and solubilizing the pore complex–lamina.

Extraction of the PCL with increasing concentrations of DOC caused progressive solubilization of the protein as indicated by both Lowry analysis and $NaDodSO_4$ gel electrophoresis. When the PCL was incubated with 2% DOC and 3% 2-mercaptoethanol for 1 h at 20 °C and then centrifuged for 5 min at 3000*g*, 90% of the PCL proteins remained in the supernatant. Since these centrifugation conditions were more than sufficient to completely pellet the intact pore complex–lamina, this result indicated that this combination of detergent and reducing agent had completely disrupted the noncovalent interactions which maintain the aggregate. The 10% of the protein which pelleted under these conditions was macroscopically fibrous, resembling the starting material. The polypeptide composition of this insoluble material was indistinguishable from that of the PCL when analyzed by $NaDodSO_4$ gel electrophoresis.

When the DOC extracts were centrifuged for 1 h at 100000*g*, a generally accepted criterion for solubilization of membrane proteins, 75% of the PCL protein remained in the supernatant. Even at the highest concentrations of DOC, 25% of the protein remained insoluble.

In the experiment shown in Figure 2, no protein remained in the 100000*g* supernatant in the absence of DOC (Figure 2, lane 0%). As the concentration of DOC increased, there was a corresponding increase in the amount of protein solubilized. The concentration of 2-mercaptoethanol was held at 3% in this experiment. The amount of each lamin that remained in the 100000*g* supernatant was determined by scanning the stained gels (Figure 2B). At low concentration of DOC there was some small differential solubilization of the lamins. The 73K species, lamin B, was the most readily

Table I: Effect of Protein Concentration on the DOC Extraction of the Pore Complex–Lamina Fraction[a]

protein concentration (mg/mL)	DOC/protein (w/w)	protein solubilized (%)
0.26	77	72.5
0.46	43	58.9
0.72	28	53.2
1.20	16	42.3
1.55	13	47.2

[a] Pore complex–lamina fraction at the indicated concentrations was extracted with deoxycholate for 1 h at room temperature (see Materials and Methods). The extracts were centrifuged for 1 h at 100000*g* (20 °C). Both supernatants and pellet were precipitated with ethanol–dextran and assayed for protein.

Table II: Effect of 2-Mercaptoethanol on the DOC Extraction of the Pore Complex–Lamina Fraction[a]

fraction	protein (%)	
	2-mercapto-ethanol absent	2-mercapto-ethanol present
3000*g* pellet	23.4	7.4
100000*g* pellet	25.5	16.0
100000*g* supernatant	51.1	76.6

[a] Pore complex–lamina at a protein concentration of 0.15 mg/mL was incubated with deoxycholate (see Materials and Methods) with or without 3% 2-mercaptoethanol. The extracts were then centrifuged at 3000*g* for 20 min. The supernatants from the low-speed centrifugation step were then centrifuged for 1 h at 100000*g* (20 °C). The supernatants and pellets were precipitated and analyzed by $NaDodSO_4$ gel electrophoresis to determine the protein concentration and distribution of PCL polypeptides.

solubilized, although only slightly greater concentrations of the detergent were required for the extraction of the 68K lamin C. However, no significant solubilization of 78K lamin A occurred until a DOC concentration of 0.6% was reached. Maximum solubility of the lamins, under these conditions, occurred at 2% DOC. At this concentration, the relative amounts of the lamins present in the extract was found to be the same as that in the PCL. Moreover, all of the polypeptides in the PCL were solubilized equally as indicated by electrophoresis of more concentrated samples of the 2% DOC extract. The resulting gel pattern (Figure 2A, lane DOC) appeared to be virtually identical with that given by unextracted pore complex–lamina (lane NPL). The PCL and its DOC extracts were also identical on two-dimensional electrophoresis (data not shown).

Effect of Protein Concentration on the Efficiency of Extraction. The concentration of protein relative to that of the detergent was also considered in determining the optimal conditions for solubilizing these proteins. In this experiment, the concentrations of DOC (2%) and 2-mercaptoethanol (3%) were held constant while the concentration of PCL was varied. The amount of protein remaining in the supernatant fraction after centrifugation for 1 h at 100000*g* was determined by the modified Lowry procedure. The results, summarized in Table I, indicated that the fraction of the total protein solubilized increased from 47% at 1.6 mg/mL PCL protein to 75% at 0.3 mg/mL protein.

Effect of 2-Mercaptoethanol on the Efficiency of Extraction. Initially, 2-mercaptoethanol was included in the extract because it greatly facilitated handling of the PCL which otherwise had a pronounced tendency to stick to glass surfaces. We subsequently found that the reducing agent altered the extent of aggregation of the PCL proteins. As shown in Table

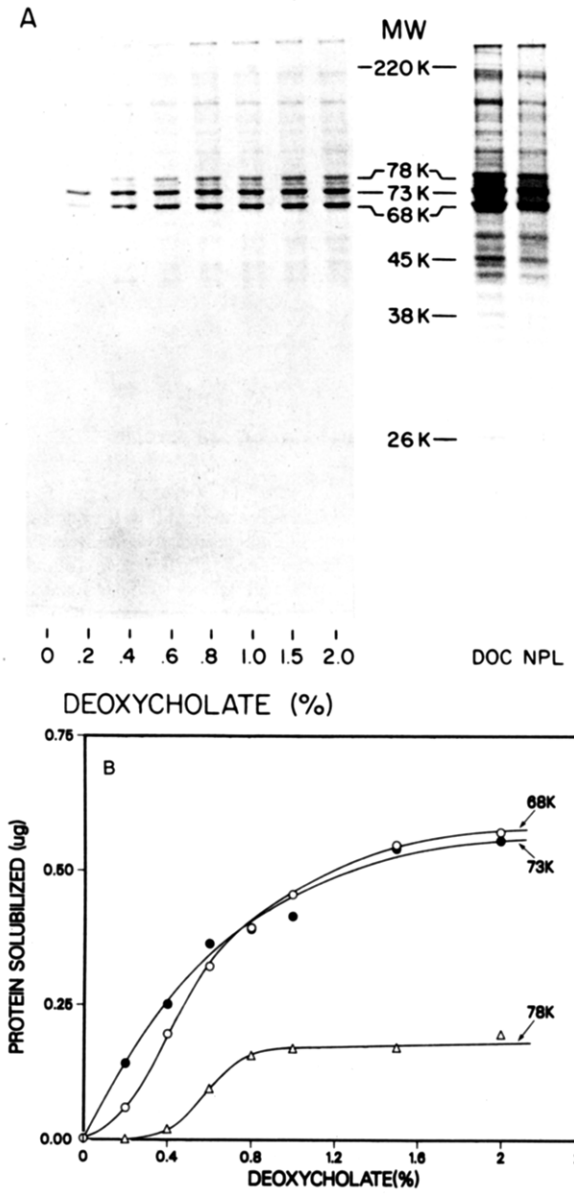

FIGURE 2: Sodium deoxycholate–2-mercaptoethanol extraction of the pore complex–lamina fraction. Pore complex–lamina fraction was extracted at a protein concentration of 0.17 mg/mL as described under Materials and Methods. The extract was then centrifuged for 1 h at 100000*g* (20 °C). The supernatants were precipitated, and 1 μg was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (A). Three times the amount of suspension from the 2% DOC extract (lane DOC) as well as unextracted pore complex–lamina (lane NPL) was also analyzed. The protein concentrations (B) of the 78 000-dalton lamin A (△), the 73 000-dalton lamin B (●), and the 68 000-dalton lamin C (○) were determined by scanning the stained gels. Lamin C comigrates exactly with bovine serum albumin, one of the standards used to calibrate the gel. The distribution of polypeptides is the same as that shown in Figure 1, but since smaller amounts of protein were applied to the gel, many minor bands are not visible in the photograph.

II, 77% of the total PCL protein was solubilized when 3% 2-mercaptoethanol was present in the extract. About 7% of the total protein was found in the pellet from the low-speed centrifugation step, while an additional 16% was brought down by centrifugation at 100000*g*. In the absence of 2-mercaptoethanol, the amount of protein pelleted by low-speed centrifugation increased 3-fold. There was also an appreciable increase in the pelleted material in the high-speed centrifugation step, and only about half of the total PCL protein was solubilized.

Comparison of the unextracted PCL with the high- and low-speed supernatants by $NaDodSO_4$ gel electrophoresis

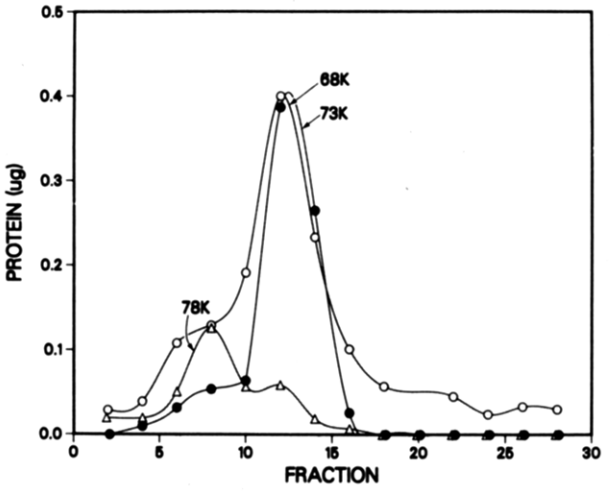

FIGURE 3: Sucrose gradient centrifugation of the deoxycholate–2-mercaptoethanol extract of the pore complex–lamina fraction. A 0.4-mL aliquot of the DOC extract (0.12 mg/mL) was layered onto a 5-mL sucrose gradient containing 0.2% deoxycholate and centrifuged as described under Materials and Methods. The protein from each 0.25-mL fraction was precipitated with ethanol–dextran and resuspended in 0.07 mL, and 0.05 mL was analyzed by $NaDodSO_4$ gel electrophoresis. The gels were scanned to determine the amount of 78 000-dalton lamin A (△), 73 000-dalton lamin B (●), and 68 000-dalton lamin C (○) present in each fraction.

revealed no differences in the polypeptide compositions (data not shown). These results suggested that 2-mercaptoethanol had reduced the state of aggregation of the PCL proteins.

Thus, the optimal conditions for the solubilization of the PCL proteins were extraction for 1 h at 20 °C with 2% DOC–3% 2-mercaptoethanol and a ratio of detergent to protein of at least 75 to 1. The extract was then centrifuged for 1 h at 100000*g*, and the supernatant containing the solubilized protein was used in all subsequent experiments.

Determination of the Sedimentation Coefficient of the Solubilized Proteins. To obtain an estimate of the size distribution of the solubilized proteins, the DOC extract was sedimented through sucrose gradients prepared in 0.2% DOC. After centrifugation, the gradient fractions were analyzed by $NaDodSO_4$ gel electrophoresis. The gradient profile showing the distribution of lamins (Figure 3) was obtained by scanning the resulting $NaDodSO_4$ gels. Lamin A sedimented most rapidly. The major peak of lamin A corresponded to a species with an $s_{20,w}$ of 5.6 S although there was a significant shoulder of this material which cosedimented with lamins B and C. Most of lamin C sedimented with a coefficient of 4.5 S, but in this case there was a shoulder of more rapidly sedimenting material. Lamin B appeared to be somewhat more homogeneous. It sedimented as a single symmetrical species with a sedimentation coefficient of 4.5 S, although there was a small amount of a more rapidly sedimenting species here as well. Other minor species not shown in Figure 3 included polypeptides with molecular weights of 220K and 150K (present in fractions 1–5), 190K, 130K, 96K, and 87K (fractions 7–9), 41K, 43K, and 47K (fraction 11), and 38K (fraction 13).

To further characterize the sedimentation behavior of the lamins, and to obtain a more reliable estimate of the sedimentation coefficients, the distance sedimented was measured as a function of centrifugation time in the presence and absence of DOC (Figure 4). For each time point the entire gradient profile was analyzed by $NaDodSO_4$ gel electrophoresis, and the peak fraction of each protein was plotted. In the presence of deoxycholate the expected linear relationship between distance sedimented and centrifugation time was observed. Lamins B and C cosedimented with an $s_{20,w}$ of 4.5 as deter-

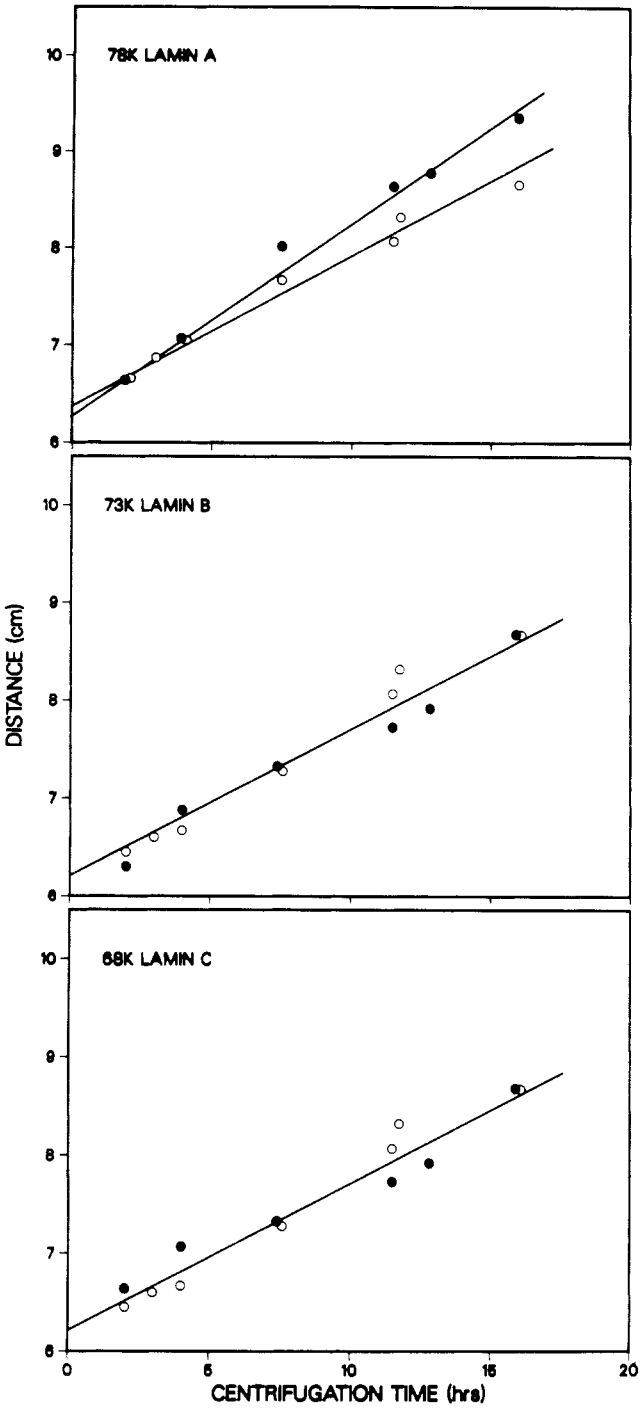

FIGURE 4: Analysis of the rate of sedimentation of the solubilized lamins in sucrose gradients. A series of sucrose gradient centrifugation runs were carried out at 234000g (20 °C) for various periods of time. A 0.40-mL aliquot of the DOC extract containing 50 μg of protein was applied to each gradient. The gradients were prepared either without deoxycholate (○) or with 0.2% deoxycholate (●). A complete analysis of each gradient profile by $NaDodSO_4$ gel electrophoresis was carried out as described in the legend to Figure 3. The distance from the center of rotation to the position of the maximum concentration of each lamin is plotted against centrifugation time.

mined from a linear least-squares fit of these data. The sedimentation of lamin A, the 78K species, was significantly more rapid with an $s_{20,w}$ of 5.6. Very similar sedimentation profiles were obtained under a variety of conditions, when 0.05–2.0% deoxycholate, 10–50 mM Tris-HCl at both pH 7.5 and pH 8.5, 0–100 mM NaCl, and 0–3% 2-mercaptoethanol were present in the gradient. However, when the DOC extract was layered onto a gradient which contained no DOC, somewhat

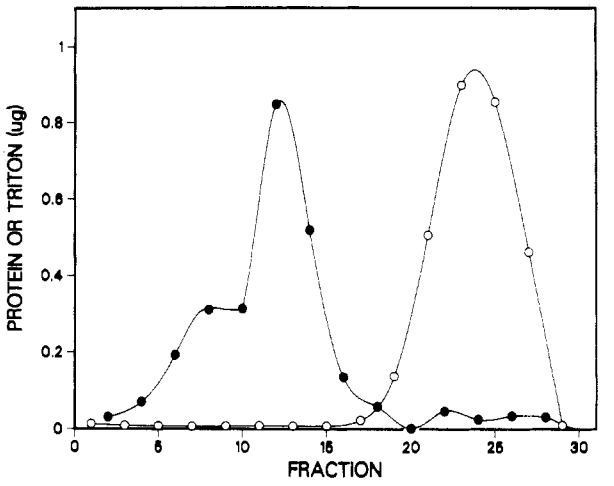

FIGURE 5: Determination of residual Triton bound to the solubilized pore complex–lamina proteins. The nuclear pore complex–lamina fraction was prepared as described under Materials and Methods by using isotopically labeled Triton X-100. The tritium-labeled DOC extract was layered onto a 10–30% sucrose gradient containing 0.2% DOC and centrifuged for 16 h at 234000g. The odd numbered fractions were counted, and the amount of Triton X-100 (○) was calculated from the specific radioactivity. The protein (●) in the even numbered fractions was determined by $NaDodSO_4$ gel electrophoresis.

different results were obtained. The sedimentation of lamins B and C was again linear, and the slope of the progress curve, and thus the sedimentation coefficients, was indistinguishable from that obtained for these species when DOC was present in the gradient. In contrast, the progress curve for lamin A indicated that this species was sedimenting somewhat more slowly than lamins B and C in the absence of DOC. The sedimentation coefficient of lamin A was calculated from the slope of the progress curve to be 4.1 S.

Determination of Tightly Bound Lipid and Detergent. No phospholipid could be detected in purified preparations of the nuclear pore complex–lamina. Given the sensitivity of the methods used for analysis, we can conclude that there could have been no more than 3.5% phospholipid associated with the PCL. Triton X-100 was used in the isolation of the PCL, so the possibility existed that residual detergent remained bound to the protein. Therefore, nuclear envelopes were extracted with [^{3}H]Triton X-100 and then carried through the remainder of the preparation. A very small fraction (1%) of the Triton used in the extraction was carried through to the PCL, although this residual detergent could be cleanly separated from the PCL proteins (Figure 5) by sucrose gradient centrifugation. We calculated that following centrifugation, Triton X-100 constituted no more than 1.2% of the material solubilized by deoxycholate. Therefore, the detergent was not tightly bound to the protein in appreciable amounts or required for solubilization of the complex. Measurement of the DOC bound to these proteins was more difficult. Attempts to remove the latter detergent by dialysis or chromatography on Sephadex G-25 resulted in immediate precipitation of the solubilized material. However, as discussed above, if the extracted proteins are sedimented through sucrose gradients that contained no deoxycholate, the bulk of the detergent could be removed without inducing aggregation of the proteins. Consequently, it should have been possible, in principle, to determine if there was residual DOC bound to these proteins in the same way Triton X-100 binding was determined. The method is much less sensitive, however, because of the large amounts of DOC present in the extract. We could calculate that the solubilized lamins, collectively, could have no more than 10% bound deoxycholate. Once we have succeeded in separating these

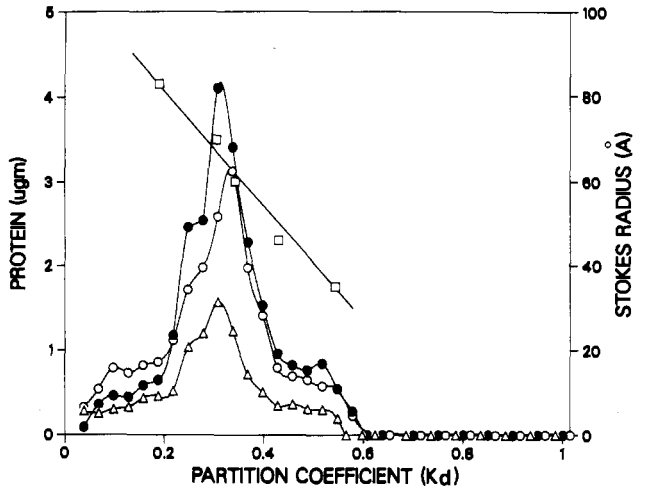

FIGURE 6: Gel filtration chromatography of the solubilized lamins. Gel filtration chromatography of a 1-mL aliquot of the DOC extract of the PCL fraction (0.12 mg/mL) was carried out on a Bio-Gel A-5m column as described under Materials and Methods. The concentration of the 78 000-dalton lamin A (△), 73 000-dalton lamin B (●), and 68 000-dalton lamin C (○) in each fraction was determined by quantitative $NaDodSO_4$ gel electrophoresis. The proteins used to calibrate the column were, reading left to right across the plot, thyroglobulin (83 Å), β-galactosidase (70 Å), catalase (52 Å), alcohol dehydrogenase (46 Å), and bovine serum albumin (35 Å), and the partition coefficients were calculated as described above.

proteins, it will be possible to measure detergent binding to the individual lamins.

Gel Filtration of the Solubilized PCL Proteins. The DOC extract was chromatographed on a Bio-Gel A-5m gel filtration column equilibrated with 1% DOC. The elution profile shown in Figure 6 was obtained by analyzing the effluent by $NaDodSO_4$ gel electrophoresis. The bulk of the three lamins eluted together from the column, although lamin C was retained somewhat longer than either lamin A or lamin B. Each protein peak had a significant leading shoulder that may be indicative of higher oligomeric forms. There was also some low level trailing of each species which may represent smaller molecular weight species or, less likely, interaction of the proteins with the agarose beads. Proteolysis which occurred during these long runs could be eliminated by the addition of phenylmethanesulfonyl chloride to a concentration of 2 mM to DOC extracts. An estimate of the size of the solubilized lamins was obtained by calibrating the column with globular proteins of a known Stokes radius. The elution position of these standards was independent of the presence of DOC in the elution buffers. The Stokes radius of the major peaks of lamins A and B was found to be 75 ± 10 Å while lamin C had a radius of 70 ± 10 Å.

Discussion

The pore complex–lamina is a macroscopic aggregate consisting of a network of nuclear pores interconnected by fibers derived from the lamina. The insolubility of this material has made its characterization difficult, and so we have tested a large number of agents in an attempt to disrupt the aggregate and solubilize the component proteins. Strong denaturants such as urea and sodium dodecyl sulfate effectively solubilize nuclear envelope proteins. We have found in these studies that moderate concentrations of urea (4 M) completely solubilized the PCL fraction. However, we were attempting to find conditions that would solubilize these proteins without disruption of their tertiary structure. Nonionic detergents and bile salts do not usually induce denaturation characteristic of anionic and cationic detergents (Makino et al., 1973).

Nonionic detergents such as Triton X-100 are generally not very effective in disrupting protein interactions (Makino et al., 1973; Helenius et al., 1979), a likely prerequisite for the disassembly of the pore complex–lamina fraction. Triton X-100 was used in the preparation of PCL fraction and did not solubilize the major structural proteins. In contrast, deoxycholate has been successfully used to disrupt networks of fibrous proteins although some protein interactions are resistant to bile salts as well (Helenius & Simons, 1975). Moreover, DOC is a relatively mild detergent. There are numerous cases (Snary et al., 1974; Lefkowitz et al., 1972; Helenius & Simons, 1971; Allan & Crumpton, 1971; Lu & Coon, 1968; Ito & Sato, 1968; Spatz & Strittmatter, 1971) in which the structure and biological functions of membrane proteins solubilized in DOC have been preserved. The selection of DOC was also prompted, in part, by an earlier report by Kirschner et al. (1977) which showed by electron microscopy that the external surface of mouse liver nuclei was stripped of recognizable pore structures by a mixture of Triton X-100 and deoxycholate. Also, when our preliminary results were reported (Havre & Evans, 1981), an observation of Long et al. (1979) was brought to our attention. These latter authors found that DOC removed two proteins of molecular weights of 62 000 and 66 000, possibly two of the lamins, from DNA-depleted nuclei. No prior attempts have been made to use DOC to solubilize the proteins of the pore complex–lamina preparations.

The combination of deoxycholate (2%) and 2-mercaptoethanol (3%) proved to be remarkably effective, causing disruption of 93% of the PCL aggregate. Of the total PCL protein, 77% appeared to be small soluble oligomers, while 16% sedimented completely during centrifugation at 100000*g*. Any particle with an $s_{20,w}$ of 22 or greater would pellet under these conditions. There was no apparent difference in the polypeptides present in the insoluble fraction, the 3000*g* supernatant (disrupted PCL), or the 100000*g* supernatant (soluble PCL proteins), so that the differential solubility of these fractions did not correspond to distinct classes of polypeptides. However, repeated extraction of the pelleted material did not significantly increase the yield of soluble PCL proteins.

Increasing the DOC concentration above 1.5% gave no appreciable increase in either the disruption of the PCL or the solubilization of the constituent proteins. The critical micelle concentration of DOC under these conditions is likely to be below 0.2% (Helenius & Simons, 1971). However, the DOC micelles are small, and the monomer concentration continues to increase (Helenius et al., 1967), probably accounting for the increased solubilization observed above the cmc. There was some slight differential solubility of the lamins at low concentrations of DOC. However, as we were interested in solubilizing the maximum amount of all of the constituent proteins, we elected to use 2% DOC in all subsequent experiments.

Increasing the ratio of deoxycholate to protein by reducing the concentration of protein significantly increased the fraction of PCL disrupted and solubilized. The maximum solubilization (77%) was observed at a protein concentration of 0.3 mg/mL corresponding to a DOC to protein ratio of 77. While this concentration of protein represents the lower practical limit for the types of experiments conducted here, it may be possible that greater solubilization could have been achieved by further reduction of protein concentration.

The major effect of 2-mercaptoethanol was on the state of aggregation of the PCL proteins. While the thiol produced

Table III: Properties of Solubilized Lamins

	lamin A	lamin B	lamin C
molecular weight of polypeptides[a]	78 000	73 000	68 000
isoelectric point	6.5	5.7	6.5
$s_{20,w}$			
DOC present	5.6	4.5	4.5
DOC absent	4.1	4.5	4.5
Stokes radius (Å)	75.0	75.0	70.0
frictional ratio[b]	2.02	2.17	2.08
calculated particle weights			
DOC present[b,c]	177 000	142 000	132 000
DOC absent[d]	111 000	142 000	132 000

[a] Determined by electrophoresis on calibrated $NaDodSO_4$-polyacrylamide gels. [b] Calculations assume a partial specific volume of 0.73. [c] Calculated by the method of Siegel & Monty (1966). [d] Calculated from the expression $M_2 = (s_{20,w,2}/s_{20,w,1})^{3/2} M_1$ where the subscripts 1 and 2 refer to values in the presence and absence of DOC, respectively.

a modest increase in the fraction of PCL disrupted (*3000g* supernatant), the fraction of the PCL protein existing as small, soluble oligomers (*100000g* supernatant) was appreciably increased. In this regard, Shelton & Cochran (1978) and Lam & Kasper (1979a,b) have reported the formation of oligomers containing two or more subunits upon oxidation of the lamin sulfhydryl residues. Our results support the contention that disulfide bonds play a role in maintaining the nuclear envelope structure.

We have begun characterization of the lamins (Table III), the major components of the PCL fractions. Although the bovine PCL has not been prepared previously, the identification of the three polypeptides with the lamins isolated from other species is unambiguous. This assignment was based on the following considerations: (1) The method of preparation of the PCL was the same as that used for rat liver preparation. (2) The amounts of these three polypeptides relative to each other and to other proteins in the PCL or nuclear envelope preparations was the same as that observed for rat liver nuclei. No other polypeptides were present in comparable amounts that could be confused with the lamins. (3) The molecular weights, the isoelectric points, and the two-dimensional gel electrophoresis of rat liver and bovine liver PCL preparations were very similar including the presence of small amounts of characteristic isoelectric variants of lamins C and B.

The molecular weights of the solubilized lamins were estimated from the $s_{20,w}$ and Stokes radius (see Table III) as described by Siegel & Monty (1966). In the presence of deoxycholate, the major components of lamins B and C were calculated to be 142 and 132 k daltons, respectively, whereas the bulk of lamin A behaves as a larger particle with a molecular weight of 177K. The frictional ratios ranged from 2.0 to 2.1, indicating that the lamin molecules are elongated. For these calculations a partial specific volume of 0.73, a value typical of proteins, was assumed. However, the presence of bound phospholipid or Triton, which have very different partial specific volumes, would appreciably alter the calculated molecular weights and frictional ratios. Analysis of the PCL fraction showed that the phospholipid content was below the level of detection (<4%). Since Triton X-100 was used in the preparation of the PCL fraction and since this material was subsequently extracted with DOC, the presence of residual phospholipid associated with the lamins is very unlikely. Similarly, no residual Triton X-100 could be detected (Figure 5), so that there was less than 1.3% of this detergent bound to the lamins. If we make the most conservative assumption that there was in fact 1.3% Triton X-100 associated with the total protein and that all of this detergent is bound to just one of the three lamins, we can calculate the partial specific volume from the weight fractions of protein and detergent. If all of the Triton X-100 was bound to lamin A, the calculated molecular weight would increase 11%. If on the other hand, the detergent was bound exclusively to either lamin B or lamin C, which are present in greater amounts, the molecular weight would increase only 4%. The calculation of the frictional ratios is much less sensitive to small changes in partial specific volume.

The effect of bound DOC is more difficult to assess since the measurements were made in the presence of this detergent. However, the partial specific volume of DOC (0.778) is very similar to that of protein. Thus, while the bound detergent would of course contribute to the mass of the particle, an underestimate of the size, due to an incorrect partial specific volume, is unlikely.

When DOC was removed rapidly by either dialysis or gel filtration, the solubilized lamins precipitated. However, the bulk of the detergent can be removed by centrifugation through sucrose gradients so that the detergent is not required to maintain the solubility of the lamins. It was not possible to use the above approach to determine the molecular weight of the lamins in the absence of detergent, since the gel filtration used to estimate the Stokes radius could not be conducted if DOC was omitted from the elution buffer. The sedimentation behavior of lamins B and C was the same in the presence and absence of DOC, so it is not likely that the detergent interacts strongly with these species or contributes significantly to the size of the particles. We conclude, therefore, that the molecular weights of lamins B and C are the same in the presence and absence of deoxycholate. In contrast, lamin A sediments more slowly in the absence of DOC. Thus, in the presence of detergent the $s_{20,w}$ is 5.6 S, but this value decreased to 4.1 S when the detergent was removed. The molecular weight can be rougly estimated (Table III), by using the expression of Martin & Ames (1961), to be 111K in the absence of DOC. The partial specific volume is assumed to be the same in the presence and absence of DOC, an assumption that is approximately valid (within 10%) because of the similarity of the partial specific volumes of DOC and protein. The decrease in the molecular weight of lamin A is probably largely due to the removal of detergent as the particle sedimented through gradients lacking deoxycholate. Moreover, the molecular weight of lamin A estimated in the presence of DOC was significantly larger than that predicted for a dimer of 78K subunits, also supporting the suggestion that the lamin A particle has bound DOC. The change in sedimentation coefficient may also be due in part to a destabilization and partial dissociation of the lamin A dimer on removal of the detergent. DOC binding to lamin A may suggest that the surface of this protein is relatively hydrophobic. However, most of the bound detergent was removed when the protein was centrifuged through sucrose. Some authors (Shelton et al., 1980b) have argued that lamin C, although present in the nuclear envelope preparations from most species, is derived from lamin A by limited proteolysis. If this finding is correct, the data presented here suggest that the hydrophobicity of lamin A must in large part reside with the 10K fragment excised in the formation of lamin C.

The calculated molecular weights of the lamins suggest that these species are dimeric, although we cannot be sure until we have separated the individual proteins whether these are homodimer or heterodimers composed of more than one type of lamin. The difference in sedimentation behavior of lamin A and elution position on the agarose column of lamin C argue

against heterodimers. In this regard, in situ cross-linking studies (Shelton & Cochran, 1978; Lam & Kasper, 1979a,b) of the proteins embedded in the nuclear envelope indicate that these proteins form homooligomers. The number of copies of the polypeptide chains was found to be different in these oligomers, suggesting that the cross-linked sulfhydryl residues are located at different subunit interfaces than those involved in the particles isolated by DOC extraction. In view of the complexity of these structures, it is likely that many different types of subunit interaction occur within the pore complex–lamina proteins.

In summary, the solubilized lamins appear to be dimeric, rod-shaped molecules, although there is evidence for the formation of higher oligomeric forms. The lamins have little or no bound phospholipid or Triton X-100. Lamins B and C do not appear to interact strongly with DOC, whereas the lamin A particle may bind substantial amounts of this detergent. The solubility of all of the lamins was maintained in the absence of DOC, if the detergent was removed slowly by sucrose gradient centrifugation.

Registry No. DOC, 302-95-4.

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Investigation of Binding between recA Protein and Single-Stranded Polynucleotides with the Aid of a Fluorescent Deoxyribonucleic Acid Derivative†

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ABSTRACT: The availability of ϵDNA, a fluorescent ssDNA derivative, has made it possible to examine quantitatively the interactions between recA protein and single-stranded polynucleotides. Fluorescence titrations of ϵDNA with recA protein and vice versa establish that each recA protein monomer covers 5.5 ϵDNA nucleotides and that the dissociation constant of the recA–ϵDNA complex is 10 nM. Fluorescence titrations of recA protein–ϵDNA mixtures with poly(dT) establish that each recA protein monomer covers 5.1 poly(dT) nucleotides and that the dissociation constant of the recA–poly(dT) complex is 0.03 nM. Observations on how the addition of ssDNA affects the fluorescence of recA protein–ϵDNA mixtures establish that the dissociation constant of the recA–ssDNA complex exceeds 20 μM. Stopped-flow kinetics in which excess recA protein binds to ϵDNA indicate that $k_2 = 6 \times 10^6$ M^{-1} s^{-1} for the process. A more approximate kinetic technique indicates that recA protein binds to ϵDNA at least one-tenth as fast as to poly(dT); the rate constant for dissociation of recA–ϵDNA exceeds that for recA–poly(dT) by at least 30-fold. ϵDNA is proven to be a versatile reagent for studying single-stranded polynucleotide–protein interactions. Not only can its own complexes with protein be investigated but also, under suitable circumstances, it can be used as a fluorescent probe to explore complexes incorporating nonfluorescent polynucleotides.

ϵDNA,[1] a highly fluorescent modified form of ssDNA, is readily prepared by treating ssDNA with chloroacetaldehyde (Lee & Wetmur, 1973; Silver & Fersht, 1982). The fluorescence of an ϵDNA solution is markedly enhanced by the addition of recA protein; subsequent introduction of ATP or ATPγS at suitable concentrations produces a further substantial rise in fluorescence. These fluorescence changes are useful for investigating the properties of recA protein. They have enabled us to establish tentative values for the stoichiometry governing the binding of recA protein to ϵDNA under various reaction conditions and to confirm that ATP and ATPγS bind to the recA–ϵDNA complex in a highly cooperative process (Silver & Fersht, 1982).

We describe here a more quantitative evaluation of the interactions between recA protein and ϵDNA and show how it is possible to use ϵDNA to probe the nature of the interactions between recA protein and other, nonfluorescent, single-stranded polynucleotides. All these experiments have been performed in the absence of any nucleoside triphosphates. We expect them to guide further efforts to study the far more complex systems incorporating NTP's. They also emphasize further the potential utility of ϵDNA for investigating protein–ssDNA interactions in other systems.

Experimental Procedures

Materials

We employed the same ϵDNA sample used previously (Silver & Fersht, 1982). ssDNA was prepared by heat denaturing highly polymerized calf thymus dsDNA (purchased from Sigma). Poly(dT) was purchased from P-L Biochemicals, Inc. One stock recA protein preparation was used for most experiments (Cotterill et al., 1982). Its purity exceeds 98%. Some experiments employed another batch isolated by Cotterill et al. of apparently greater purity. As far as we can tell, the two samples of recA protein used here and also the one employed in our earlier work behave identically in the fluorescence experiments described.

Methods

All fluorescence titrations were performed by adding small volumes of reactants to 1.0 mL of standard buffer contained in a cuvette thermostated at 25 °C, mixing by gentle shaking, and recording the fluorescence with a Perkin-Elmer MPF-44B instrument. Standard buffer consisted of pH 7.5 20 mM Tris-HCl holding 10 mM $MgCl_2$ and 1 mM dithiothreitol

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[1] Abbreviations: ss, single stranded; ds, double stranded; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); NTP, nucleotide triphosphate; poly(ϵA), poly(1,N^6-ethenoadenylic acid); ϵDNA, the product obtained by treating ssDNA with chloroacetaldehyde, which contains 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine residues; recA, recA protein in hyphenated expressions and equations; p(dT), poly(dT) in equations; SSB, *Escherichia coli* single-strand binding protein; Tris, tris(hydroxymethyl)aminomethane.

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