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Expression of Chicken Lamin B_2 in *Escherichia coli*: Characterization of its Structure, Assembly, and Molecular Interactions

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Abstract. Chicken lamin B_2, a nuclear member of the intermediate-type filament (IF) protein family, was expressed as a full-length protein in *Escherichia coli*. After purification, its structure and assembly properties were explored by EM, using both glycerol spraying/low-angle rotary metal shadowing and negative staining for preparation, as well as by analytical ultracentrifugation. At its first level of structural organization, lamin B_2 formed "myosin-like" 3.1S dimers consisting of a 52-nm-long tail flanked at one end by two globular heads. These myosin-like molecules are interpreted to represent two lamin polypeptides interacting via their 45-kD central rod domains to form a segmented, parallel and unstaggered 52-nm-long two-stranded α-helical coiled-coil, and their COOH-terminal end domains folding into globular heads. At the second level of organization, lamin B_2 dimers associated longitudinally to form polar head-to-tail polymers. This longitudinal mode of association of lamin dimers is in striking contrast to the lateral mode of association observed previously for cytoplasmic IF dimers. At the third level of organization, these polar head-to-tail polymers further associated laterally, in an approximately half-staggered fashion, to form filamentous and eventually paracrystal-like structures revealing a pronounced 24.5-nm axial repeat. Finally, following up on recent studies implicating the mitotic cdc2 kinase in the control of lamin polymerization (Peter, M., J. Nakagawa, M. Dorée, J. C. Labbé, and E. A. Nigg. 1990. *Cell.* 61:591–602), we have examined the effect of phosphorylation by purified cdc2 kinase on the assembly properties and molecular interactions of the bacterially expressed lamin B_2. Phosphorylation of chicken lamin B_2 by cdc2 kinase interferes with the head-to-tail polymerization of the lamin dimers. This finding supports the notion that cdc2 kinase plays a major, direct role in triggering mitotic disassembly of the nuclear lamina.

LAMINS, having molecular masses between 60 and 75 kD, are members of the intermediate-type filament (IF)[1] protein family (McKeon et al., 1986; Fisher et al., 1986; Franke, 1987). In situ, they form a karyoskeletal structure, known as the nuclear lamina. This fibrillar meshwork lines the inner nuclear membrane (Gerace et al., 1978; Krohne and Benavente, 1986; Aebi et al., 1986; Gerace and Burke, 1988; Nigg, 1989; Paddy et al., 1990) and is thought to interact with interphase chromatin (Gerace et al., 1978; Hancock, 1982; Lebkowski and Laemmli, 1982; Paddy et al., 1990; Glass and Gerace, 1990). In mammals, lamins have been classified as either A-, or B-type, based on protein chemical and primary sequence criteria (see Gerace and Burke, 1988; Höger et al., 1988; Peter et al., 1989; Nigg, 1989). In addition, mammals express a C-type lamin, but this protein is identical to lamin A except for its COOH terminus, and probably arises from alternate RNA splicing (McKeon et al., 1986; Fisher et al., 1986; Riedel and Werner, 1989). In birds, three structurally distinct lamin isotypes have been identified; according to the homologies of their amino acid sequences (Vorburger et al., 1989*a*; Peter et al., 1989) and their protein chemical properties (Lehner et al., 1986; Stick et al., 1988), they have been designated as lamins A, B_1, and B_2. Whereas lamin B_2 is the quantitatively predominant B-type lamin in chicken, it represents a minor component in most mammalian tissues thus far investigated; conversely, lamin B_1 is a minor component in most avian tissues, but it is closely related to the predominant B-type lamin in mammals (Lehner et al., 1986). The existence of B_1- and B_2-type lamin polypeptides in both mammals and amphibians has recently been confirmed by protein sequence analysis of purified murine lamin B (Weber et al., 1990), and by cDNA cloning of murine and *Xenopus laevis* lamin B_2 (Höger et al., 1990).

Lamins exhibit structural features common to all IFs (Aebi

1. *Abbreviations used in this paper*: IPTG, isopropyl-β-D-thiogalactopyranoside; MES, 2-morpholino-ethanesulfonic acid.

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et al., 1986, 1988; Parry et al., 1986, 1987; Stewart, 1990; Moir et al., 1990). Specifically, they contain a conserved rod domain consisting of four α-helical coils (i.e., 1A, 1B, 2A, 2B) connected by linkers and flanked by end domains of variable size and sequence (McKeon et al., 1986; Fisher et al., 1986; Parry et al., 1986). Compared with vertebrate cytoplasmic IFs, coil 1B in lamins has a 42-amino acid extension, yielding a 45- instead of a 40-kD rod domain. From an evolutionary point of view, it is intriguing that an identically increased rod length is observed also in cytoplasmic IFs of invertebrates (Weber et al., 1988, 1989). At their first level of structural organization, two lamin polypeptides associate via their rod domains into parallel, unstaggered two-stranded α-helical coiled-coil dimers (see Parry et al., 1986; Krohne et al., 1987). In the electron microscope lamin dimers resemble "myosin-like" molecules in that they reveal a 50–53 nm long tail segment flanked at one end by two globular "heads." (We note that the terms "head" and "tail" are occasionally used to designate NH_2 and COOH termini when describing the tripartite organization of IF proteins. These designations should not be confounded with the morphological descriptions used here. In particular, we emphasize that the "head" structures discussed here, i.e., those that can be seen by EM, are formed by the COOH-terminal end domains of lamins.) (Aebi et al., 1986, 1988; Moir et al., 1990). Under appropriate in vitro conditions, lamin dimers assemble into IF-like structures, and ultimately into paracrystalline arrays with a characteristic 24.5-nm axial repeat (Goldman et al., 1986; Aebi et al., 1986; Parry et al., 1987; Moir et al., 1990).

During mitosis, lamins depolymerize and repolymerize in a time frame closely corresponding to disassembly and reformation of the nuclear envelope (Gerace et al., 1978; Krohne et al., 1978; Gerace and Blobel, 1980). Whereas A-type lamins become completely solubilized upon disassembly, B-type lamins remain associated with membranous structures (Gerace and Blobel, 1980; Stick et al., 1988; see also Burke and Gerace, 1986). Subcellular fractionation studies have revealed that after lamina depolymerization, lamins A and C in mammalian cells are dissociated into dimers or tetramers (Gerace and Blobel, 1980; Dessev et al., 1990). Disassembly of the nuclear lamina has long been proposed to be the consequence of hyperphosphorylation of the lamin polypeptides by cell cycle–specific protein kinases (Gerace and Blobel, 1980; Gerace et al., 1984; Miake-Lye and Kirschner, 1985; Ottaviano and Gerace, 1985; Dessev et al., 1988). Strong support for this notion stems from the finding that cdc2 kinase, a highly conserved regulator of the cell cycle (Nurse, 1990), phosphorylates lamins at M phase–specific sites (Peter et al., 1990; see also Ward and Kirschner, 1990), and, concomitantly, is able to depolymerize the nuclear lamina upon incubation with nuclei (Peter et al., 1990). Moreover, mutations affecting mitotic phosphorylation sites have been shown to interfere with lamina disassembly in vivo (Heald and McKeon, 1990). Site-specific phosphorylation has been shown to induce disassembly, or inhibit assembly, of cytoplasmic IFs both in vitro (see Inagaki et al., 1987; Geisler and Weber, 1988; Kitamura et al., 1989; Hisanaga et al., 1990; Chou et al., 1990) and in mitotic cells (see Chou et al., 1989; Lamb et al., 1989).

The expression of cDNA clones encoding either cytoplasmic IF proteins (see Magin et al., 1987; Quinlan et al., 1989; Stewart et al., 1989; Hatzfeld and Weber, 1990; Coulombe and Fuchs, 1990) or nuclear lamins (Ward and Kirschner, 1990; Moir et al., 1990) in bacteria offers a powerful tool to systematically investigate the structure, assembly, and molecular interactions of both wild-type and mutated IF polypeptides in vitro.

Here we describe the expression of full-length chicken lamin B_2 in *Escherichia coli* using an expression plasmid containing the T7 promoter (Rosenberg et al., 1987). The purified recombinant protein is characterized in terms of its in vitro structure, assembly, and molecular interactions. We have also performed preliminary experiments exploring the effect of phosphorylation by the cdc2 kinase on the molecular interactions of this bacterially expressed chicken lamin B_2.

Materials and Methods

Construction of a Chicken Lamin B_2 Expression Plasmid

The chicken lamin B_2 cDNA (Vorburger et al., 1989*a*) was cloned into the expression vector pAR3038 (Rosenberg et al., 1987), which allows selective expression of the cloned cDNA under the control of the T7 promoter. To express lamin B_2 from its proper ATG start codon, an NdeI restriction site was introduced into the 5′ end of the lamin B_2 cDNA (Fig. 1). For this

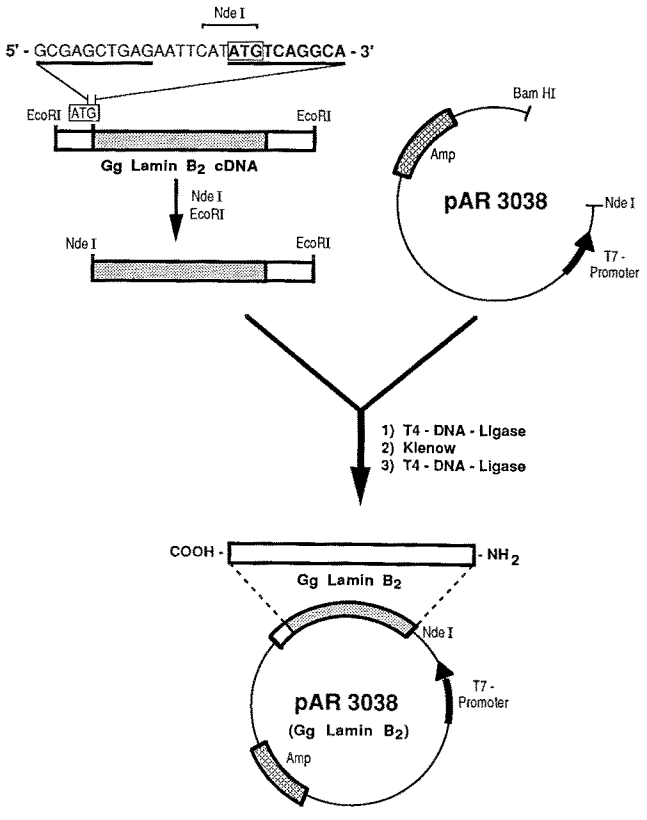

Figure 1. Construction of a chicken lamin B_2 bacterial expression plasmid. For insertion of the cDNA into the NdeI site of the expression vector pAR3038 (Rosenberg et al., 1987), oligonucleotide-directed site-specific mutagenesis was used to create an NdeI site within the translational start codon ATG of the lamin B_2 cDNA. Ligation of the EcoRI site at the 3′ end of the lamin B_2 cDNA with the BamHI site of the vector was achieved after creating blunt ends using the Klenow fragment of DNA polymerase I.

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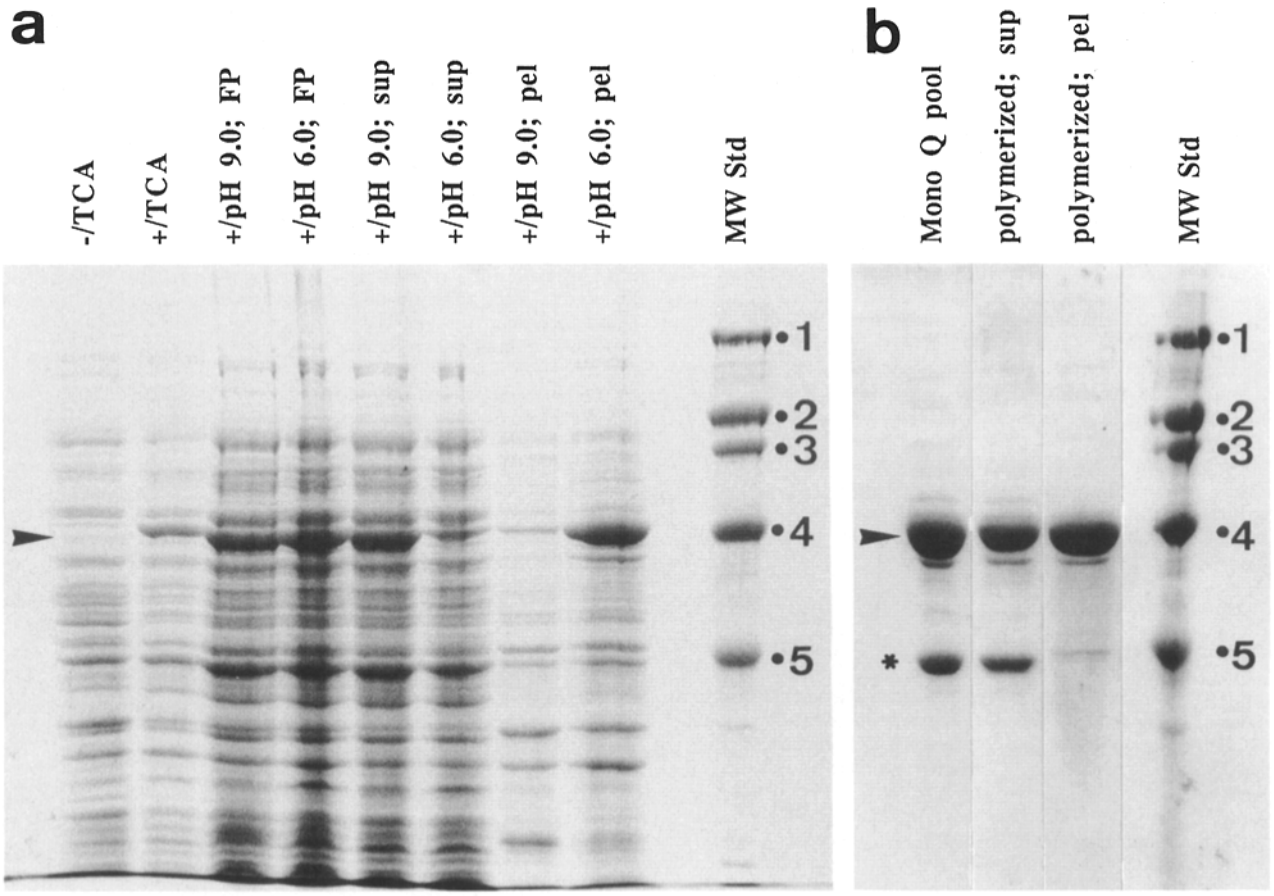

Figure 2. Expression in *E. coli* and extraction/purification of recombinant chicken lamin B_2. (*a*) Expression and crude extraction of recombinant lamin B_2 monitored by SDS-PAGE (10.5%). The equivalent of 150 μl of bacterial culture was loaded onto the gels. Uninduced bacteria (−) and induced bacteria (+). Lane *1* (*−/TCA*): uninduced bacteria containing the expression plasmid, treated with 10% TCA without lysing the cells. No expression of lamin B_2 was detected. Lane *2* (*+/TCA*): induced bacteria expressing lamin B_2 (*arrowhead*) treated with 10% TCA without lysing the cells. Lane *3* (*+/pH 9.0; FP*): induced bacteria resuspended in high-pH (pH 9.0) buffer before breaking them up in a French press. Lane *4* (*+/pH 6.0; FP*): induced bacteria resuspended in low-pH (pH 6.0, i.e., lysis buffer: 25 mM MES, 150 mM NaCl, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 6.0) buffer before breaking them up in a French press. Lanes *5* (*+/pH 9.0; sup*) and *7* (*+/pH 9.0; pel*): under high-pH conditions, centrifugation at 10,000 *g* for 30 min at 4°C yielded most of the lamin B_2 in the supernatant with only a small amount pelleting. Lanes *6* (*+/pH 6.0; sup*) and *8* (*+/pH 6.0; pel*): under low-pH conditions, centrifugation at 10,000 *g* for 30 min at 4°C yielded most of the lamin B_2 in the pellet with little lamin left in the supernatant. This pH-dependent aggregation effect was therefore used for one-step extraction of recombinant lamin B_2 from the bacterial lysates. (*b*) Purification of recombinant lamin B_2 (*arrowhead*) monitored by SDS-PAGE (10.5%). Lane *1* (*Mono Q pool*): chicken lamin B_2 after solubilizing the low-pH extract (lane *8* [*+/pH 6.0; pel*]) with 6 M urea (i.e., with "solubilization" buffer: 20 mM Tris, 6 M urea, 2 mM EGTA, 1 mM DTT, pH 9.0) and running it over a mono Q anion exchange FPLC column. To remove the major ~42-kD band contaminating the mono Q pool (*), we took advantage of its different polymerization properties relative to those of the intact lamin B_2 polypeptide. For this purpose, the pool was dialyzed at room temperature first for 3 h into equilibration buffer (25 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 8.5) and then for 1 h into "polymerization" buffer (25 mM MES, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 6.5), before it was centrifuged at 100,000 *g* for 1 h at 4°C. As documented in lanes *2* (polymerized; *sup*) and 3 (polymerized; *pel*), most of the ~42-kD contaminating polypeptide remained in the supernatant, while the pellet contained almost exclusively intact lamin B_2. For further use, the pellet was solubilized in 6 M urea. Gel standards (*MW Std; a* and *b*): (*1*) myosin, 200 kD; (*2*) β-galactosidase, 116 kD; (*3*) phosphorylase b, 97 kD; (*4*) BSA, 66 kD; (*5*) ovalbumin, 43 kD.

purpose, the oligonucleotide 5′-GCGAGCTGAGAATTCATATGTCAGGCA-3′ was used for site-directed mutagenesis (Kunkel et al., 1987), taking advantage of the mutagenesis kit provided by Bio-Rad Laboratories (Richmond, CA). The mutation was confirmed by dideoxy sequencing (Sanger et al., 1977), and the construct was verified with diagnostic restriction digests. The expression plasmid was transformed into *E. coli JM109* made competent with $CaCl_2$. Expression was induced by infection with bacteriophage λ Ce6 (Studier et al., 1986), carrying the T7 RNA polymerase gene. The same expression plasmid was also transformed into *E. coli* strains *CAG456* (Baker et al., 1984) and *LC137* (Goff et al., 1985), which exhibit reduced proteolysis activity when grown at 30°C. Recombinant DNA procedures were performed by standard techniques (Sambrook et al., 1989).

Purification of Chicken Lamin B_2 after Expression in E. coli

An overnight culture of bacteria transformed with the lamin B_2-containing expression plasmid was diluted 1:100 into fresh LB-medium (10 g Bacto-Tryptone, 5 g Bacto-Yeast Extract (both from Difco, Detroit, MI), and 5 g NaCl in 1 liter H_2O), supplemented with 10 mM $MgSO_4$ and 0.2% mal-

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tose. Cells were grown to an OD_{600} of ~1.0 in the presence of 50 μg/ml ampicillin before they were infected with bacteriophage λ Ce6 at a multiplicity of infection (m.o.i.) of 7. After another 3 h at 37°C, the cells were pelleted and resuspended in one-tenth the original volume in 25 mM 2-morpholino-ethanesulfonic acid (MES), 150 mM NaCl, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 6.0 ("lysis" buffer). Next, the cells were broken up with a French press at a pressure of 20,000 p.s.i. and centrifuged at 10,000 *g* for 10 min at 4°C. The pellet containing the insoluble lamin was washed another two times with lysis buffer before it was solubilized in 20 mM Tris, 6 M urea, 2 mM EGTA, 1 mM DTT, pH 9.0 ("solubilization" buffer), using a Dounce homogenizer. To remove insoluble material, the sample was centrifuged at 10,000 *g* for 10 min at 4°C before the supernatant was loaded onto a mono Q 5/5 anion exchange FPLC column (Pharmacia, Uppsala, Sweden). The column was eluted with a linear 0–300 mM KCl gradient in 20 mM Tris, 6 M urea, 2 mM EGTA, 1 mM DTT, pH 9, and 0.5-ml fractions were collected. Those fractions enriched in intact lamin B_2 (i.e., those eluting around 200 mM KCl) were pooled and dialyzed at room temperature, first for 3 h against 25 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 8.5 ("equilibration" buffer), and then for 1 h against 25 mM MES, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 6.5 ("polymerization" buffer). At this stage, the material was centrifuged for 1 h at 100,000 *g* at 4°C and the pellet, which contained highly purified intact lamin B_2, was resuspended in 6 M urea and used for subsequent studies.

PAGE and Immunoblotting

For SDS-PAGE, samples were run on 10.5% polyacrylamide gels and stained with Coomassie brilliant blue R (Laemmli, 1970). For immunoblotting, the protein was electrophoretically transferred to a nitrocellulose sheet (BA 85; Schleicher & Schuell, Dassel, Germany) as described by Towbin et al. (1979). After transfer, the nitrocellulose was blocked with 5% milk powder in TBS for 30 minutes. As the primary antibody, we used E-3, a monoclonal anti-lamin B_2 (Lehner et al., 1986) at a dilution of 1:2,000; the secondary antibody was horseradish peroxidase–conjugated rabbit anti-mouse IgG (DAKO-Immunoglobulins, Glostrup, Denmark). Antibody-conjugates were visualized with 4-chloro-1-naphthol (Fluka, Buchs, Switzerland).

In Vitro Phosphorylation of Chicken Lamin B_2 by cdc2 Kinase

Purified lamin B_2 solubilized in 6 M urea was equilibrated by dialysis in 25 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 mM $MgCl_2$, 100 μM ATP, pH 7.5 ("kinase" buffer) for 3 h at room temperature. Next, aliquots were incubated at 30°C with highly purified cdc2 kinase (specific activity: 400 pM/μl per min) isolated from starfish oocytes. Specifically, it was obtained from a 70% ammonium sulfate precipitate after the P11-phosphocellulose step of a previously described procedure (Labbé et al., 1989) and kindly provided by Dr. M. Dorée (Centre National de la Recherche Scientifique and Institut Nationale de la Santé et de la Recherche Medicale, Montpellier, France). Reactions were stopped after 0, 15, and 30 min by addition of EDTA to 10 mM. Polymerization of kinase treated lamin B_2 was assayed by first dialyzing aliquots against equilibration buffer for 3 h at room temperature, followed by dialysis against polymerization buffer for 1 h at room temperature. Spin-down experiments were performed with the 0-, 15-, and 30-min samples by centrifugation at 100,000 *g* for 1 h at room temperature. In parallel, aliquots of the 0-, 15-, and 30-min samples were prepared for electron microscopy as described below.

Electron Microscopy

For low-angle rotary metal shadowing, a 15-μl aliquot of each sample was mixed with glycerol to a final concentration of 5–30% and sprayed onto freshly cleaved mica. The mica piece was next placed en face on the rotary table of a freeze-etch apparatus (BA 511 M; Balzers AG, FL-9496 Balzers, Fürstentum Liechtenstein) and dried in vacuo at room temperature for at least 1 h. Finally, the dried sample was rotary-shadowed with platinum carbon at an elevation angle of ~5°C (Fowler and Aebi, 1983).

For negative staining, a 5-μl aliquot of the sample was adsorbed for 1 min to a glow-discharged (Aebi and Pollard, 1987) carbon-coated collodion film on a copper grid. Then the grid was washed on one to three drops of either distilled water or buffer before it was sequentially placed on three drops of 0.75% uranyl formate (pH 4.25) for ~10 s each. Excess liquid was drained with filter paper followed by suction with a capillary applied to the edge of the grid which was then permitted to air-dry.

Specimens were examined in a Hitachi H-7000 transmission electron microscope operated at 100 kV. Electron micrographs were recorded on Kodak SO-163 electron image film at nominal magnifications of either 20,000 or 50,000. Magnification calibration was performed according to Wrigley (1968) using negatively stained catalase crystals.

Analytical Ultracentrifugation

Both sedimentation velocity and low-speed sedimentation equilibrium measurements were performed in an analytical ultracentrifuge (model E; Beckman Instruments Co., Fullerton, CA) equipped with the UV absorption optics system and a photoelectrical scanning device (Chervenka, 1970; Rickwood, 1984). Sedimentation velocity runs were carried out in a rotor (An-D; Beckman Instruments) at 56,000 rpm using a 12-mm double-sector Epon cell. With buffers containing urea, runs were performed at 52,000 rpm using a 12-mm double-sector Kel-F cell. For low-speed sedimentation equilibrium runs the same cell types were used but only filled up to 3 mm column height. Sedimentation coefficients ($s_{20,w}$) were determined according to the method of Schachman (1959). For these calculations, a partial specific volume (v) of 0.73 ml/g was used, and $s_{20,w}$ was corrected using the tables published by Kawahara and Tanford (1966). To evaluate the different M_r species contained in multicomponent solutions, rotor speeds ranging from 5,600 to 18,000 rpm were used (Chernyak and Magretova, 1982). To calculate M_rs, a program that adjusts the baseline absorbance such as to obtain the best linear fit of $\ln A$ vs. r^2 (written by H. Berger and A. Lustig, Biocenter, Basel) was used.

Results

Expression of Chicken Lamin B_2 in E. coli

In initial experiments, we found that transformation of the lamin B_2 expression plasmid into the bacterial strain *BL21-(DE3)* (Studier et al., 1986) was not possible, presumably reflecting a lethal effect of lamin B_2 expression on this host. Considering that this *E. coli* strain is a lysogen, containing the gene coding for the T7 RNA polymerase under the control of the inducible lac UV5 promoter, lethality might be explained if lamin B_2 were toxic, by low levels of expression occurring in the absence of its inducer, isopropyl-β-D-thiogalactopyranoside (IPTG).

As an alternative, we therefore proceeded to introduce the gene encoding the T7 RNA polymerase into the bacterial strain *JM109* by infection with bacteriophage λ Ce6 (Studier et al., 1986). Even under these circumstances we found that a portion of cells died during growth and expression, an observation which may indicate some level of toxicity of lamin B_2. Nevertheless, in fully induced cells the 67-kD lamin B_2 was found to account for ~5% of the total soluble protein, and ~20 mg of lamin B_2 could be purified from a 1-liter culture.

Lysis of *E. coli* under appropriate conditions resulted in soluble recombinant protein, indicating that the expressed protein had not aggregated into bacterial inclusion bodies. Specifically, as illustrated in Fig. 2 *a*, lysis of the bacteria in "high-pH" (pH 9) buffer yielded the majority of the lamin B_2 in the supernatant after 10-min centrifugation at 10,000 *g*. In contrast, lysis of induced bacteria in "low-pH" (pH 6) buffer led to effective aggregation of the expressed protein, rendering it readily pelletable (Fig. 2 *a*). To facilitate preparative purification of lamin B_2, we took advantage of its pH-dependent aggregation and therefore adjusted the pH of the "lysis" buffer to 6.0 (25 mM MES, 150 mM NaCl, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 6.0).

Immunoblotting of the type of gel shown in Fig. 2 *a* stained with a monoclonal antibody against chicken lamin β_2 indi-

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cated that 10–20% of the expressed lamin B_2 was truncated, most likely by endogenous proteolysis, and revealed three distinct bands between 40 and 60 kD (data not shown). Even bacteria, which after expression were directly TCA treated without lysing the cells, yielded a similar amount of truncated product. In attempts to minimize this truncation, the lamin B_2 expression vector was transformed into *CAG456* (Baker et al., 1984) and *LC137* (Goff et al., 1985) bacteria, both of which lack the *lon* protease due to its noninducibility. However, in *LC137* cells lamin B_2 was not expressed, and although expression occurred in *CAG456* cells, truncation was only moderately reduced (data not shown). We therefore decided to express lamin B_2 in *JM109* cells as described above and to remove the truncated products during extraction/purification of the intact recombinant protein (see below).

Purification of the Recombinant Chicken Lamin B_2 Protein

Taking advantage of the aggregation of lamin B_2 in low-pH lysis buffer, the expressed protein was partially purified by washing the pelleted material several times with lysis buffer (Fig. 2 *a*). The washed material was then redissolved in "solubilization" buffer (20 mM Tris, 6 M urea, 2 mM EGTA, 1 mM DTT, pH 9) and fractionated by FPLC on a mono Q anion exchange column eluted with a 0–300-mM KCl linear salt gradient. The lamin-containing fractions eluted around 200 mM KCl; although the truncated lamin bands were significantly attenuated in these fractions, a major ~42-kD band which did not react with our mAb against chicken lamin B_2 (data not shown) accumulated in these fractions (Fig. 2 *b*). In attempts to get rid of this ~42-kD contaminating band, the pooled fractions were first dialyzed for 3 h against "equilibration" buffer (25 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 8.5) and then for ≥1 h against "polymerization" buffer (25 mM MES, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 6.5). Under these conditions, the ~42-kD band remained quantitatively in the supernatant after high-speed centrifugation (150,000 *g* for 3 h; Fig. 2 *b*). For further use, this pellet was resuspended in 6 M urea.

At its First Level of Structural Organization, Chicken Lamin B_2 Forms "Myosin-like" Dimers

As documented in Fig. 3, EM of glycerol-sprayed/rotary metal shadowed samples of purified chicken lamin B_2 in equilibration buffer revealed predominantly "myosin-like" molecules consisting of a 52 ± 2-nm-long tail with a pair of globular heads at one end. Most likely, these myosin-like molecules represent lamin dimers with two lamin polypeptides being arranged in a parallel and unstaggered fashion such that their 45-kD central rod domains form a segmented two-stranded α-helical coiled coil, and their COOH-terminal end domains protrude as globular heads. A small amount (~10–20%) of these dimers appeared as short, two to three repeats long "head-to-tail" polymers (i.e., head-to-tail tetramers and hexamers: see arrows, Fig. 3); in contrast, no significant "side-by-side" aggregation (i.e., side-by-side tetramers) of these dimers was found. In the analytical ultracentrifuge, the same material (~0.2 mg/ml) yielded an average M_r of 170 kD by sedimentation equilibrium, and an average $s_{20,w}$ of 3.9 by sedimentation velocity. We also performed an analytical ultracentrifugation analysis of lamin B_2 samples (~0.4 mg/ml) equilibrated in solubilization buffer with urea concentrations ranging from 6 to 3 M. Specifically, the following oligomeric species predominated: monomers (M_r 88 kD and $s_{20,w}$ 2.1) in 6 M urea, dimers (M_r 130 kD and $s_{20,w}$ 3.1) in 4.5 M urea, and tetramers (M_r 250 kD and $s_{20,w}$ 4.2) in 3 M urea. EM of the same samples after glycerol spraying/rotary metal shadowing revealed predominantly myosin-like dimers in 6 and 4.5 M urea, and a mixture of dimers and head-to-tail tetramers in 3 M urea (data not shown). None of the buffer conditions tried (see also below), including lowering the glycerol concentration to 5%, led to significant lateral aggregation of two lamin dimers into side-by-side tetramers as has commonly been observed with cytoplasmic IF polypeptides (Quinlan et al., 1984, 1986; Eichner et al., 1985).

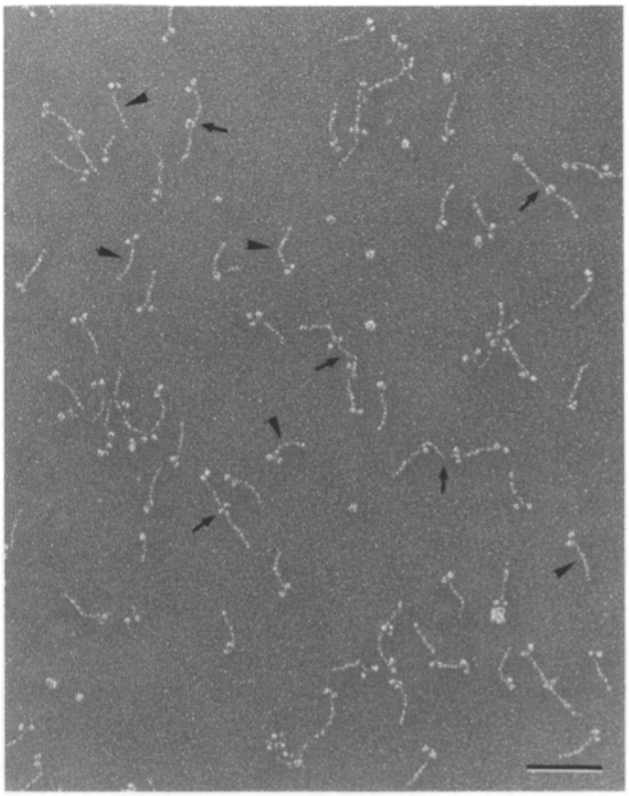

Figure 3. At its first level of structural organization the bacterially expressed chicken lamin B_2 forms "myosin-like" dimers. Purified recombinant chicken lamin B_2 was equilibrated for 3 h at room temperature in 25 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 8.5, and glycerol-sprayed/rotary metal shadowed. This condition yielded predominantly myosin-like dimers (*arrowheads*) consisting of a 52 ± 2-nm tail segment (representing a two-stranded α-helical coiled-coil of two parallel/unstaggered rod domains) flanked at one end by two globular heads (each head representing one COOH-terminal end domain). In this buffer, the lamin B_2 dimers exhibited a concentration-dependent tendency to form two to three molecules long head-to-tail polymers (*arrows*), a finding that was confirmed by low-speed sedimentation equilibrium centrifugation (see text). Bar, 100 nm.

Chicken Lamin B_2 Dimers Assemble Linearly into Polar "Head-to-Tail" Polymers

Recombinant lamin B_2 assembly in vitro was assayed under

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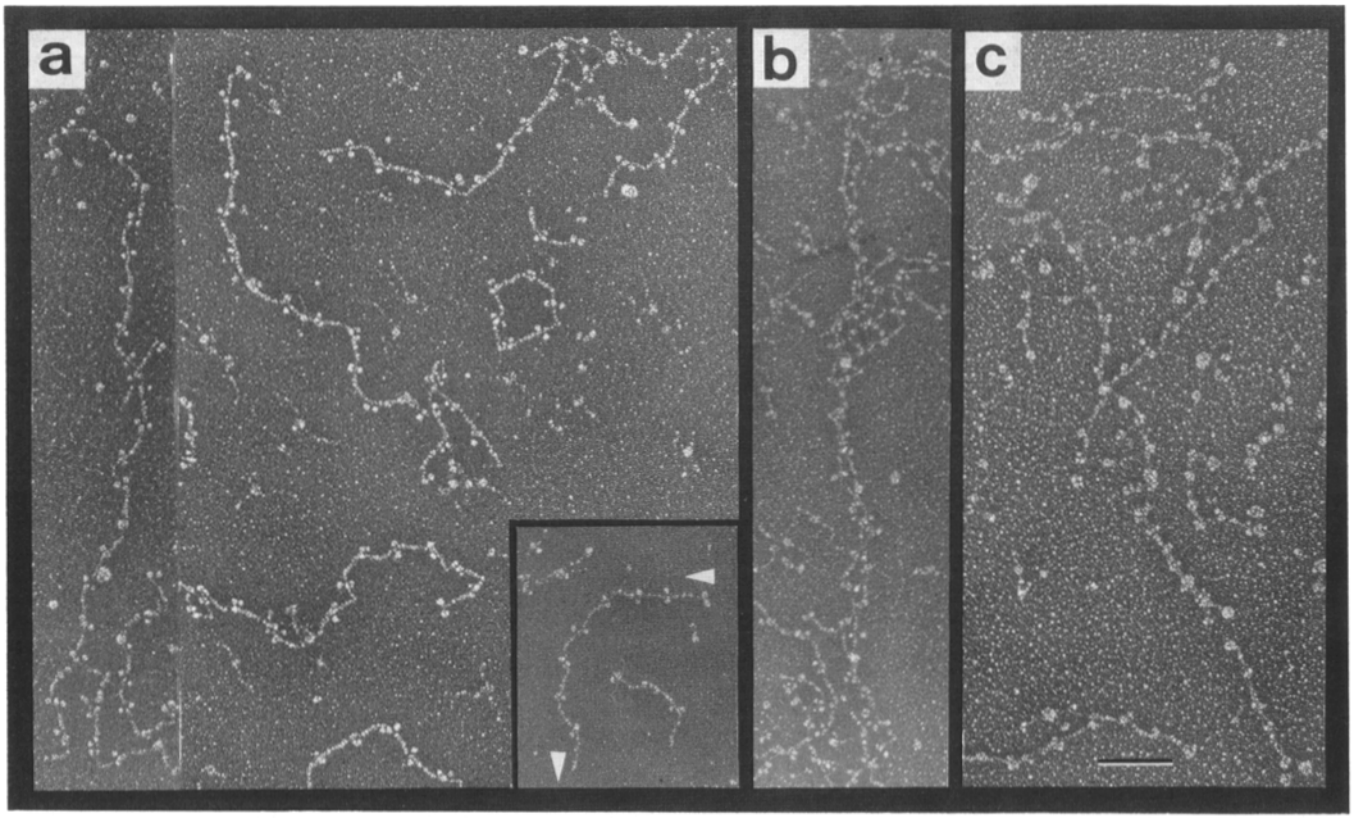

Figure 4. Chicken lamin B_2 dimers associate longitudinally into polar head-to-tail polymers which further aggregate laterally in a half-staggered, probably antiparallel fashion. For EM, samples were prepared by glycerol spraying/low-angle rotary metal shadowing. (*a*) Dialysis of chicken lamin B_2 dimers against 25 mM MES, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 6.5 for ≥1 h at room temperature yielded polar (*arrowheads, inset*) head-to-tail polymers. (*b*) Lowering the NaCl concentration to 100 mM caused the head-to-tail polymers to loosely laterally associate with a tendency to stagger. (*c*) When lamin B_2 was exposed to conditions favoring paracrystal formation (see Fig. 5), thin filamentous structures with a characteristic 24–25-nm "beading" repeat were predominantly revealed after glycerol spraying/rotary metal shadowing. Bar: (*a–c*) 100 nm.

different buffer conditions. Variations included pH values ranging from 6 to 9, and ionic strengths ranging from 0 to 500 mM NaCl. Dialysis of purified lamin B_2 into equilibration buffer (see Materials and Methods) yielded predominantly dimers (see above and Fig. 3). As illustrated in Fig. 4 *a*, when such samples were dialyzed for 1–3 h at room temperature against polymerization buffer, longitudinal aggregation of lamin dimers into polar (as indicated by arrowheads) head-to-tail polymers was observed in the EM after glycerol spraying/rotary metal shadowing. When the ionic strength of the polymerization buffer was lowered from 150 to 50–100 mM NaCl, the head-to-tail polymers started to loosely, laterally aggregate (Fig. 4 *b*). The same types of structures (Fig. 4, *a* and *b*) were also observed when the corresponding samples were prepared for EM by negative staining (data not shown).

Ca^{2+} Causes Chicken Lamin B_2 to Form Filamentous and Paracrystal-like Structures

As illustrated in Fig. 5, inclusion of 10–25 mM $CaCl_2$ in the polymerization buffer revealed filamentous (Fig. 5, *a* and *b*) and paracrystal-like (Fig. 5 *c*) structures in a time-dependent fashion when the samples were negatively stained for EM. Specifically, after 45 min, predominantly loose webs of IF-like structures were observed (Fig. 5 *a*) with apparent filament diameters between 5 and 10 nm. After 1 h, the filaments started to exhibit a characteristic 24–25-nm "beading" repeat, and the average filament diameter had increased (Fig. 5 *b*). Typically after 2 h, a significant fraction of the material had aggregated into paracrystal-like structures with a pronounced 24.5 ± 0.5-nm axial repeat (Fig. 5 *c*). In contrast, when the 1–2-h samples (i.e., Fig. 5, *b* and *c*) were prepared for EM by glycerol spraying/rotary metal shadowing, comparably thin filamentous structures, in many places revealing a 24–25-nm beading repeat, were found (Fig. 4 *c*). Supposedly, the strong shearing forces occurring during glycerol spraying had caused the paracrystal-like structures to break up laterally, probably preferentially decaying into half-staggered pairs of head-to-tail polymers of lamin dimers.

As documented in Fig. 6, significantly more extensive and regular paracrystal formation was obtained by first dialyzing recombinant lamin B_2 for 3 h against equilibration buffer, followed by 1–2 h dialysis at room temperature against 25 mM Tris, 1 mM DTT, 1 mM PMSF, pH 8.5, containing 10–25 mM $CaCl_2$. Under these conditions, practically 100% of the material was found aggregated into paracrystals with a highly regular 24.4 ± 0.3-nm axial repeat (Fig. 6, *a* and *b*). Unlike the low-pH paracrystals (Figs. 4 *c* and 5 *c*), these high-pH paracrystals were stable enough to withstand brief "sweeping" of a 25-μl sample drop (i.e., by centrifugation) over a freshly cleaved piece of mica in the presence of

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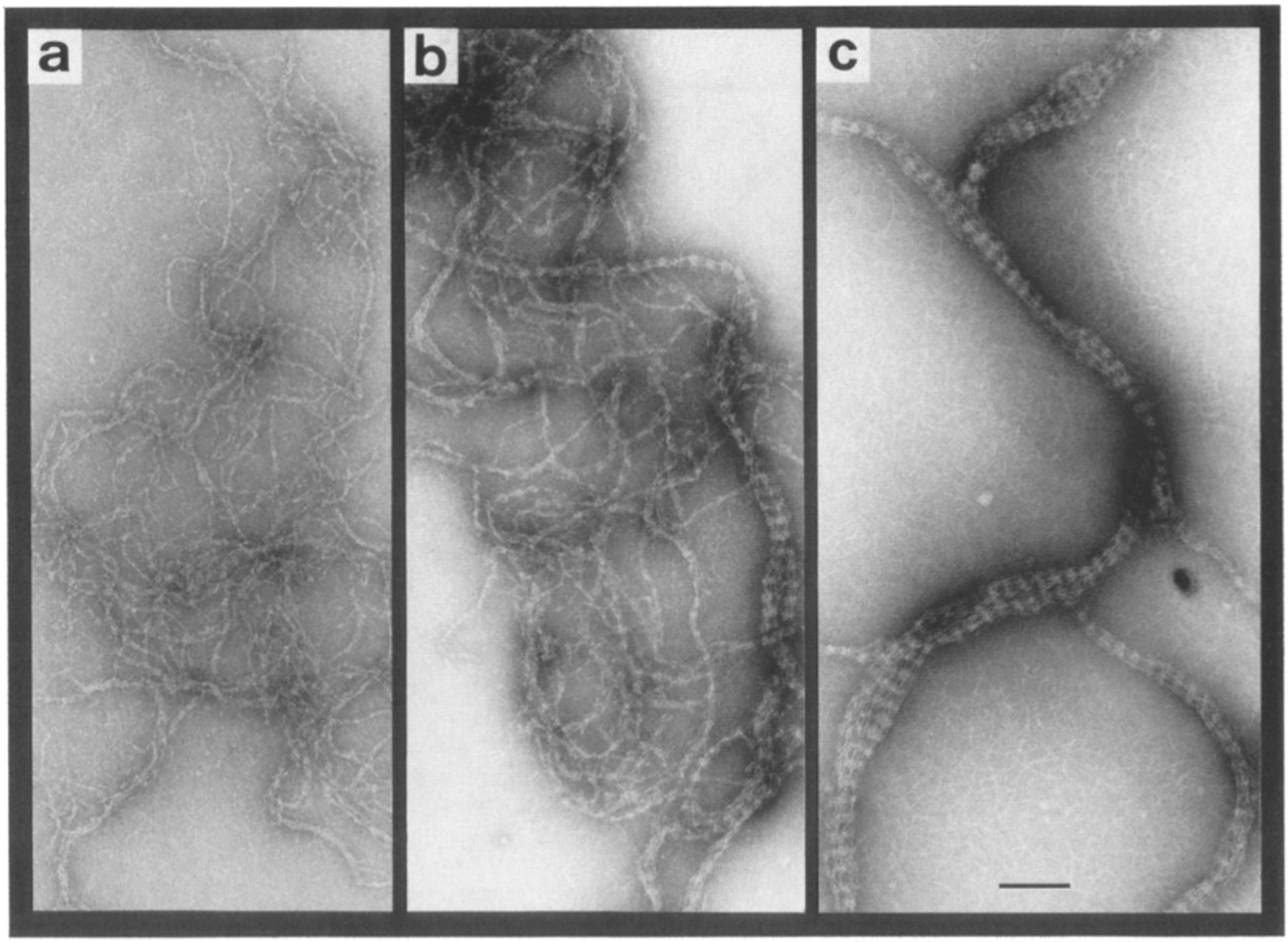

Figure 5. Ca/pH 6.5 induces the formation of filamentous and paracrystal-like structures. Shown are negatively stained filamentous and paracrystal-like structures assembled at room temperature from chicken lamin B_2 dimers by dialysis against 25 mM MES, 150 mM NaCl, 1 mM DTT, 1 mM PMSF, 25 mM $CaCl_2$, pH 6.5. Samples were analyzed at different times of dialysis: (*a*) 45 min; (*b*) 1 h; (*c*) 2 h. Bar, (*a–c*) 100 nm.

30% glycerol (Nave et al., 1989) before in vacuo drying/low-angle rotary metal shadowing (Fig. 6 *c*). By this preparation, the axial repeat was 23.9 ± 0.7 nm.

While at high pH substitution of Mg for Ca did not induce formation of any paracrystal-like structures, at low pH a few small and poorly ordered paracrystals were found with Mg (data not shown). Also, by lowering the pH to 6.0, we did, among all the aggregates, observe a small number of paracrystal-like structures in the absence of divalent cations (data not shown). The requirement of divalent cations for effective paracrystal formation by lamin B_2 is in contrast to the previous finding that rat liver lamins A plus C (Aebi et al., 1986) as well as bacterially expressed human lamin C (Moir et al., 1990) readily formed large and well-ordered paracrystals in the absence of divalent cations. While at low pH paracrystals formed in the presence of 150 mM NaCl, high-pH paracrystals only occurred in the absence of NaCl or KCl.

In Vitro Phosphorylation of Chicken Lamin B_2 by cdc2 Kinase Does Not Interfere with Dimer Formation But Abolishes Linear Assembly into Polar Head-to-Tail Polymers

Recent evidence strongly points to a direct link between the cdc2 kinase, a universal mitotic inducer, and mitotic phosphorylation of lamins (Peter et al., 1990) and cytoplasmic intermediate filament proteins (Chou et al., 1990). In particular, cdc2 kinase was shown to phosphorylate lamins in vitro at M phase-specific sites and, concomitantly, to trigger disassembly of the nuclear lamina when incubated with isolated nuclei (Peter et al., 1990). The sites phosphorylated on lamin proteins during mitosis were mapped to regions flanking the central rod domain (Ward and Kirschner, 1990; Peter et al., 1990), and by mutation analysis, a subset of those sites was found to be critical for lamina disassembly in vivo (Heald and McKeon, 1990). In the light of these recent findings, it was of considerable interest to examine the effect of phosphorylation by the cdc2 kinase on the in vitro assembly properties of our bacterially expressed lamin B_2.

For this purpose, lamin B_2 was equilibrated in "kinase" buffer (25 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 mM $MgCl_2$, 100 μM ATP, pH 7.5), and phosphorylation reactions were started by the addition of highly purified cdc2 kinase isolated from starfish oocytes (Labbé et al., 1989). After 0, 15, and 30 min, the kinase reaction was stopped by addition of EDTA to 10 mM, and assembly was started by dialysis into equilibration buffer fol-

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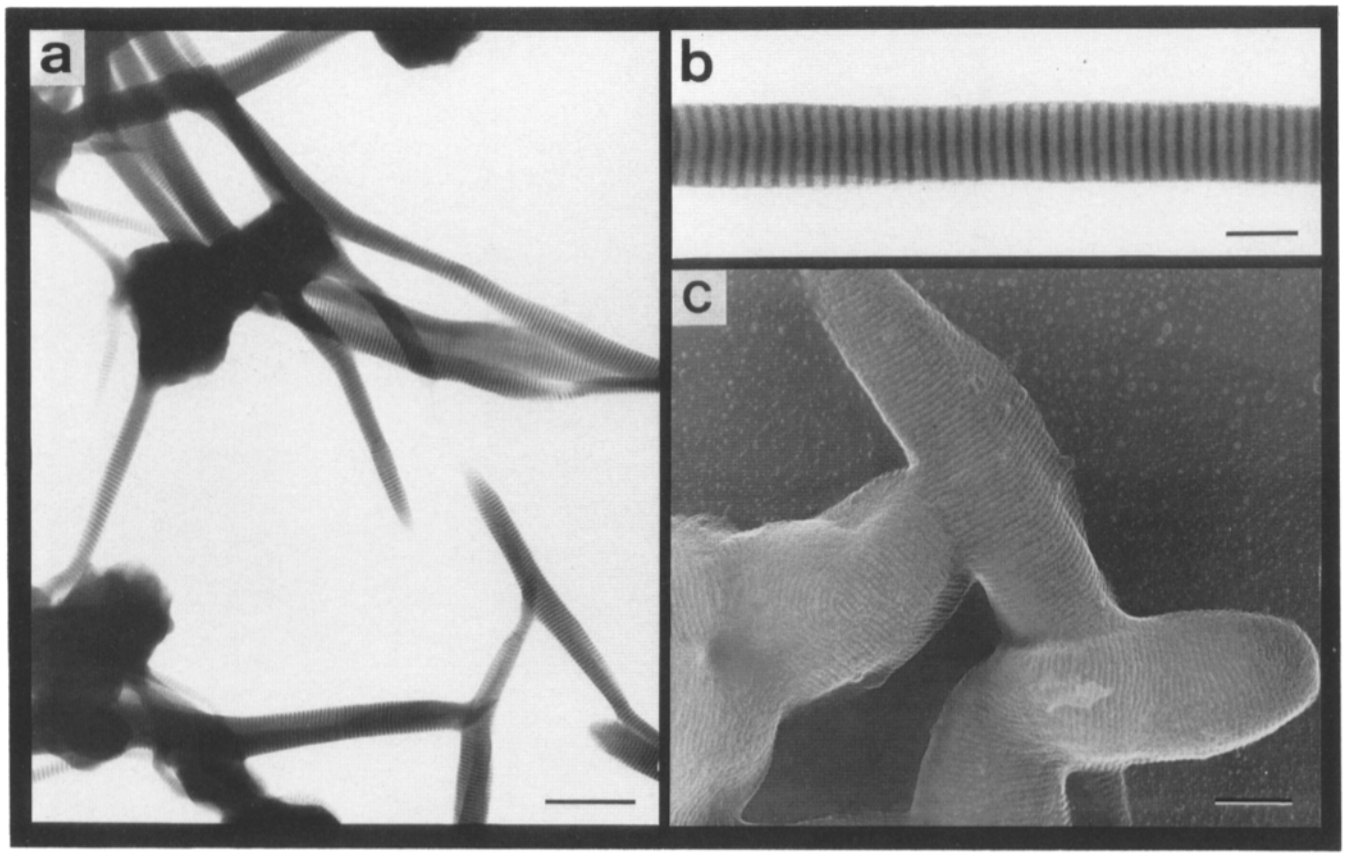

Figure 6. Ca/pH 8.5 causes lamin B_2 dimers to aggregate into highly ordered large-scale paracrystalline arrays. Shown are negatively stained and metal shadowed paracrystalline arrays formed at room temperature from chicken lamin B_2 dimers by dialysis against 25 mM Tris, 1 mM DTT, 1 mM PMSF, 25 mM $CaCl_2$, pH 8.5 for 2 h. (*a*) Negatively stained low-mag overview. (*b*) Negatively stained high-magnification view of a paracrystal stretch. (*c*) A 25-μl sample drop briefly swept (i.e., by centrifugation) over a freshly cleaved piece of mica in the presence of 30% glycerol, dried in vacuo, and low-angle rotary metal shadowed with platinum-carbon. Bars: (*a*) 500 nm; (*b*) 100 nm; and (*c*) 250 nm.

lowed by dialysis into polymerization buffer (see Materials and Methods). As documented in Fig. 7 *a*, spin-down experiments clearly showed that phosphorylation prevented the formation of pelletable head-to-tail polymers of lamin dimers, with maximal effects being reached by 30 min of kinase reaction. Moreover, whereas EM of glycerol-sprayed/rotary metal shadowed samples readily revealed assembly of lamin B_2 dimers into polar head-to-tail polymers with the sample corresponding to time point 0 of the kinase reaction (Fig. 7 *b*), head-to-tail polymerization was significantly attenuated with the sample that had been exposed to cdc2 kinase for 30 min (Fig. 7 *c*).

The above presented results remained unchanged when the samples, after stopping the kinase reaction, were first denatured in solubilization buffer containing 6 M urea, before dialysis into equilibration buffer followed by dialysis into polymerization buffer (data not shown). In particular, phosphorylation of the bacterially expressed chicken lamin B_2 by the cdc2 kinase did not interfere with dimer formation.

Discussion

Purification of Bacterially Expressed Chicken Lamin B_2

Using recombinant DNA methodology, we have expressed chicken lamin B_2 in *E. coli*. This enabled us to produce recombinant lamin B_2 in milligram quantities necessary for investigating structure–function relationships in vitro. We emphasize that unlike the recently bacterially expressed human lamin C (Moir et al., 1990), the lamin B_2 polypeptide used in these experiments was not a fusion construct but the full-length protein with translation being initiated at the proper ATG start codon (see Materials and Methods and Fig. 1). Purification of the expressed protein was facilitated by our observation that low-pH (i.e., 6.0) buffers induced selective aggregation of lamin B_2. Although a denaturing agent (6 M urea) was required to redissolve the aggregated material, such treatments are not generally thought to represent a problem with proteins refolding as easily as IF polypeptides. A ~42-kD, probably bacterial, polypeptide copurified with the intact lamin B_2 band during FPLC, but could readily be removed upon inducing head-to-tail polymerization and subsequent centrifugation. Under these conditions, intact lamin B_2 predominantly pelleted, whereas the ~42-kD polypeptide quantitatively remained in the supernatant.

Unlike lamin B isolated from rat liver nuclear envelopes (Aebi et al., 1986), the recombinant chicken lamin B_2 did not significantly aggregate or precipitate in the absence of detergent in the pH range 6.5 to 9.0 in the presence of ≥150 mM NaCl, unless millimolar amounts of Ca^{2+} or Mg^{2+} were present (see below). At least in part, this difference may be

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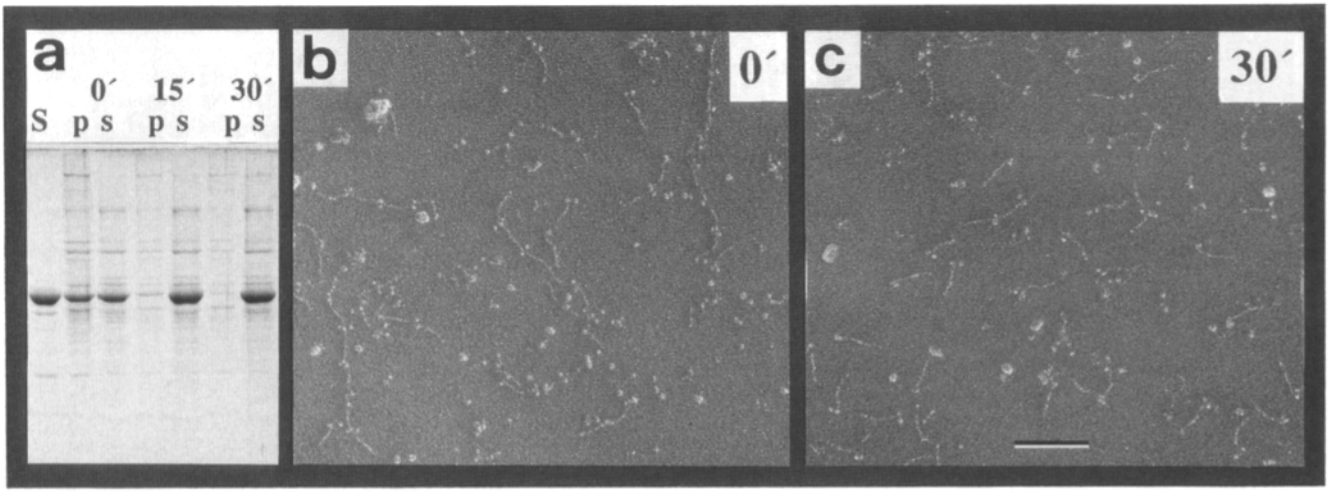

Figure 7. In vitro phosphorylation of recombinant chicken lamin B_2 dimers abolishes their assembly into head-to-tail polymers. Chicken lamin B_2 was incubated at 30°C with cdc2 kinase (isolated from starfish oocytes; Labbé et al., 1989) before the material was assembled into polar head-to-tail polymers. Aliquots were removed after 0, 15, and 30 min of incubation with cdc2 kinase, and the reaction was stopped by addition of EDTA to 10 mM. These were then dialyzed at room temperature, first for 3 h against 25 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 8.5, and then for ⩾1 h against 25 mM MES, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 6.5. (*a*) Spin-down assay (100,000 *g* for 1 h: *p*, pellet; *s*, supernatant) to evaluate the effect of lamin B_2 phosphorylation (for 0, 15, 30 min) on head-to-tail polymerization. *S*, bacterially expressed chicken lamin B_2 standard. (*b* and *c*) Electron micrographs of negatively stained specimens reveal that, in contrast to a 0-min control (*b*), head-to-tail polymerization of the dimers is practically abolished after 30 min of lamin B_2 phosphorylation by cdc2 kinase (*c*). Bar, (*b* and *c*) 100 nm.

due to the fact that, most likely, the bacterially expressed protein has not been subject to posttranslational modifications such as isoprenylation (Vorburger et al., 1989*b*) and carboxyl methylation (Chelsky et al., 1987).

In Vitro Assembly Properties of Bacterially Expressed Chicken Lamin B_2

Upon dialysis into a basic-pH buffer of near physiological ionic strength (i.e., pH 8.5, 150 mM NaCl), the lamin B_2 polypeptides dimerized to "myosin-like" molecules revealing a 52-nm long tail and a pair of globular heads at one end. Almost certainly, the long tail represents the parallel and unstaggered association of two lamin polypeptides via coiled-coil interaction of their α-helical rod domains, and the globular heads are formed by their COOH-terminal end domains. Structurally, these bacterially expressed chicken lamin B_2 dimers appeared indistinguishable from the previously described rat liver lamin A plus C and B dimers (Aebi et al., 1986), and the bacterially expressed human lamin C dimers (Moir et al., 1990).

The second detectable step in the hierarchical order of chicken lamin B_2 assembly was the longitudinal association of dimers into polar head-to-tail polymers. In fact, a similar behavior was previously observed with rat liver lamin dimers (see Fig. 3 *b* of Aebi et al., 1986; and U. Aebi, unpublished). This observation is remarkable as all hitherto studied cytoplasmic IF proteins appear to first undergo lateral association of dimers into tetramers, and possibly octamers, before significant longitudinal association of these building blocks to form mature, 8-12-nm-diam IFs (see Ip et al., 1985; Eichner et al., 1985; Aebi et al., 1988; Stewart, 1990). Thus, our finding that lamin B_2 dimers are capable of forming polar head-to-tail polymers as distinct assembly intermediates may hint at a novel mechanism for lamin assembly and dynamics.

At their third level of structural organization, which occurred most effectively in the presence of Ca^{2+}, varying numbers of polar head-to-tail polymers associated approximately half-staggered laterally into IF-like structures of heterogeneous diameter. Whether this Ca^{2+}-specificity stems from the fact that the tail protein of B-type lamins contains a sequence element resembling the typical loop sequence of several Ca^{2+} binding proteins (Höger et al., 1988) remains to be seen. It is conceivable, though not proven, that under these conditions adjacent head-to-tail polymers associate in an antiparallel fashion thus revealing apolar filaments, a property which may be generally true for IFs (see Aebi et al., 1988; Stewart, 1990) but would distinguish them from actin filaments and microtubuli. As with isolated rat liver lamins A plus C, in vitro, these lamin B_2 IF-like structures were not stable over time, nor did they form the sort of near-orthogonal meshworks as had been observed for the *Xenopus* oocyte nuclear lamina in situ (Aebi et al., 1986). Instead, at steady state, they assumed a variety of filamentous and paracrystalline polymorphs with a characteristic 24.5-nm axial periodicity (Aebi et al., 1986; Parry et al., 1987). Thus far, the lamins are the only IF polypeptides which, when intact, readily assemble into paracrystalline arrays in vitro (Goldman et al., 1986; Aebi et al., 1986; Parry et al., 1987; Moir et al., 1990). In certain embryonic epithelial cells, however, similar looking paracrystalline arrays of tonofilaments with a distinct 22-nm axial periodicity have been described (Kallman and Wessels, 1967). Also, paracrystalline arrays, albeit with different axial periodicities, have been obtained in vitro (*a*) after limited proteolysis of human epidermal keratin extracts (Aebi et al., 1983; Eichner et al., 1985), and (*b*) with a bacterially expressed fragment corresponding to the α-helical rod domain of glial fibrillary acidic protein (Stewart et al., 1989).

It is remarkable that chicken lamin B_2 forms structurally

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similar paracrystal-like structures at pHs as different as 6.5 and 8.5 as long as 10–25 mM Ca^{2+} is present. Their stability, however, is quite different: The low-pH paracrystals appear to be very loosely held together laterally, i.e., they break up into thin filamentous structures (see Fig. 4 *c*) when centrifuged with ≥10,000 *g* or prepared for EM by glycerol spraying. In contrast, the high-pH paracrystals are extremely stable with regard to such treatments. Based on these pH-dependent breakup patterns, it is conceivable that the pH primarily affects the strength of the lateral intersubunit interactions (i.e., lowering the pH weakens them) without, however, having a significant effect on the strength of the longitudinal intersubunit bonds.

In Vitro Phosphorylation of Recombinant Chicken Lamin B_2 by cdc2 Kinase Interferes with the Head-to-Tail Polymerization of Dimers

As documented by both a centrifugation assay as well as EM of glycerol-sprayed/rotary metal shadowed samples, phosphorylation of chicken lamin B_2 by highly purified starfish cdc2 kinase profoundly interfered with lamin assembly. The major inhibitory effect of phosphorylation was observed at the level of longitudinal association of the lamin dimers into polar head-to-tail polymers, rather than at the level of dimer formation as has recently been speculated (Moreno and Nurse, 1990). As shown by tryptic phosphopeptide mapping (Peter M., E. Heitlinger, M. Häner, U. Aebi, and E. A. Nigg, manuscript in preparation), phosphorylation of bacterially expressed lamin B_2 by cdc2 kinase in vitro occurred at two M phase–specific sites flanking the central rod domain previously characterized in vivo (Peter et al., 1990). Moreover, we recently found that phosphorylation of preformed polar head-to-tail polymers causes them to break up into dimers. This cdc2 kinase induced disassembly is dependent on ATP hydrolysis and abolished with head-to-tail polymers assembled from lamin B_2 polypeptides altered by site-specific mutagenesis (S→A) at the two critical phosphoacceptor sites, one including serine 16 and the other serines 384, 386, 388, and 389 (Peter et al., manuscript in preparation). Also, the effect of phosphorylation by cdc2 kinase on lamin B_2 assembly could be reversed by phosphatase treatment (Peter et al., manuscript in preparation).

It is tempting to interpret these observations in view of the finding that major mitotic phosphorylation sites on lamin polypeptides lie within highly conserved motifs that flank the central α-helical rod domain (Ward and Kirschner, 1990; Peter et al., 1990). Considering that dimerization of lamins, and of IF polypeptides in general, occurs through α-helical coiled-coil formation involving the central rod domains of two monomers (see Parry et al., 1986; Krohne et al., 1987; Conway and Parry, 1988), it is conceivable that phosphorylation of sites outside the rod domain does not affect this association step. On the other hand, it is not surprising that phosphorylation of the NH_2- and COOH-terminal end domains interferes with the head-to-tail association of dimers. Together, these results confirm and extend the conclusion that phosphorylation of lamins by cdc2 kinase may play a major role in controlling the state of polymerization of the nuclear lamina during mitosis (Peter et al., 1990).

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