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In Vitro Posttranslational Modification of Lamin B Cloned from a Human T-Cell Line†

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Autoimmune diseases are characterized by spontaneously occurring autoantibodies which have proven to be useful reagents for the characterization of specific nuclear proteins. Using a monoclonal autoantibody (72B9) derived from a murine lupus strain, we have cloned a cDNA from the human T-cell line MOLT-4, which encodes nuclear lamin B. The identity of the encoded protein as lamin B was established by both biochemical and immunological criteria. Inspection of the deduced amino acid sequence of lamin B revealed the presence in coil 1B of the α-helical domain of a leucine heptad repeat region. Analysis of mRNA in HL60 and MOLT-4 cells, which express only lamin B, or HeLa cells, which express all three major lamins (A, B, and C), together with the comigration of in vitro-translated product with isolated HeLa cell lamin B by two-dimensional gel electrophoresis, suggests that a single lamin B is expressed in mammalian somatic cells. In vitro translation with the cDNA clone revealed an EDTA-sensitive posttranslational modification which resulted in an increase in the apparent molecular weight to that equivalent to the native in vivo-synthesized lamin B protein. This in vitro modification included incorporation of a product of mevalonolactone and required an intact carboxy terminus.

Many eucaryotic cells contain a cytoskeleton composed of 10- to 15-nm filaments and a karyoskeleton (nuclear envelope) made up of a meshlike lattice of 10-nm filaments. These structures, particularly the cytoplasmic filaments, are classically known as intermediate filaments (IF). IF proteins comprise a multigene family which may number 30 or more proteins in a mammalian species (for reviews, see references 3 and 61). This heterogeneous family comprises, at present, five principal types, of which the first four are tissue-specific cytoplasmic filaments. Type V IF proteins are the nuclear lamins, which have only recently been recognized as a distinct class of intermediate filament proteins (for reviews, see references 14 and 16).

The lamins form a meshwork of filaments on the inner, or nucleoplasmic, surface of the nuclear envelope. The true diversity of the type V IF proteins, or lamins, has not yet been completely defined. Currently, mammalian species appear to contain at least three major lamins, called A (M_r 70), B (M_r 67), and C (M_r 60), of which two (A and C) arise by alternative splicing of the same gene (13, 43), and two minor lamins, D and E (26), whose molecular weight varies from species to species. All five lamins are thought to share some homology, since a human autoantibody recognizes all five (26) and a monoclonal antibody reactive against the common domain of all intermediate filament proteins recognizes lamins A, B, and C (36, 52), as well as minor acidic lamins (51), which could represent lamins D and E.

Three amphibian lamins have been cloned and sequenced from *Xenopus laevis*; one of these shows close sequence homology to human lamin A and is thought to be the amphibian equivalent of mammalian lamin A (72). The other two, L_I (31) and L_{III} (63), share certain sequence homologies but are no more closely related to each other than to lamin A and appear to be distinct lamins. LMD_0, a lamin cloned and sequenced from *Drosophila melanogaster* (19), is larger than *X. laevis* L_I but smaller than human and amphibian lamin A, and its sequence is sufficiently divergent to suggest that it is also a distinct lamin. Recently the *X. laevis* L_I sequence was used to isolate a mouse lamin clone (FML11-1) whose protein sequence bears striking homology to the amphibian lamin L_I (24). Although it lacks immunological identification, the acidic nature and distinct sequence of this lamin clearly distinguish it from mammalian lamins A and C and suggest that it is lamin B (24). Two acidic avian lamin proteins, B_1 and B_2, have been described whose deduced amino acid sequences suggest that they represent structurally distinct B-type lamins (50, 68). However, whereas B_1 appears immunologically related to mammalian lamin B, B_2 is immunologically related to lamin A (37, 64). It therefore appears that most nuclear lamins fall into two dominant but related types, A and B, which are themselves composed of subtypes.

Although IF proteins form an insoluble skeleton within the cell, their functional significance is unclear. It is thought that they play some part in cellular differentiation (reviewed in reference 15). However, cell growth and division can proceed in the absence of most IF proteins, suggesting that their presence is not essential (23). The one exception to this appears to be lamin B, which is found in all somatic cells, even those lacking lamins A and C (20, 35, 62) and other IF proteins (48, 49). This, together with the fact that the lamins appear to have arisen very early in eucaryote cell evolution (61), has led to the suggestion that the nuclear lamins, and particularly lamin B, may be the ancestors of the cytoplasmic IF proteins (14, 61, 69). At the least, it is apparent that the cytoplasmic IF proteins and lamins do not have different ancestors (4).

Autoantibodies to the three major mammalian lamins have

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been found in the sera of patients with certain immunological disorders such as systemic lupus erythematosus (53), scleroderma (44), and autoimmune liver disease (34, 65, 70). These sera have proved to be useful reagents for the characterization of lamins in mammalian and other species (25, 42, 72, 73) and for the cloning of human lamins A and C (43). In this report we describe our use of a murine monoclonal autoantibody, called 72B9, to clone a nuclear lamin by using a λgt11 cDNA expression library prepared from the human leukemia T-cell line MOLT-4. A unique feature of this study is that the monoclonal antibody used reacts in immunofluorescence studies with the nucleolus and targets the M_r 34 protein component of the nucleolus-restricted U3 small nuclear ribonucleoprotein particle (54). We show, immunologically, that MOLT-4 cells express only lamin B and that the cloned cDNA encodes a protein reactive with anti-lamin B antibodies. In vitro translation revealed that the expressed protein requires a posttranslational modification to achieve the apparent molecular weight of native in vivo-synthesized lamin B. Several distinctive features of the deduced protein sequence of human lamin B and the other mammalian lamins are discussed.

MATERIALS AND METHODS

Cell lines, cDNA library, and Antisera. MOLT-4, HL60, and HeLa cell lines were obtained from the American Type Culture Collection, Rockville, Md., and were maintained in RPMI 1640 medium or Dulbecco modified Eagle medium containing 10% fetal calf serum. The MOLT-4 λgt11 cDNA expression library was constructed by K. Ogata and D. J. Noonan, Scripps Clinic and Research Foundation. Plasmid DNA M9, encoding the nuclear antigen SS-B/La, was obtained as previously described (5). Murine monoclonal autoantibody 72B9, generated from the spleen of a (NZB × NZW)F_1 lupus mouse, has been described previously (54). Guinea pig antibodies to rat lamins A and C and to rat lamin B were as described previously (17) and were the kind gift of Larry Gerace, Scripps Clinic and Research Foundation. Human sera containing polyclonal autoantibodies to lamin B or the nuclear protein SS-B/La were from the serum bank of W. M. Keck Autoimmune Disease Center, Scripps Clinic and Research Foundation, and were characterized by Western immunoblotting.

Antibody screening of cDNA library. The MOLT-4 cDNA library was screened by using antibody by the method of Young and Davis (74) with cell culture supernatant from hybridoma 72B9. All screening was done with duplicate filters, and positive bacteriophages were plaque purified. When polyclonal sera were used to assess the identity of expressed protein, they were first adsorbed against nitrocellulose filters lifts from RY1090 bacterial cultures to reduce nonspecific antibody binding to bacterial antigens. Adsorbed antibody and ^{125}I-protein A were diluted in phosphate-buffered saline (PBS)-Tween 20 containing 3% nonfat milk before use.

DNA hybridization screening of the cDNA library. The MOLT-4 cDNA library was screened essentially as described by Benton and Davis (2). Hybridization conditions were as described for genomic analysis (see below). The large (1.3-kilobase-pair [kb]) DNA insert of LAM-1 was isolated by *Eco*RI digestion and agarose gel electrophoresis, and radiolabeled probe was prepared by using random primers and Klenow fragment.

Subcloning of DNA fragments. Plaque-purified lambda phages from the MOLT-4 cDNA library screenings were used to infect 100-ml cultures of *Escherichia coli* LE392 cells, and lambda DNA was isolated and purified as described previously (41). cDNA inserts were excised from lambda DNA and cloned into pBluescript (KS−) by using the *Eco*RI restriction site. Following transformation (21) and growth in DH5α cells, plasmid DNA was purified on a cesium chloride gradient for DNA sequencing.

DNA sequencing. Nucleotide sequence analysis of the plasmid DNAs were performed by the dideoxy method of Sanger et al. (56) and the double-stranded sequencing procedure of Chen and Seeburg (8). The major portion of the sequencing was done with the DNA polymerase Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). Regions difficult to resolve were sequenced with *Taq* polymerase (Promega Biotec, Madison, Wis.) at 70°C. M13 sequencing primer was obtained from U.S. Biochemical. T3, T7, and additional 18-, 19-, and 20-mer oligonucleotide primers were synthesized by using a DNA synthesizer (model 380B; Applied Biosystems, Inc., Foster City, Calif.) and β-cyanoethyl chemistry.

Genomic analysis. Genomic DNA was prepared from HeLa cells and human blood leukocytes, and 20-μg aliquots were digested to completion with restriction enzymes and separated by electrophoresis through 0.8% agarose. Fractionated DNA was transferred to a nylon membrane (ONCOR, Gaithersburg, Md.) by the method of Southern (60). To prepare a cDNA probe, pLAM-2 DNA was cut with restriction enzymes *Eco*RI and *Acc*I to yield a 720-base-pair (bp) fragment beginning 50 bp 3′ to the TAA stop codon and extending to the 3′ end of pLAM-2. The fragment was then purified by agarose gel electrophoresis, and radiolabeled cDNA was prepared by using random primers and Klenow fragment. The Probe was hybridized to the nitrocellulose blot at 42°C with 50% formamide and then washed three times in 0.1% sodium dodecyl sulfate (SDS)–0.15 mM sodium citrate–0.015 M NaCl at 55°C. Washed blots were exposed to X-ray film for 18 h.

Northern (RNA) analysis. Total cellular RNA was isolated from cultures of HeLa, MOLT-4, and HL60 cells by the single-step method of Chomczynski and Sacchi (9). Approximately 10 μg of each of the total cellular RNAs was fractionated by electrophoresis through 1% agarose containing 2.2 M formaldehyde and transferred to nitrocellulose. The nitrocellulose blot was hybridized for 15 h at 42°C with 50% formamide to a radiolabeled cDNA probe containing the 2 kb of LAM-2 clone sequence 3′ to the *Eco*RI site and washed at 60°C as described above.

In vitro translations. RNA was transcribed from 1 μg each of pLAM-2 and pM9 DNA with T3 polymerase (Stratagene Inc., La Jolla, Calif.) after linearization with *Bam*HI, *Dra*I, or *Ssp*I.

Electrophoresis in 0.8% agarose–2.2 M formaldehyde gels was used to confirm that the RNA transcripts were the same size as the parent cDNA. Before translation in vitro, transcribed RNAs and cellular RNAs were phenol extracted and ethanol precipitated. A 1-μg portion of either pLAM-2 or pM9 RNA transcript was added to a 50-μl mix of rabbit reticulocyte lysate containing amino acids (Promega Biotec), [^{35}S]methionine (ICN Radiochemicals, Irvine, Calif.), and RNasin (Stratagene), and the reaction mixture was incubated at 30°C for 1 h. In separate experiments, EDTA, RNase A (Sigma Chemical Co., St. Louis, Mo.) and cold L-methionine were added to translation mixtures as described in Results. To measure the incorporation of mevalonolactone into lamin B, 50 μCi of *RS*-[5-^{3}H(N)]mevalonolactone (37.9 Ci/mmol; Du Pont Co., Wilmington, Del.) in ethanol was vacuum dried in a 1.5-ml microcentrifuge tube

before addition of rabbit reticulocyte lysate containing amino acids including cold L-methionine, RNasin, and appropriate pLAM-2 RNA transcripts. The reaction mixture was incubated at 30°C for 2 h. Total translations (5 to 20 μl) were separated by electrophoresis through SDS–15% polyacrylamide gels and then subjected to fluorography and autoradiography.

Immunoprecipitation of in vitro translations. Protein A-Sepharose-facilitated immunoprecipitation by antibody was performed as described previously (6), with the following minor modification. Antibodies adsorbed to protein A-Sepharose were mixed with 5 to 20 μl of radiolabeled translation product in a total volume of 500 μl and then washed and analyzed by SDS-polyacrylamide gel electrophoresis (15% acrylamide) (PAGE), or two-dimensional gel electrophoresis (see below).

Immunoprecipitation of HeLa cell lamin B. For analysis on two-dimensional gels, lamin B was immunoprecipitated from HeLa cell extracts with anti-lamin B antiserum as described previously (45). Briefly, five confluent 100-mm dishes of cells were washed twice with PBS and then scraped into 5 ml of 0.4% SDS–5 mM triethanolamine (pH 7.4)–0.1 M NaCl–2 mM EDTA. This cell lysate was heated at 60°C for 10 min and then cooled, sonicated briefly, and clarified by centrifugation. To the supernatant were added, in the following order, 0.5 ml of 20% SDS, 0.05 ml of Trasylol, 0.01 ml of a solution containing 1 mg each of leupeptin and pepstatin per ml in dimethyl sulfoxide, and 0.25 ml of protein A-Sepharose beads to which had been adsorbed 25 μl of anti-lamin B antibodies. The mixture was incubated for 8 h at 4°C, and then the beads were washed five times with 0.5% Triton X-100–0.1% SDS–0.05 M triethanolamine (pH 7.4)–0.1 M NaCl–2 mM EDTA and twice with 0.01 M triethanolamine (pH 7.4) to remove unbound material. Protein remaining bound to the beads was eluted with 8 M urea, 2% ampholytes, 5% β-mercaptoethanol and 1% Nonidet P-40 at 37°C for 15 min and stored at −80°C until use.

Two-dimensional gel electrophoresis. HeLa cell and in vitro-translated lamin B preparations were loaded onto the acidic end of 3-mm-diameter NEPHGE tube gels containing 2% Ampholines (LKB, Bromma, Sweden) and overlaid with a solution of 2 M urea and Ampholines (0.2% and pH 5 to 8, 0.2% and pH 3 to 10, 0.2% and pH 2 to 11). Gels were run for 10 min at 200 V, 20 min at 300 V, and 5 h at 400 V. Following electrophoresis, the gels were equilibrated with Laemmli sample buffer and layered onto an SDS–15% polyacrylamide gel, overlaid with agarose, run at a constant current of 20 mA per gel through the stacking gel, and then resolved at 25 to 30 mA per gel. Unless gels were to be used for Western blot transfers (see below), they were stained with Coomassie blue, and gels containing radioactively labeled in vitro-translated lamin B were fluorographed, dried, and exposed against X-ray film.

Western blotting. Whole-cell suspensions were obtained from cultures and washed with PBS, mixed with Laemmli sample buffer, and sonicated to reduce viscosity due to DNA. Samples were electrophoresed on SDS-15% polyacrylamide gels and transferred to nitrocellulose as described previously (66). Two-dimensional gel transfers were stained with Ponceau red, and those containing in vitro translation products were then autoradiographed. Before immunoblotting, two-dimensional transfers were rinsed in PBS to remove the Ponceau red. Immunoblotting was performed with antibodies diluted in PBS-Tween 20 containing 3% nonfat milk powder. Bound antibody was detected with ^{125}I-protein A.

Computer analysis of nucleic acid and protein sequences. DNA and protein sequences were analysed by the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package (Version 5.3) (11). Data bases used were the NBRF. Protein (Release 19.0, December 88) and NBRF.Nucleic acid (Release 34.0, November 88). Lamin protein sequences were aligned by using the CLUSTAL4 software as described by Higgins and Sharp (23a).

RESULTS

Cloning and sequencing of lamin cDNA. Murine monoclonal antibody 72B9 was used to screen 510,000 plaques from a MOLT-4 λgt11 expression library. Five plaques showed positive reactions, but only one retained reactivity through plaque purification. Excision of this cDNA (LAM-1) with *Eco*RI revealed two fragments, of 260 and 1,300 bp. Both fragments were cloned into Bluescript plasmid DNA, with the larger fragment being further restricted by using *Pst*I, *Xba*I, and *Sph*I, sites and these fragments were also cloned into Bluescript. cDNA fragments were sequenced from their termini, and the deduced amino acid sequences were used to search the National Biomedical Research Foundation (NBRF) protein data bank for homologous sequences. Close sequence homology was found with human lamins A and C, suggesting that the cloned cDNA encoded a laminlike protein. This cDNA, however, lacked both an ATG start site and a poly(A) tail, indicating that it was not a full-length clone.

Screening additional plaques with the ^{32}P-radiolabeled 1,300-bp fragment of LAM-1 resulted in the isolation of a 2,872-bp cDNA (LAM-2). This insert was cloned into Bluescript by using both partial and complete *Eco*RI digestion, resulting in three different insert sizes, a complete 2,872-bp insert (pLAM-2) and two partial fragments of 770 bp (pLAM-2a) and 2,102 bp (pLAM-2b). These clones were used to obtain the complete nucleotide sequence of this cDNA in both directions.

LAM-2 cDNA contains a 341-nucleotide 5′ untranslated region followed by a 1,758-nucleotide coding sequence and an 873-nucleotide 3′-untranslated sequence. The complete nucleotide sequence and its deduced amino acid sequence are shown in Fig. 1. A 16-nucleotide sequence beginning at nucleotide −12 contains the first ATG triplet preceded by an almost perfect Kozak consensus sequence for initiation of translation in vertebrates (27, 28). A second, in-frame, ATG begins at nucleotide +22. This is also preceded by a sequence which resembles the Kozak consensus sequence. A polyadenylation site (AATAAA) begins 17 nucleotides upstream from a 22-nucleotide string of A's at the 3′ end of LAM-2, which we believe begins the poly(A) tail. An internal *Eco*RI restriction site begins at nucleotide residue +426, which explains the presence of two fragments when both LAM-1 and LAM-2 are cut from λ DNA.

Several features of the primary protein sequence of LAM-2 are also shown in Fig. 1. The sequence KRKRV, which has features of a nuclear localization signal, occurs at amino acid residues 415 to 419. Consistent with other lamin sequences, except lamin C, the carboxy terminus of human lamin B ends in a conserved sequence motif, C(A/S)IM. LAM-2 contains alanine instead of serine.

The intermediate filament character of the lamin B sequence is clearly indicated by the three regions of heptad repeats (coils 1A, 1B, and 2) (Fig. 1) which are characteristic of the α-helical domains of all IF proteins. Also found, toward the end of coil 2 at residues 380 to 384, is the

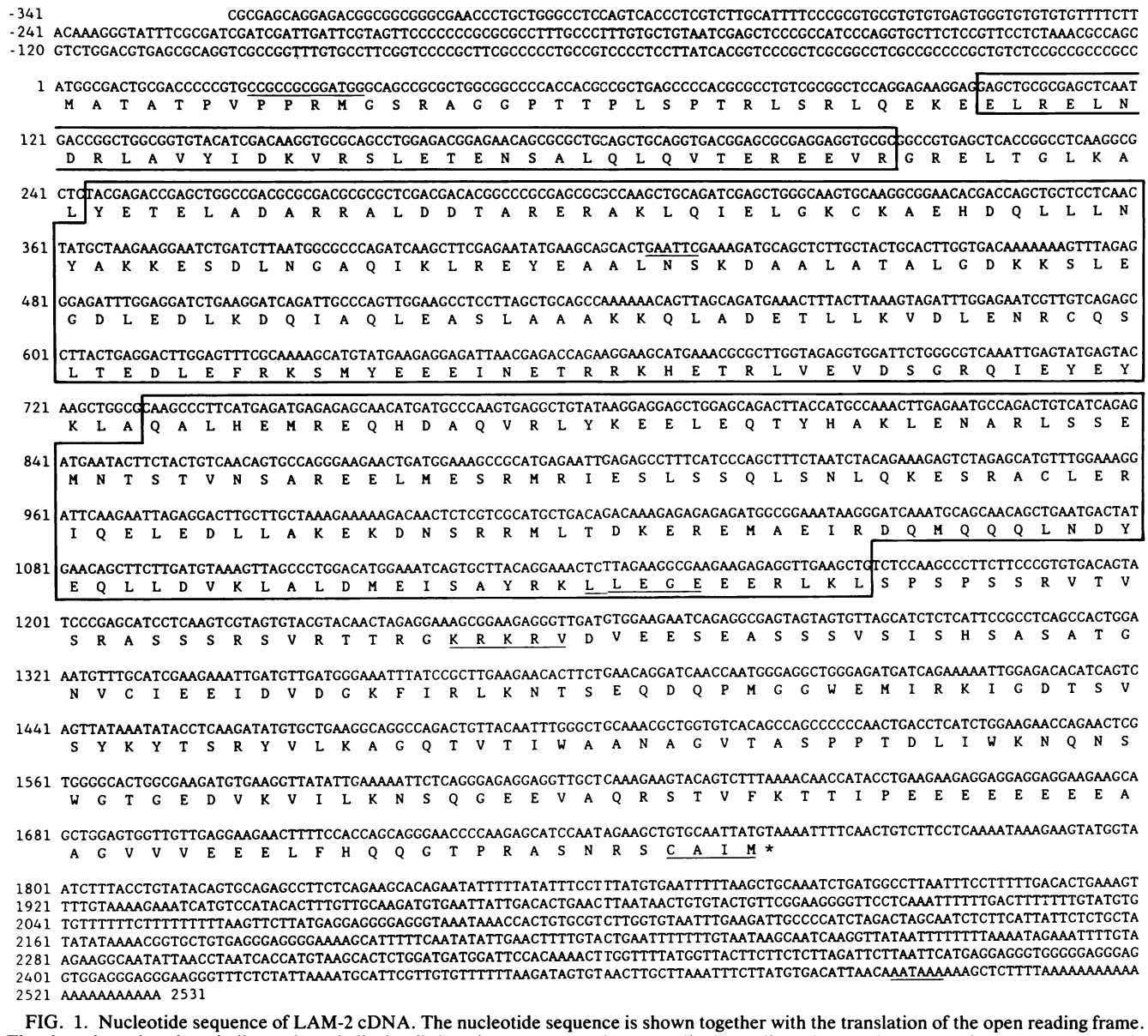

FIG. 1. Nucleotide sequence of LAM-2 cDNA. The nucleotide sequence is shown together with the translation of the open reading frame. The three boxed regions indicate the α-helical coil domains 1A, 1B, and 2 respectively. Indicated toward the end of coil 2 in the protein sequence is the LLEGE pentapeptide, which is well conserved among IF proteins. Also underlined within the protein sequence is the nuclear localization signal KRKRV and the C-terminal consensus sequence CAIM. Features of the nucleotide sequence indicated are a second Kozak consensus sequence for initiation of translation beginning at nucleotide +22 and the internal *Eco*RI restriction site at residue +426. The polyadenylation signal is also shown underlined.

sequence LLEGE, which is well conserved among IF proteins (4). Another striking feature of the lamin B sequence is an acidic carboxy tail which contains a string of eight glutamic acid residues (552 to 559).

Identification of the encoded protein as lamin B. Beginning at the first ATG, a long open reading frame encodes a 586-amino-acid protein with a calculated molecular weight of 66,334. Comparison of this sequence with that of other nuclear lamins (Fig. 2) revealed a 72% similarity with human lamins A and C, a 70% similarity with amphibian lamin L_{III}, and a 55% similarity with *D. melanogaster* lamin LMD_0. This is in sharp contrast to the 85% similarity with amphibian lamin L_I and a 97% similarity with the protein sequence of the mouse FML11-1 cDNA.

Since proteins encoded by amphibian lamin L_I or mouse FML11-1 cDNAs have not been analyzed by using anti-lamin B-specific antibodies, we sought to confirm our encoded protein as lamin B by immunoprecipitation of the in vitro-translated product from LAM-2 RNA with anti-lamin B antibodies. Figure 3 shows the translated products from pLAM-2 and pM9 (SS-B/La) RNA (Fig. 3A) and the immunoprecipitation of these polypeptides by a variety of antibodies (Fig. 3B and C). Translation of LAM-2 RNA resulted in two polypeptides of approximately 71 and 68 kilodaltons, both of which were precipitated by anti-lamin B antibodies only. Neither anti-lamin A and C antibodies, nor monoclonal antibody 72B9 reacted with the lamin B polypeptides. As expected from the control M9 RNA product, only anti-SS-B/La antibodies precipitated this approximately M_r 47 polypeptide. Final confirmation of the identity of LAM-

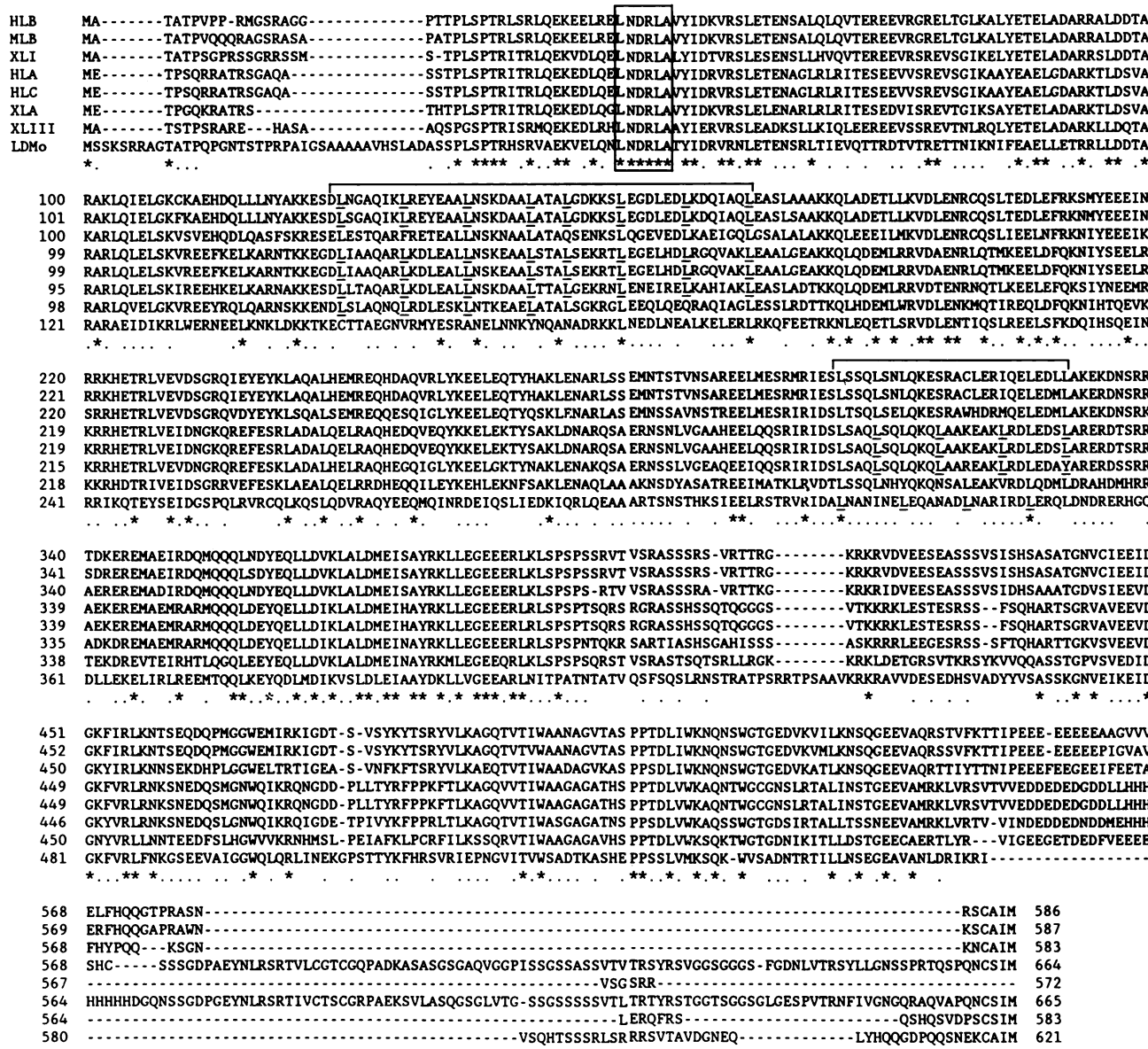

FIG. 2. Comparison of lamin protein sequences. Shown are the protein sequences of HLB (human lamin B), MLB (mouse lamin B), XLI (*Xenopus* lamin L_I), HLA (human lamin A), HLC (human lamin C), XLA (*Xenopus* lamin A), XLIII (*Xenopus* lamin L_{III}) and LMD_0 (*Drosophilia* lamin). Sequences were aligned by using the CLUSTAL4 programme of Higgins and Sharp (23a). Gaps introduced by the alignment are shown as dashed lines. Beneath the aligned sequences an asterisk indicates an identical match in all eight sequences, whereas a period indicates a conservative match in all eight sequences. The conserved sequence LNDRLA is boxed, and the regions containing leucine heptad repeats are bracketed with the leucines underlined in the respective sequences.

2-expressed protein as lamin B came from comparison of the in vitro-translated product with HeLa cell lamin B following immunoprecipitation with anti-lamin B antibodies and two-dimensional gel electrophoresis (Fig. 4). In vitro-translated lamin B and HeLa cell lamin B migrated to the same acidic pI and were resolved as proteins of equivalent molecular weight. The ability of anti-lamin B antibodies to immunoprecipitate both the M_r 71 and M_r 68 polypeptides clearly demonstrates that both are in-frame products of the LAM-2 sequence.

In vitro processing of lamin B. Several explanations could account for the appearance of two in vitro translation products of LAM-2: the use of the two ATG start codons (Fig. 1), inefficient termination of translation, or a posttranslational processing even similar to that described for chicken lamin B_2 (68). The first of these alternatives was tested by cleaving the 3′ end of LAM-2. Cleavage with *Dra*I, which removes the C-terminal 40 amino acids, resulted in a single protein band (Fig. 5A), suggesting that aberrant initiation of translation was not the cause of the two translation products. Cleavage of the LAM-2 sequence with *Ssp*I, which removes all but 87 nucleotides of 3′ untranslated sequence, still gave two protein products (Fig. 5A), indicating that an intact C terminus was required for the production of the two products but that the 3′ untranslated sequence was largely unnecessary.

The EDTA-sensitive in vitro posttranslational event recently described for chicken lamin B_2 (68) involves the processing of a higher-molecular-weight form to a lower-molecular-weight form. The in vitro translation of human

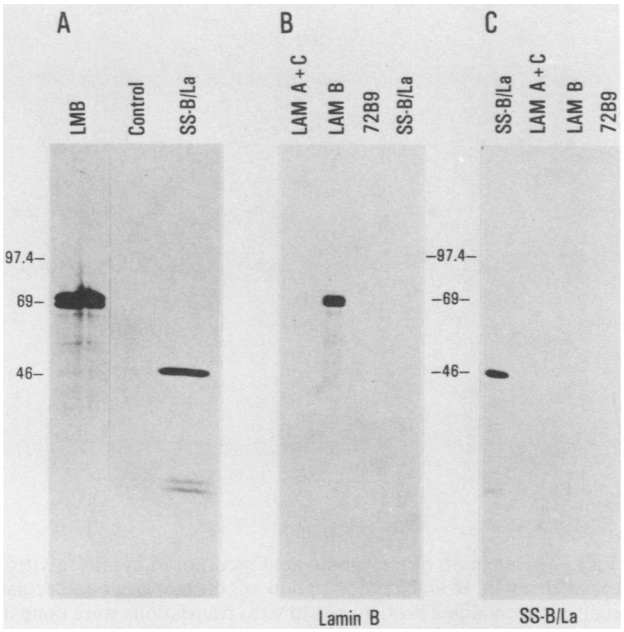

FIG. 3. In vitro translation and immunoprecipitation of lamin B. (A) In vitro translations of RNA transcripts from pLAM-2 and pM9 (SS-B/La) cDNAs resolved by SDS-PAGE. Lamin B (LMB) produced a doublet of M_r 71 and 68, while SS-B/La gave a single predominant band at M_r 47. The in vitro translation extract in the absence of RNA contained no translation products (Control). (B and C) Antibodies to lamins A and C (LAM A+C), anti-lamin B (LAM B), monoclonal antibody 72B9, and antibodies to the nuclear protein SS-B/La were used to immunoprecipitate in vitro-translated lamin B (B) and SS-B/La (C), and the immunoprecipitates were resolved by SDS-PAGE.

lamin B was also sensitive to EDTA (Fig. 5B). However, in contrast to chicken lamin B_2, the presence of 1 mM EDTA during in vitro translation of human lamin B completely blocked the appearance of the higher-molecular-weight protein. Comparison of the accumulation of the two protein bands after 15 and 120 min of in vitro translation showed an increase in the higher-molecular-weight form with time (Fig. 5C). Addition of 1 mM EDTA after 15 min of translation reduced the intensity of the higher-molecular-weight band, suggesting that the lower-molecular-weight protein was undergoing a posttranslational modification, resulting in an increase in apparent molecular weight.

The relationship between the two in vitro-translated lamin B products was examined in a pulse-chase experiment in which cold L-methionine and RNase A were added after 15 min of in vitro translation and the fate of the radiolabeled protein was monitored over the subsequent 105 min. The level of the lower-molecular-weight form gradually decreased over time, whereas that of the higher-molecular-weight form increased (Fig. 6), clearly demonstrating a precursor-product relationship. Control experiments (results not shown) in which RNA was added after the cold L-methionine and RNase A additions failed to produce radiolabeled protein, thereby confirming that radiolabeled products were produced only during the first 15 min of incubation.

The requirement of the posttranslational modification for an intact C terminus suggested that this may be the site of the modification. In common with a number of proteins, notably Ras, most lamin sequences end in a Cys-A-A-X box, where Cys is cysteine, A is an aliphatic amino acid, and X is any amino acid (38). The presence of a Cys-A-A-X box identifies

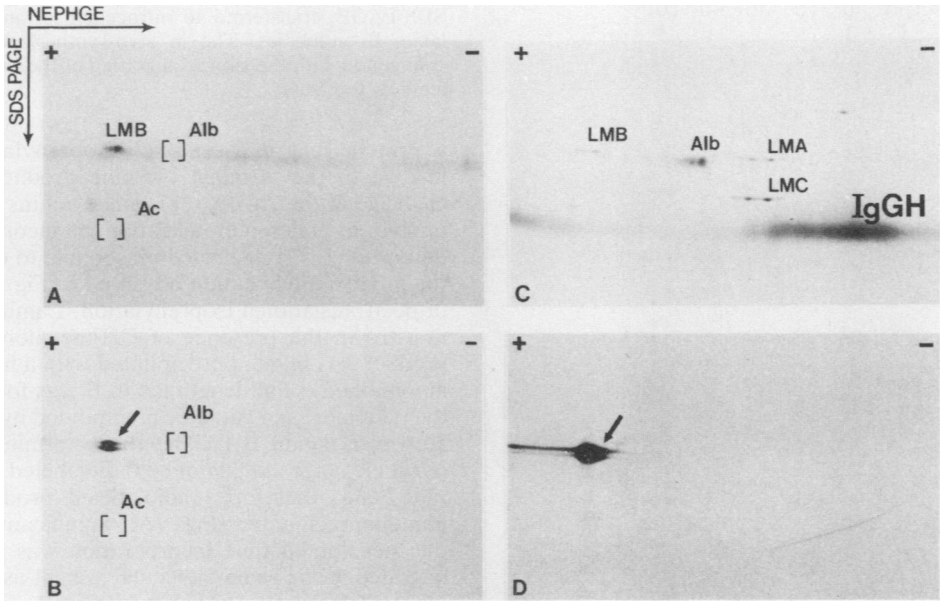

FIG. 4. Two-dimensional gel analysis of HeLa and in vitro-translated lamin B. (A and B) HeLa cell and [^{35}S]LAM-2 in vitro-translated lamin B were purified by immunoprecipitation with anti-lamin B antibodies. The purified HeLa lamins (A) and LAM-2 translation products (B) were run separately on NEPHGE/SDS-PAGE gels with added protein markers actin (Ac) and serum albumin (Alb). Separated proteins were transferred to nitrocellulose and stained with Ponceau red to identify markers. HeLa cell lamin B (panel A) was identified with guinea pig anti-lamin B antibodies. Before immunoblotting, the Ponceau red was removed by a PBS wash. The nitrocellulose transfer containing LAM-2 translation product (panel B) was autoradiographed following Ponceau red staining. (C and D) To eliminate ambiguities due to running separate gels, purified HeLa lamins were mixed with ^{35}S-labeled LAM-2 translation product. Panel C shows the Coomassie blue-stained HeLa proteins, and panel D the autoradiograph of the same gel showing the LAM-2 translation product. Abbreviations: LMA, LMB, and LMC, lamins A, B, and C, respectively; Alb, albumin; IgGH, heavy chain of immunoglobulin G.

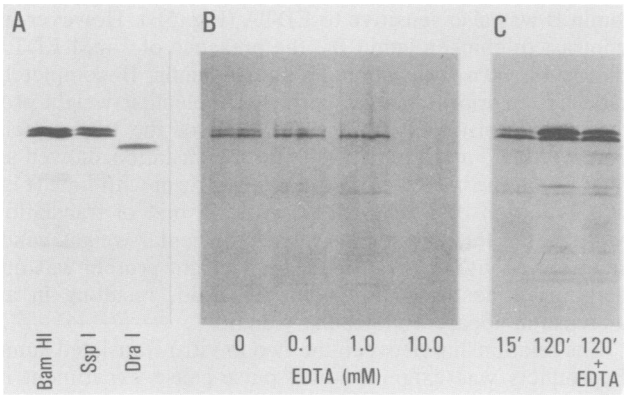

FIG. 5. Processing of in vitro-translated lamin B. (A) pLAM-2 cDNA was cleaved with *Bam*HI, *Ssp*I, or *Dra*I to allow transcription of RNA containing the complete 3′ untranslated region (*Bam*HI), 87 nucleotides of the 3′ untranslated region (*Ssp*I), or lacking the sequence for the C-terminal 40 amino acids (*Dra*I). RNA transcripts were then translated in vitro in the presence of L-[^{35}S]methionine and resolved by SDS-PAGE. (B) The effect of EDTA on in vitro translation of full-length lamin B (*Bam*HI) was determined by adding increasing concentrations of EDTA to the translation mix before RNA addition. (C) The effect of time and EDTA on the accumulation of the two lamin B products was examined by halting translation after 15 min (15′) or 120 min (120′), or by the addition of 1 mM EDTA after 15 min and then halting translation at 120 min (120′ + EDTA).

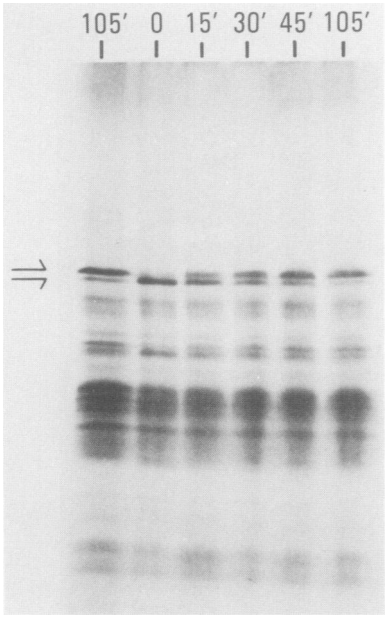

FIG. 6. Demonstration of a precursor-product relationship between the two in vitro-translated lamin B products. Full-length lamin B RNA was translated for 15 min in the presence of L-[^{35}S]methionine, and then unlabeled L-methionine (2 mM) and RNase A (200 μg) were added to halt further translation of radiolabeled protein. The fate of the already radiolabeled lamin B products was then monitored over time by sampling the incubation mixture at 15-min intervals. Time zero identifies the sample taken immediately after the addition of unlabeled L-methionine and RNase A.

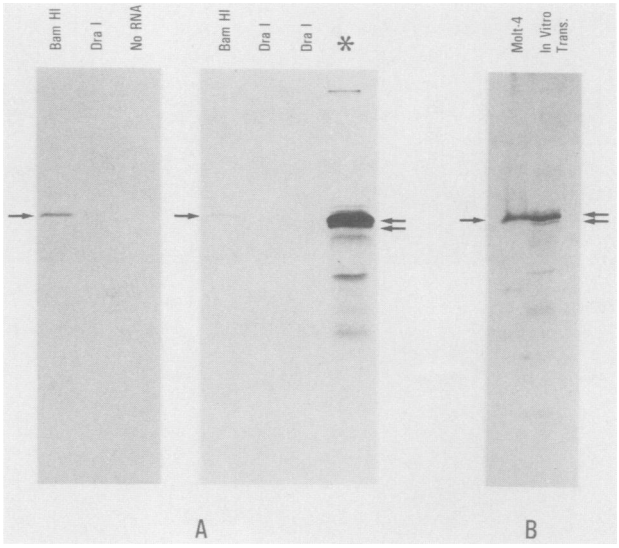

FIG. 7. Detection of mevalonic acid incorporation into in vitro-translated lamin B and determination of the apparent molecular weight of the modified protein. (A) In vitro translations were done in the presence of [^{3}H]mevalonolactone, and total translations or immunoprecipitations were resolved by SDS-PAGE. The left-hand segment shows the single band produced following translation of full-length lamin B RNA (*Bam*HI). No radiolabeled proteins were found following translation of RNA from *Dra*I-cut LAM-2, or in the absence of RNA. The right-hand segment shows the immunoprecipitation with anti-lamin B antibodies of proteins in the above translations. As expected, only the full-length lamin B translation (*Bam*HI) yielded an immunoprecipitable product. When compared with [^{35}S]methionine-labeled in vitro-translated full-length lamin B (*), the mevalonic acid-modified protein comigrated with the slower migrating of the two ^{35}S-labeled products. (B) MOLT-4 cell lysate and [^{35}S]methionine in vitro-translated lamin B were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-lamin B antibodies. The in vivo-synthesized MOLT-4 lamin B comigrated with the slower migrating of the two in vitro-translated lamin B products.

a protein that may undergo isoprenylation via thioester linkage to the terminal cysteine produced by proteolytic cleavage of the A-A-X (38). Since lamins have been shown, in vivo, to undergo modification via incorporation of mevalonic acid (1, 71) it seemed reasonable to determine whether the in vitro modification observed in Fig. 6 was the product of posttranslational isoprenylation. Lamin B was translated in vitro in the presence of [^{3}H]mevalonolactone, and the product was immunoprecipitated with a human anti-lamin B autoantibody. Full-length lamin B was found to incorporate the radiolabel and could be precipitated by the autoantibody. However, lamin B lacking the C-terminal 40 amino acids (*Dra*I cleavage) could not be radiolabeled with mevalonolactone, and, therefore, radiolabeled product could not be immunoprecipitated (Fig. 7A). Significantly, the product of the mevalonolactone incorporation was a single band that migrated at the same molecular weight as the higher-molecular-weight in vitro-translated lamin B.

To confirm that posttranslationally modified lamin B is the correlate of native in vivo-synthesized lamin B, radiolabeled in vitro-translated lamin B was compared by SDS-PAGE with lamin B of MOLT-4 cells detected by Western blotting. Both proteins migrated at equivalent molecular weight (Fig. 7B).

The in vitro posttranslational modification of lamin B is therefore due, at least in part, to the incorporation of a

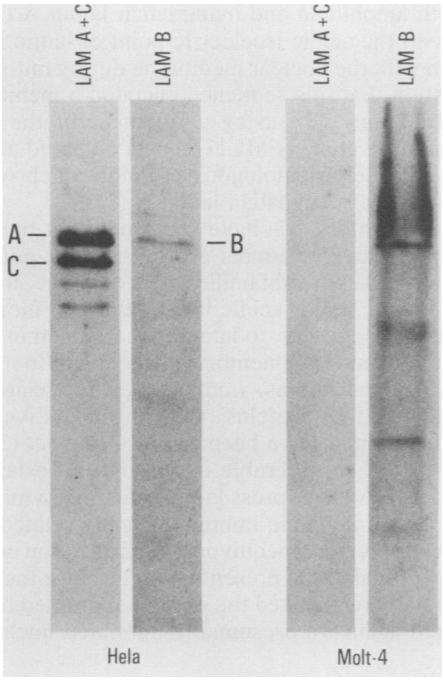

FIG. 8. Detection of lamins in human MOLT-4 and HeLa cells. Sonicated total-cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose before detection of lamins with guinea pig antibodies to lamins A and C (LAM A+C) and lamin B (LAM B). A, B, and C indicate lamins A, B, and C, respectively.

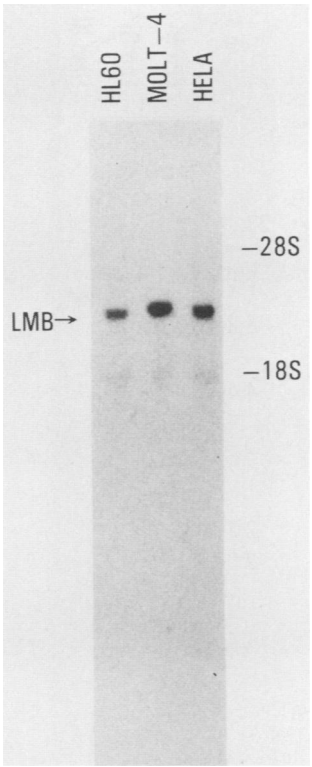

FIG. 9. Northern (RNA) analysis. Total cellular RNA was isolated from human cell lines HL60, MOLT-4, and HeLa. Following agarose-formaldehyde gel electrophoresis and transfer to nitrocellulose, blots were probed with the 3′-^{32}P-labeled 2-kb fragment of LAM-2 DNA. The position of lamin B (LMB) mRNA is indicated, as are the positions of rRNA markers (28S and 18S).

mevalonic acid derivative, requires an intact C terminus, and produces a protein with an apparent molecular weight equivalent to that of native in vivo-synthesized lamin B.

MOLT-4 cells express only lamin B. Several studies have shown that there is differential expression of nuclear lamins A and C in lymphocyte and other cell lines, but that lamin B is always present (20, 35, 48, 49, 62). Since MOLT-4 cells are a T lymphocyte-derived cell line, we reasoned that they may contain only lamin B. Immunofluorescence studies with whole-cell preparations (not shown) and Western blot analysis with whole-cell extracts showed the absence of lamins A and C in MOLT-4 cells (Fig. 8). In contrast, HeLa cells contained all three lamins.

Lamin B mRNA expression in human cell lines. The Western blot and immunofluorescence studies described above confirm the constitutive expression of lamin B and the differential expression of lamins A and C that have been reported by others. To determine whether a single species of lamin B is present in human cell lines known to express either lamin B alone or all three major lamin species, total cellular RNA was probed with the 3′ 2-kb LAM-2 fragment produced by *Eco*RI restriction of pLAM-2 (Fig. 9). For each of the three cell lines examined, MOLT-4, HL60, and HeLa, a single band of 3 kb was identified. No additional bands were seen with the HeLa cell RNA, even though this cell line does express lamins A and C.

Genomic analysis. Human lamin B appears to be encoded by a single gene (Fig. 10). Total human DNA cut with *Eco*RI or *Hin*dIII yielded a single band at approximately 6.2 and 8.2 kb, respectively. The sequence of the DNA probe used in this study should be highly specific for the lamin B gene, and on the basis of the intron positions of other IF proteins (18), this sequence should not be interrupted by intervening sequences. The presence of a single band indicates that this sequence occurs only once and that a single gene encodes lamin B.

Features of lamin protein sequences. In comparing other lamin sequences with that of human lamin B, we observed several conserved sequence motifs (Fig. 3). The most highly conserved region in the eight lamin sequences shown in Fig. 3 and in the chicken lamins A, B_1 and B_2 (50, 68) and mouse lamin C (55) is a hexapeptide, LNDRLA, which is found at the beginning of coil 1A. This hexapeptide was also found in four type 1 keratins (human keratin K 14, mouse keratin K 10, bovine 54K cytoskeletal keratin, and sheep 48K microfibrillar keratin) and the cytoskeletal IF-like A protein of the nematode *Ascaris lumbricoides* (69). A less well conserved sequence, PLSPTR (24), precedes the coil 1A region in all lamins except *Xenopus* L_{III} and chicken lamin B_1.

An additional feature of the lamin sequences is the presence in coil 1B, in all three mammalian lamins, amphibian lamin A, and (not shown) chicken lamin A of a leucine heptad repeat which is separated by three residues from another, less consistent leucine heptad repeat; only five residues separate the first and second leucines in this second repeat. The first leucine heptad repeat also occurs in the *Xenopus* L_{III} lamin and (not shown) the chicken lamin B_1. Another leucine heptad repeat is found in coil 2 of the mammalian lamins A and C and (not shown) chicken and mouse lamin A. In the amphibian lamin A, the position of the fourth leucine in the heptad repeat is occupied by a tyrosine. A leucine heptad repeat also occurs in coil 2 of the *Drosophilia* lamin LMD_0, but it is slightly out of register with that of the type A lamins. One striking feature of these leucine

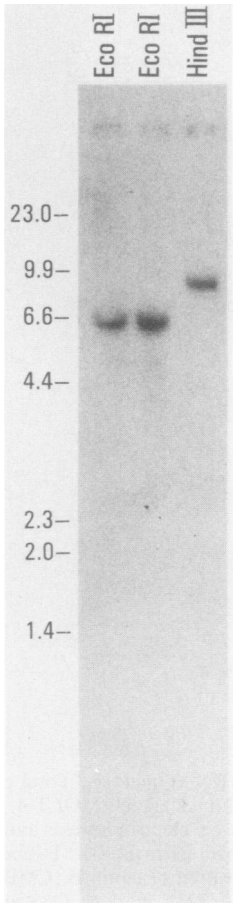

FIG. 10. Southern blot analysis of human DNA with the 3′ probe of LAM-2. Aliquots (20 μg) of HeLa cell (left lane) and human leukocyte DNA were digested with an excess of the indicated restriction endonucleases. DNA fragments were resolved by electrophoresis through 0.8% agarose and then transferred to a nylon membrane. The blot was hybridized with ^{32}P-probe prepared from a 720-bp fragment released from LAM-2 following digestion with *Eco*RI and *Acc*I. The fragment extended from residue 2152 to the 3′ end of the LAM-2 sequence.

heptad repeats is their central location within the respective coils.

DISCUSSION

Using a monoclonal autoantibody which was previously shown to be reactive with the nucleolar antigen fibrillarin (46, 54), we have isolated clones from a human T-cell cDNA library coding for a nuclear lamina protein which we have shown to be lamin B. Owing to reports of acidic lamins of both the major A and B types as well as minor species (see below), unambiguous identification of the lamin encoded by LAM-2 cDNA was sought not only from biochemical evidence but also by recognition of the translated product by lamin B-specific antibodies. This adds supporting evidence from immunological identification that this cDNA-encoded protein is indeed lamin B.

Previous studies, which have described the homologous B-type lamins of the mouse and amphibian, used biochemical and protein sequence data for identification. In the first of these studies it was found that the cDNA of *X. laevis* lamin L_I encoded a protein sequence considerably different from that of both amphibian and mammalian lamin A (31). This, together with the acidic isoelectric point of lamin L_I and its association with the nuclear membrane during mitosis, led to the suggestion that this sequence encoded a member of the lamin B subfamily. By using a fragment of the lamin L_I cDNA, a murine clone (FML11-1) was obtained and shown to encode a closely homologous protein, which was therefore considered a mammalian lamin B (24).

Some investigators (37) have cautioned that the physicochemical properties of lamins are not a clear guide to the classification of lamin subfamilies. For example, it has been shown that some major acidic lamins that are more closely related, immunologically, to lamin A than to lamin B remain associated with nuclear membrane fragments (64). In addition, minor acidic lamins, immunologically related to the major lamins and IF proteins but with distinctive protease digestion fragments, have been found in the rat (26, 51). It was therefore of considerable importance to establish that our cloned cDNA did express lamin B and not a minor lamin species or an acidic lamin immunologically related to lamin A. The necessity for unequivocal identification was made even more critical in the present study because the antibody which originally recognized the protein expressed by LAM-1 was directed against a presumably unrelated nucleolar protein.

The evidence in support of the protein expressed by LAM-2 as lamin B is fourfold. First, in vitro-translated protein from LAM-2 RNA was immunoprecipitated by antibodies against mammalian lamin B, but not by antibodies against the other major mammalian lamins, A and C. Second, the MOLT-4 cell line, which was the source of mRNA for the cDNA library, was shown by Western blotting and immunofluorescence to express only lamin B. Third, analysis of the mRNA expression of several human cell lines showed the presence of a single 3-kb species in cell lines previously shown to express only lamin B (HL60 [49]) or all three major lamins (HeLa [20, 49]). This mRNA expression was supported by detection of protein by Western blot and immunofluorescence. Finally, the amino acid sequence of LAM-2 was significantly different from that of the immunologically identified products of cDNAs encoding mammalian and amphibian lamin A sequences. These observations therefore provide further support, by virtue of immunological specificity and other data, that the cDNA for lamin L_I of *X. laevis* (31) and the murine cDNA FML11-1 (24) encode lamin B.

In vitro translation of RNA transcribed from pLAM-2 cDNA resulted in two prominent proteins bands, both of which were immunoprecipitated by anti-lamin B antibodies. Changing the amount of RNA transcript added to the translation mix did not affect the expression of the two proteins (data not shown), nor did cleavage within the 3′ untranslated region of the sequence. However, cleavage with *Dra*I to produce RNA lacking the coding sequence for the C-terminal 40 amino acids resulted in the synthesis of a single protein band, suggesting that the second protein band was the result of posttranslational processing requiring the C terminus of lamin B.

The recently described in vitro processing of chicken lamin B_2 (68) and that described here for human lamin B share several features. Both are sensitive to EDTA, and a clear precursor-product relationship can be demonstrated. Unlike chicken lamin B_2, however, human lamin B undergoes an increase rather than a decrease in apparent molecular size as judged by SDS-PAGE.

In vivo posttranslational modifications such as phosphor-

ylation (45), methyl esterification (7) and ADP-ribosylation (59) of lamin B have been described; however, none of these appear to require an intact C terminus. Lamin B has also been shown to undergo a novel type of posttranslational modification involving incorporation of a product of mevalonic acid (1, 71). For such a modification to occur, an intact C-terminal consensus sequence termed the CAAX box (39) is required. When [^{3}H]mevalonolactone was added to rabbit reticulocyte lysate, intact lamin B could be shown to be radiolabeled, but the C-terminal-deficient protein produced by *Dra*I cleavage could not. Immunoprecipitation of the labeled protein showed that the posttranslational modification occurred only to the more slowly migrating of the two in vitro-translated lamin B products. In addition, in vivo-synthesized lamin B of MOLT-4 cells comigrated with the same slower-migrating protein band.

The functional significance of the attachment of a mevalonic acid derivative to lamin B awaits elucidation. However, since Ras proteins require attachment of a C-terminal mevalonic acid derivative to localize into the plasma membrane (22, 38), a similar modification may enable lamin B to attach the nuclear lamina to the nuclear envelope (1, 38, 71). This is supported by recent studies with *Xenopus* lamin L_I (the amphibian lamin B), which clearly demonstrate the requirement of the conserved cysteine in the lamin B CAAX box for nuclear envelope association (30).

The chemical identification of the mevalonic acid derivative attached to lamin B is unknown. Recent studies suggest that farnseol, a derivative of mevalonate, is the isoprenoid involved in this novel posttranslational modification of mammalian cell proteins (38, 40). The ability to achieve such a modification in the rabbit reticulocyte lysate system may aid in the identification of the attached moiety.

Our studies indicate that the lamin B described here is the major, more probably the only, lamin B expressed in human somatic cells. Thus, it appears unlikely that the lamin B, which is the only major lamin, in HL60 cells (49) and T lymphocytes (20) is different from lamin B expressed in cell types (e.g., HeLa) which also express lamins A and C, because each contains a single mRNA of equivalent size that hybridizes to LAM-2 cDNA. This is supported by two-dimensional gel electrophoresis of immunoprecipitated lamin B, which showed that the LAM-2-encoded protein migrated identically with the lamin B of HeLa cells. In addition, Southern blotting suggests that a single gene contains the coding information for lamin B. Furthermore, the anti-lamin antibodies used in this study were able to identify a single protein of equivalent molecular weight in both MOLT-4 and HeLa cells.

Many of the features of the lamin B sequence (24, 31) and of lamin protein sequences in general, particularly those identifying lamins as members of the IF family of proteins (13, 36, 43, 47), have been noted before and will not be further discussed here. However, we do wish to note several sequence motifs which appear to have gone unreported.

As members of the IF protein family, the lamins contain several regions of heptad repeats. It has long been recognized that heptad repeats are a classic feature of an α-helix and that the interlocking of two α-helices generates a coiled-coil structure which promotes stabilization in many different types of proteins (reviewed in reference 10). However, unlike cytoplasmic IF proteins, most of the lamins contain one or more leucine heptad repeats, with the number and location of these leucine repeats appearing to distinguish between A- and B-type lamins (Fig. 3). This "periodic array of at least four leucines," or leucine zipper (32), was originally proposed as a motif representing the dimerization domain of a new class of DNA-binding proteins. Subsequent studies have shown that this region is indeed necessary for dimerization but does not appear to be directly involved in DNA binding (29, 33, 57, 58, 67). Whether the leucine heptad repeats in lamins are involved in lamin dimerization and/or interaction with other nuclear proteins (12) is unclear. The significance of leucine residues as opposed to other hydrophobic amino acids in these heptad repeats is not yet known; however, it should be noted that the leucine heptad repeat found in coil 1B is contained within the additional six-heptad repeat that characterizes these nuclear IF proteins from their cytoplasmic counterparts.

The one additional striking feature of the lamin sequences is the conserved hexapeptide LNDRLA (Fig. 3). The significance of this sequence is unclear at present, but it is the most highly conserved region in all lamin sequences so far described. The presence of this region in four type 1 keratins may indicate a structure-function relationship with lamins.

The relationship between lamin B and monoclonal antibody 72B9 remains unexplained. Monoclonal antibody 72B9 immunoprecipitates the nucleolus-restricted U3 small nuclear ribonucleoprotein particles (46); however, it could not immunoprecipitate in vitro-translated lamin B. In the initial selection from the MOLT-4 cDNA library, we found that the protein expressed by LAM-1 was recognized by 72B9 and a polyclonal human serum containing autoantibodies to lamin B, but not by the guinea pig antibodies used in this study (data not shown). LAM-1 does not contain coding sequence for the N-terminal 55 amino acids or the C-terminal 18 amino acids of lamin B. Attempts to immunoblot fusion protein from LAM-1 cDNA following SDS-PAGE have so far been unsuccessful.

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