Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 4374–4378, July 1983
Cell Biology

Autoimmune response directed against conserved determinants of nuclear envelope proteins in a patient with linear scleroderma

(rheumatic diseases/nuclear lamins/*Drosophila* nuclear lamins/IgG clonality)

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Communicated by Bruce M. Alberts, April 25, 1983

ABSTRACT **We have studied the autoantibodies in the serum of a patient with linear scleroderma that specifically recognize the nuclear envelope of cultured cells. These antibodies bind to conserved determinants of nuclear lamins, the predominant mammalian nuclear envelope proteins. Of the three mammalian nuclear lamin proteins (P70, P68, and P60), only P70 and P60 bind the autoantibodies. In addition, two proteins of the *Drosophila* embryonic nuclear matrix, P70 and P68, bind these autoantibodies. We have used nuclear matrices to isolate the autoantibodies from the patient's serum that react to the nuclear lamins. At least three different IgG heavy chains were found to be involved in this autoimmune response to nuclear lamins, indicating that this response is not due to the expansion of a single B-cell clone.**

A strong correlation exists between systemic rheumatic diseases and the presence of circulating autoantibodies to protein–nucleic acid complexes (1). The targets of these autoantibodies include nuclear ribonucleoprotein particles (2), nucleosomes (3), nucleoli (4), kinetochores (5), and others. The range of targets for particular disease syndromes are often limited; for example, systemic lupus erythematosis is often associated with autoantibodies to nuclear ribonucleoprotein particles (2), whereas most scleroderma CREST patients produce autoantibodies to centromeric regions of chromosomes (kinetochores; ref. 5). Surprisingly, little is known of the autoimmune responses or their targets on a molecular level.

In an attempt to associate the immunofluorescence pattern of sera from patients with specific autoimmune diseases, we discovered a high-titer serum directed against the nuclear envelope in a patient with linear scleroderma. We designate this serum as LS-1. We have chosen here to characterize this autoimmune response more precisely by identifying the antigens recognized by this serum and by determining the nature and complexity of the antibody response. We have found that this autoimmune response is polyclonal and directed against two of the three predominant polypeptides of the nuclear envelope, known as nuclear lamins (6).

Using immunofluorescence and immunotransfers of Na$DodSO_4$ gels, we also have detected nuclear lamins in *Drosophila melanogaster* embryonic nuclear matrices. This demonstrates that nuclear lamins are present in invertebrates and that these autoantibodies from LS-1 serum must be directed against highly conserved determinants.

MATERIALS AND METHODS

Autoantibodies. The autoantibodies of LS-1 serum were obtained from a 24-yr-old female patient with linear scleroderma. She had clinically typical, cutaneous linear sclerotic lesions involving the left arm and the posterior right calf and thigh. There were no clinical indications of systemic disease.

Tissue Culture Cells. Chinese hamster ovary (CHO) cells were originally obtained from the Cell Culture Facility at the University of California, San Francisco. They were maintained in α modified Eagle's medium (without nucleosides) supplemented with 5% fetal calf serum and 5% calf serum in a 10% CO_2/90% air incubator at 37°C.

D. melanogaster embryonic cells of the Kc line were grown at 26°C in Echalier's medium (7).

Immunofluorescence Microscopy. CHO and Kc cells were grown on glass coverslips (12-mm diameter, Corning), fixed in 1% formaldehyde in phosphate-buffered saline (P_i/NaCl) for 5 min at 22°C, and made permeable to antibodies by washing in 0.1% Triton X-100 (Sigma) in P_i/NaCl. The cells were first exposed to LS-1 serum diluted 1:200 and subsequently to a rhodamine-conjugated goat anti-human IgG (Cappel Laboratories, Cochranville, PA). Cells were photographed with a Zeiss photomicroscope with a ×100 planapo objective.

Immunohistochemical Microscopy. CHO cells were grown in polystyrene tissue culture dishes (Falcon) and processed by a standard immunoperoxidase technique (8). After postfixation with osmium tetroxide and dehydration in ethanol, the cells were embedded in a mixture of Epon and Araldite; 1-μm sections were obtained by using glass knives.

CHO Nuclear Matrix Preparation. CHO cells were grown to confluence on 150-mm diameter tissue culture dishes and extracted in cold P_i/NaCl containing 0.1% Nonidet P-40 (Sigma), 15 mM 2-mercaptoethanol, and 1 mM aminoacetonitrile as a protease inhibitor. The cells then were exposed to 10 units of micrococcal nuclease (Worthington) per ml in the same buffer with 10 mM $CaCl_2$ for 10 min at 22°C, followed by 3 M NaCl in a buffer containing 80 mM KCl, 5 mM EDTA, 0.1% Triton X-100, 15 mM 2-mercaptoethanol, 1 mM aminoacetonitrile, and 15 mM Pipes (pH 7.4), and pelleted at 2,000 × *g* for 10 min, and solubilized in Na$DodSO_4$ sample buffer.

***Drosophila* Embryonic Nuclear Matrix Preparation.** Two grams of *Drosophila* embryos from a 16-hr collection were dechorionated in 50% Chlorox for 90 sec. Embryonic nuclei were obtained essentially as described (9) by using four strokes of a glass homogenizer in 10 ml of an extraction buffer containing 50 mM Tris·HCl (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 250 mM sucrose, 15 mM 2-mercaptoethanol, and 1 mM aminoacetonitrile. The nuclei were separated from soluble material by two 10-min centrifugations at 1,000 × *g*. The pellet was resuspended in 5 ml of P_i/NaCl containing 5 mM $CaCl_2$, 20 units of micrococcal nuclease per ml, 15 mM 2-mercaptoethanol, 1 mM aminoacetonitrile, and 0.1% Triton X-100, followed by incubation for 15 min at 22°C. The preparation was then centri-

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Abbreviations: CHO cells, Chinese hamster ovary cells; P_i/NaCl, phosphate-buffered saline.

fuged for 10 min at 1,000 × *g*, and the pellet was resuspended in 3 M NaCl/0.1% Triton X-100/45 mM KCl/5 mM EDTA/15 mM Pipes, pH 7.2, for 5 min at 0°C. After centrifugation for 10 min at 3,000 × *g*, the pellet was solubilized in $NaDodSO_4$ sample buffer.

Isoelectric Focusing, $NaDodSO_4$ Gel Electrophoresis, and Protein Transfer. The CHO cell and *Drosophila* nuclear matrix preparations were fractionated by $NaDodSO_4$ gel electrophoresis alone or in combination with nonequilibrium pH gradient electrophoresis by using standard procedures (10–12). After fractionation, the proteins were electrophoretically transferred to nitrocellulose essentially as described (13). The nitrocellulose was then exposed to a 1:5,000 dilution of LS-1, followed by an ^{125}I-labeled (14) goat anti-human IgG Fc-fragment-specific IgG (Cappel Laboratories), dried, and subjected to autoradiography.

Immunoabsorption of Anti-Lamins IgGs. Nuclear matrices from 10^7 CHO cells were incubated for 1.5 hr in a 1:1,000 dilution of LS-1 serum and subsequently washed extensively by repeated centrifugation and resuspension in P_i/NaCl. The entire CHO nuclear matrix preparation, together with the absorbed antibodies, were processed for Western transfer as above.

RESULTS

Immunocytochemical Localization of the LS-1 Serum Antigen. The series of immunofluorescence and phase-contrast micrographs (Fig. 1) showed a cell cycle-dependent staining of CHO cells with LS-1 serum. Interphase cells (Fig. 1 *A* and *B*) showed a preferential staining of the nuclear envelope. This pattern of staining disappeared during chromosome condensation at prophase (Fig. 1 *C, D, E,* and *F*). There was virtually no staining of the nucleus at metaphase (Fig. 1 *G* and *H*), but it reappeared at telophase (Fig. 1 *I* and *J*). The observed staining coincided with the breakdown and reformation of the nuclear envelope during the cell cycle (15, 16). We could further confirm that LS-1 serum specifically recognizes the nuclear envelope of interphase cells by observing sectioned nuclei processed by immunoperoxidase techniques (Fig. 2). Thin sectioning clearly revealed the localization of the antibody to the nuclear envelope by demonstrating the reaction at the periphery of the nucleus.

LS-1 serum yielded an identical cell cycle-dependent immunofluorescence pattern in a *D. melanogaster* embryonic cell line (Fig. 3 *A* and *B*). The characteristic nuclear envelope staining was absent from the mitotic cell in the field (arrow in Fig. 3*B*).

Identification of the Polypeptide Antigens to LS-1 Serum. The immunofluorescence results above are similar to those of previous reports that used antibodies to nuclear lamins derived from heterologous immunization (6, 17, 18). To test the possibility that the target of these autoantibodies was the nuclear lamins, we isolated a crude preparation of nuclear matrices that could be prepared rapidly by a solid-phase extraction of CHO cells attached to plastic dishes. These extracted nuclei stained brightly with LS-1 serum with a pattern identical to that in Fig. 1. Although this preparation left a large amount of the intermediate filament protein, vimentin, and actin as determined by $NaDodSO_4$ gels (Fig. 4, lane 1), it was also highly enriched in the three nuclear lamin proteins (P70, P68, and P60). When a $NaDodSO_4$ gel of this preparation was electrophoretically transferred to nitrocellulose and probed with LS-1 serum, the major reacting proteins comigrated with the P70 and P60 lamins, also known as lamins A and C (Fig. 4, lane 2) (6). Only a slight recognition of a protein comigrating with the P68 lamin (lamin B) was detected. In addition, there appeared to be spe-

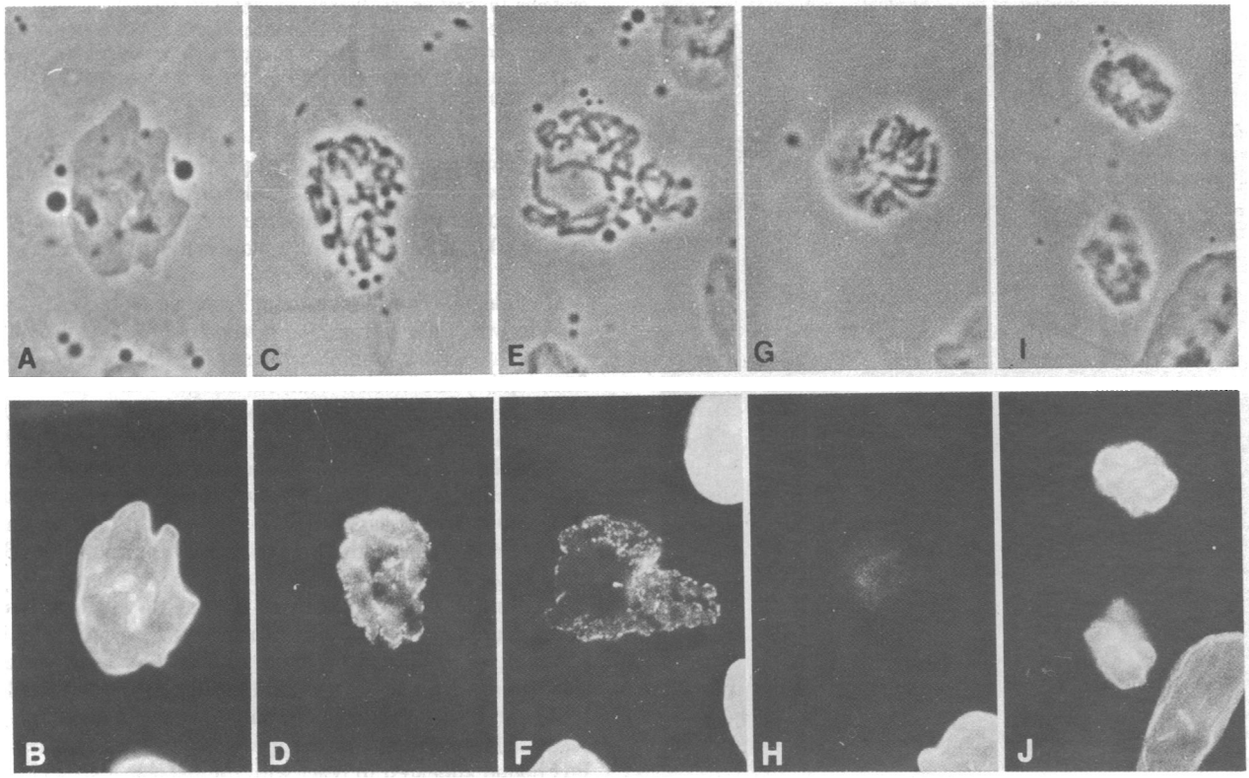

FIG. 1. Immunofluorescence localization of LS-1 serum in the CHO cell cycle. (*A, C, E, G,* and *I*) Immunofluorescence micrographs. (*B, D, F, H,* and *J*) Corresponding phase-contrast micrographs. (*A* and *B*) Interphase cell with preferential staining of the nuclear envelope. (*C, D, E,* and *F*) Granular staining pattern in prophase cells in which the nuclear envelope is disintegrating. (*E* and *F*) Mitotic cell and the lack of discrete staining with the serum. (*G* and *H*) Two daughter cells during reformation of the nuclear envelope and the coincident return of autoantibody staining. (×2,500.)

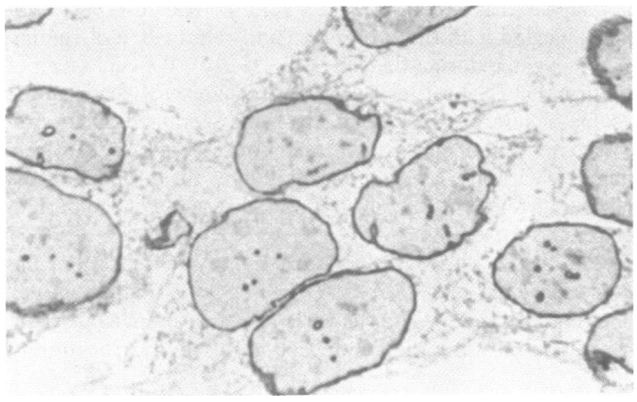

FIG. 2. Immunoperoxidase localization of LS-1 serum staining. CHO cells were fixed, permeabilized with detergents, and exposed to both the LS-1 serum and a peroxidase-conjugated goat anti-human IgG. The cells were then embedded in plastic and cut into 1-μm sections. The staining is restricted to the nuclear envelope or invaginations of the nuclear envelope. (×2,500.)

cific binding to a protein that migrated just above the P70 band at a molecular weight of 71,000.

To resolve the question of whether LS-1 serum weakly recognizes the P68 (B) lamin, the nuclear matrix preparation was first fractionated in one dimension by nonequilibrium pH gradient electrophoresis and in the second dimension by molecular weight by $NaDodSO_4$ gel electrophoresis. Such fractionation has been shown to separate the P70 and P60 lamins from the more acidic P68 lamins (Fig. 5*A*; ref. 19). When transferred to nitrocellulose and probed with LS-1 serum, it was clear that the P68 lamin was not recognized by LS-1 serum (Fig. 5*B*). A

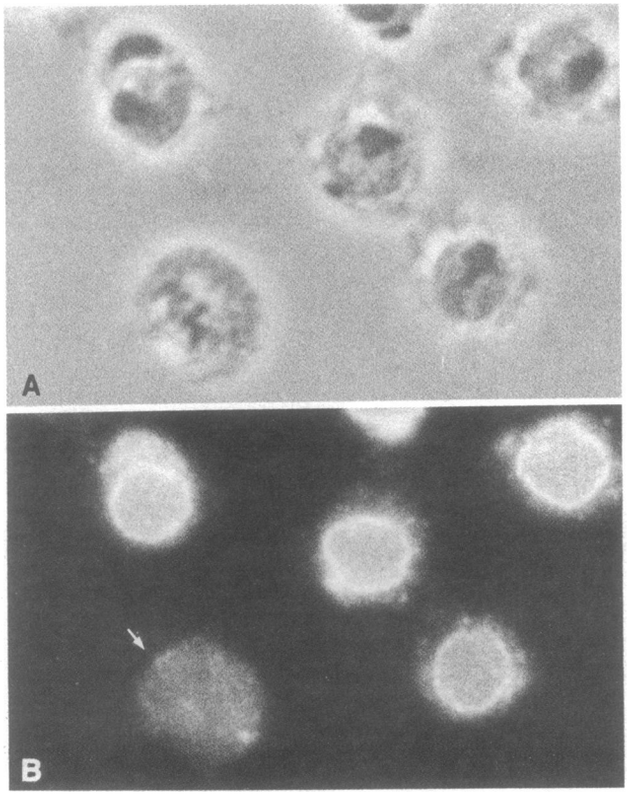

FIG. 3. Immunofluorescence detection of autoantibody binding to *Drosophila* embryonic Kc cells. (A) Immunofluorescence micrograph. (B) Corresponding phase-contrast micrograph. The arrow points to a mitotic cell that lacks discrete staining. (×2,500.)

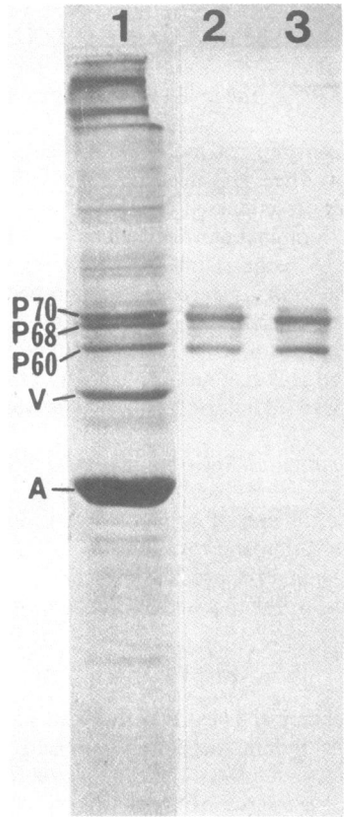

FIG. 4. Fractionation of CHO cell nuclear matrix proteins and immunological detection of LS-1 serum target antigens. Lanes: 1, Coomassie blue stain of the CHO cell nuclear matrix proteins fractionated on an 8.5% polyacrylamide gel: P70, P68, and P60 are the nuclear lamin proteins (migration at 70,000, 68,000, and 60,000 daltons, respectively), V is the intermediate filament protein vimentin, and A is actin; 2, autoradiograph of the corresponding protein transfer of the contents of lane 1, after probing with LS-1 serum. A reactive species migrated at ≈71,000 daltons, just above the P70 band.

series of species with similar charge properties appeared to migrate between the P70 and P60 lamins (Fig. 5*B*). This technique also detected a reproducible, discrete spot that migrated at ≈71,000 daltons (P71) with a more acidic pI than that of P70 lamin.

Homologous Nuclear Lamin Proteins Detected in *Drosophila* with LS-1 Serum. To further define the presence of nuclear lamins in *Drosophila*, nuclear matrices were prepared from embryonic nuclei. $NaDodSO_4$ gel electrophoresis revealed a reproducible doublet of bands migrating at 68,000 and 70,000 daltons (Fig. 6, lanes 1 and 2), which was not solubilized by 3 M NaCl. Treatment of the nuclear matrix with 3 M NaCl solubilized all but the P70/P68 doublet and a protein migrating at 65,000 daltons (Fig. 6, lane 2). When transferred to nitrocellulose and probed with LS-1 serum, only the doublet at 68,000 daltons was detected.

Analysis of the Complexity of the IgG to Nuclear Lamins in the Autoimmune Response. To analyze the number of IgG-secreting species produced in the autoimmune response to nuclear lamins, LS-1 serum was immunoadsorbed to the insoluble CHO cell nuclear matrix preparation, and unbound IgG was separated from the matrix by repeated centrifugation. The only IgG bound appeared to react with the nuclear matrix (Fig. 1). The specifically-bound autoantibodies, together with the entire nuclear matrix preparation, were fractionated in polyacrylamide gels in two dimensions and transferred to nitrocellulose. The transfer was probed with an ^{125}I-labeled goat anti-human

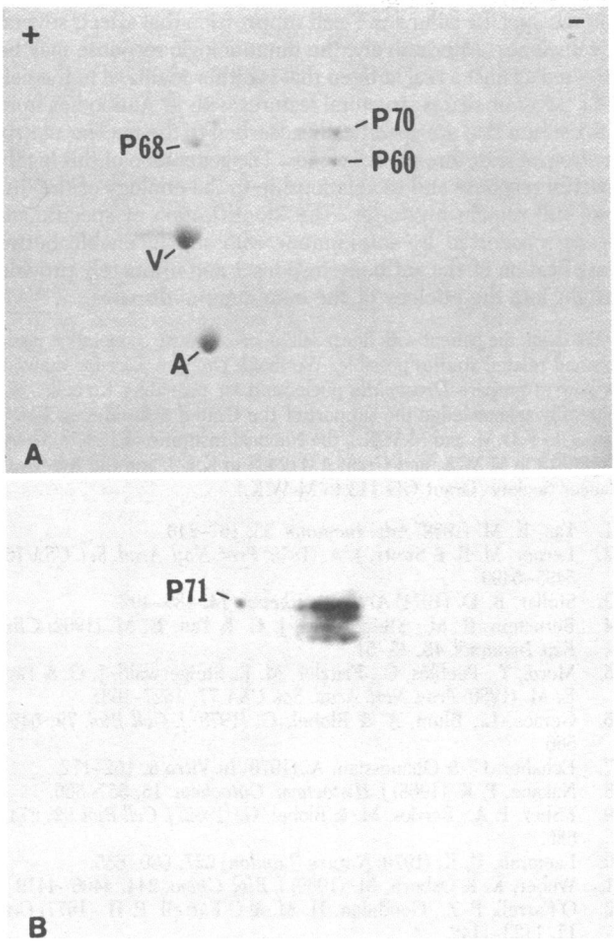

FIG. 5. Two-dimensional gel fractionation of CHO cell nuclear matrices and immunological detection of target antigens. (*A*) Coomassie blue-stained gel of CHO nuclear matrix preparation. P70, P68, and P60 refer to the nuclear lamin proteins; V is the intermediate filament protein vimentin; and A is actin. (*B*) Corresponding transfer of proteins in *A* after probing with LS-1 serum. Note the series of reactive species migrating between lamins P70 and P60. P71 refers to the polypeptide with an apparent molecular size of 71,000 daltons. The region containing the P68 lamins is devoid of antibody binding.

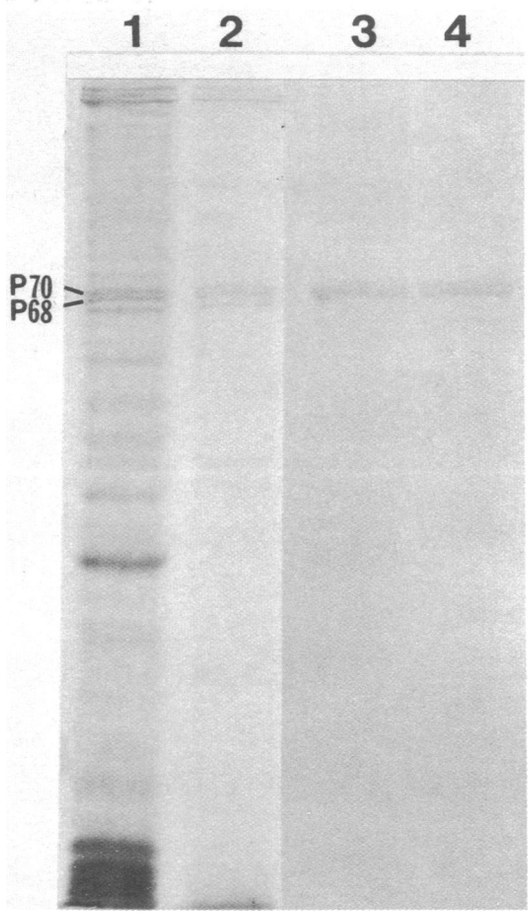

FIG. 6. Fractionation of *Drosophila* embryonic nuclear matrices and detection of LS-1 serum target antigens. In a Coomassie blue-stained 8.5% polyacrylamide gel of the *Drosophila* embryonic nuclear matrix (lanes 1 and 2), P70 and P68 refer to the proteins that migrate at 70,000 and 68,000 daltons. Lanes: 1, insoluble proteins of the *Drosophila* nuclei after treatment with micrococcal nuclease; 2, remaining insoluble proteins after treatment of the material of lane 1 with 3 M NaCl; 3 and 4, duplicates of lanes 1 and 2, respectively, after transfer to nitrocellulose and immunological detection of lamins with LS-1 serum.

IgG Fc-fragment (Fig. 7*B*). Three distinct families of spots were detected, probably representing at least three different heavy chains with differential glycosylation. The interchain disulfide bonds of the heavy chains appeared to be resistant to the reducing conditions used and, therefore, migrated as dimers of ≈100,000 daltons as reported by others (20, 21). Total LS-1 serum revealed only a smear of IgG heavy chains (Fig. 7*A*).

DISCUSSION

Various patterns of immunofluorescent staining have been detected in sera from patients with the scleroderma spectrum of autoimmune diseases. Linear scleroderma is a relatively rare form characterized by cutaneous linear sclerosis of an extremity or the scalp. The underlying muscle and bone are frequently affected. Clinical and serological features of systemic connective tissue disease often occur (22). We have shown that a 24-year-old female patient with linear scleroderma possesses a high-titer antiserum against the nuclear envelope. This serum specifically reacts with a 70,000- and a 60,000-dalton prominent protein in the nuclear envelope known as lamin A and lamin C, respectively.

The three nuclear lamin polypeptides (P70, P68, and P60) have been compared through immunological and peptide mapping techniques (23–26). Lamin polypeptides P70 and P60 appear to be similar and different from P68 by these criteria. This is supported by the observation that LS-1 serum reacts with both P70 and P60 and not P68 on two-dimensional immunotransfers. The heterogeneity of the P70 and P60 proteins is also clearly manifest on the immunotransfers. In addition, LS-1 serum reacts with a protein with a more acidic and discrete pI, which migrates at 71,000 daltons. The high specificity of LS-1 serum suggests that this protein is a member of the nuclear lamin family, but further experiments are needed to rule out the possibility that LS-1 serum has antibodies to nonlamin proteins. This P71 protein is a minor component of the interphase nuclear matrix, and it is not known whether it is a precursor or a modified form of the P70 and P60 lamins, or if it has some specific function.

The lamin proteins have been detected in vertebrate cells from mammalian, avian, and amphibian origin (27–29). Studies of isolated nuclear matrices in *Drosophila* previously have failed to reveal characteristic nuclear lamins (9). LS-1 serum reacts strongly with the nuclear envelope in *Drosophila*. Immunotransfers reveal the characteristic lamins at 70,000 and 68,000

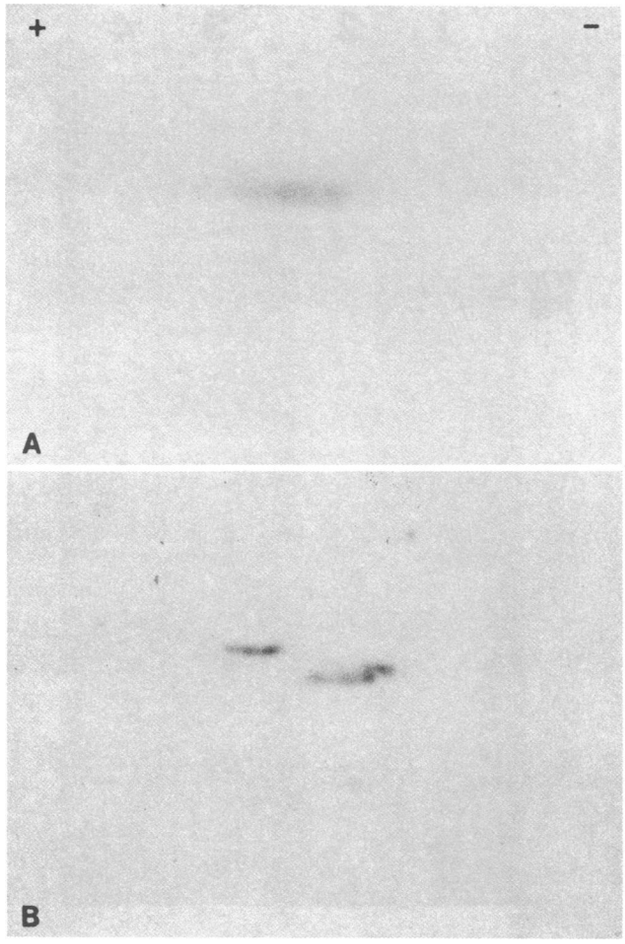

FIG. 7. (*A*) Immunological detection of total IgG heavy chains in LS-1 serum. Total LS-1 serum was fractionated in two dimensions, transferred to nitrocellulose, and probed with an ^{125}I-labeled goat anti-human IgG Fc fragment-specific antibody. (*B*) Immunological detection of the specific heavy chains involved in the nuclear lamins autoimmune response. Nuclear lamin autoantibodies were isolated from LS-1 by specific absorption onto CHO cell nuclear matrices as described. The CHO nuclear matrix, together with the absorbed IgGs, were fractionated in two dimensions on polyacrylamide gels, transferred to nitrocellulose, and probed with an ^{125}I-labeled goat anti-human IgG Fc fragment antibody. At least three families of heavy chain were detected.

daltons that are observable in rapidly isolated crude nuclear matrices (Fig. 4). This suggests a high conservation of some determinants of the lamin proteins that may represent functional domains in these proteins.

The expression of high-titer antibodies against specific cellular antigens in a variety of autoimmune diseases is baffling. Various models of the autoimmune response would depend on whether there is a clonal response from a single B-cell clone or whether the response is polyclonal, possibly reflecting some antigen-specific failure of T-cell suppression that affects several B-cell clones. Alternatively, the immunologic response may be directed against a real antigen that is either identical to the cellular target or shares structural features with it. Antibodies from LS-1 serum that are specifically adsorbed to the nuclear matrix are from at least three B-cell clones. The generation of this highly specific response and its relationship to the etiology of the disease still remain mysteries. The identification of specific antigens recognized by autoimmune sera should enable better classification of the antibody responses and ultimately provide insight into the etiology of the autoimmune diseases.

We thank the patient with linear scleroderma whose cooperation made this and related studies possible. We thank Dr. Tim Karr for showing us how to prepare *Drosophila* nuclei and for providing Kc cells. We gratefully acknowledge the support of the United Scleroderma Foundation (to F.D.M. and M.W.K.), the National Institutes of Health (Grant GM26875 to M.W.K. and Grant AM12433 to K.F.), and the American Cancer Society (Grant CD 113 to M.W.K.).

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