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Monoclonal antibodies against myofibrillar components of rat skeletal muscle decorate the intermediate filaments of cultured cells

(hybridoma/indirect immunofluorescence/myofibrils/Colcemid/perinuclear capping)

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ABSTRACT **Monospecific antibodies were produced *in vitro* by fusing mouse myeloma cells with spleen cells from a BALB/c mouse immunized with rat skeletal myofibrils. After cloning 3 times on agarose, two stable clones were obtained and chosen for further characterization. The first clone, JLB1, produced an antibody that recognizing an antigen distributed in the M-line region and on either side of the Z line of myofibrils. The second clone, JLB7, produced an antibody reacting only with an antigen located at the M-line region of myofibrils. Both JLB1 and JLB7 antibodies decorate the typical intermediate filaments of a variety of cultured cells. Colcemid treatment of cells before reaction with both antibodies resulted in the coiling or capping (or both) of the fibers around the nucleus. Brief treatment of cells with cytochalasin B did not affect the integrity of the fibers stained by both antibodies whereas, under the same conditions, microfilament bundles visualized by another monoclonal antibody (JLA20) against actin were disassembled into many aggregates in the cytoplasm. Identical staining patterns of the intermediate filaments are obtained by double-label immunofluorescence microscopy of the same cell stained with these monoclonal antibodies and rabbit autoimmune serum (which has been shown to react with the components of the intermediate filaments). By using immunoprecipitation, protein bands at 210,000 and 95,000 daltons from chicken embryo fibroblasts were identified as the potential antigens recognized by JLB1 and JLB7 monoclonal antibodies, respectively. The widespread occurrence of these antigenic determinants in different cultured cells suggests the highly conservative property of these intermediate-filament components.**

Monoclonal antibodies produced by the Köhler and Milstein technique (1) have the potential for enhancing the immunofluorescent localization of specific proteins in cells. In particular, they can be generated by immunization with complex mixtures of proteins as well as with highly purified proteins. As long as a given protein is antigenic, its injection into an experimental animal will lead to the presence in the spleen of antibody-producing cells of complementary specificity. Given the ability to screen large numbers of the resulting hybridomas, it should be possible to obtain clones that produce pure antibodies against minor proteins that often are difficult to separate from other proteins. Moreover, the natural immunity to structural proteins found in normal animals (2–4) does not interfere or mask the specific antibody. Equally advantageous is the fact that monoclonal antibodies can be produced continuously in large amounts and maintained with constant specificity. Thus they should be useful tools for the purification of antigens, in conjunction with affinity chromatography.

I have immunized a mouse by using the crude myofibril fraction from rat skeletal muscle. Spleen cells from the animal were fused with mouse myeloma cells to produce hybridoma clones. Then, they were screened for the production of antibodies that can show the fibrillar patterns on gerbil fibroma cells by indirect immunofluorescence. Here, I report on the properties of two stable hybridoma clones whose respective antibodies react not only with the specific myofibril components but also with the intermediate filaments in a variety of cultured cells.

MATERIALS AND METHODS

Cell Cultures. The cells used included gerbil fibroma cells (American Type Culture Collection 146), L8 myoblasts, neuroblastoma (N18TG2), chicken embryo fibroblasts, mouse 3T3 cells, human skin fibroblasts (202D13), BSC-1 cells, and PtK-1 cells. The cells were grown in Dulbecco's modified Eagle's medium/10% fetal calf serum. For indirect immunofluorescence, the cells were grown on 12-mm round glass coverslips for 1 or 2 days before use. (Gerbil fibroma cells, L8 myoblasts, neuroblastoma, and chicken embryo fibroblasts were obtained from K. Burridge. 3T3 cells, human skin fibroblasts, and BSC-1 cells were obtained from G. Albrecht-Buehler. PtK-1 cells were obtained from E. B. Lane.)

Preparation of Myofibrils. Rat back and leg muscles were cut into strips, tied to wooden applicator sticks, and stored in 50% glycerol (vol/vol) in relaxing buffer (0.1 M KCl/2 mM $MgCl_2$/2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)/1 mm dithiothreitol/10 mM Tris·HCl, pH 6.8) at −20°C for at least 1 week. Myofibrils were prepared from these glycerinated muscles by the method of Etlinger *et al.* (5) and stored in 50% glycerol in relaxing buffer at −20°C. For indirect immunofluorescence, the myofibrils were washed 3 times with phosphate-buffered saline (P_i/NaCl/KCl) (137 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/8 mM Na_2HPO_4, pH 7.3) and allowed to settle onto coverslips.

Cell Fusion. A female BALB/c mouse was immunized intraperitoneally with rat myofibrils homogenized in an equal volume of complete Freund's adjuvant. After 3 weeks, the mouse was boosted intraperitoneally with rat myofibrils suspended in P_i/NaCl/KCl. At the end of the 4th week, the mouse was given an intravenous injection with myofibrils in P_i/NaCl/KCl. Three days later, the animal was sacrificed and the spleen was used as a source of immune cells for fusion to the mouse myeloma cells P3/NS1/1-Ag4-1 (NS1; a generous gift of E. B. Lane). Cell fusion was carried out according to the method of Kennett *et al.* (6), except that no attempt was made to remove

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*,-tetraacetic acid; P_i/NaCl/KCl, phosphate-buffered saline; CPK, creatine phosphokinase.

erythrocytes from spleen cells before fusion, 40% polyethylene glycol was used during fusion, and the selective medium (hypoxanthine/aminopterin/thymidine) used after fusion was Dulbecco's modified Eagle's medium/20% fetal calf serum/100 μM hypoxanthine/25 μM thymidine/0.4 μM aminopterin. Indirect immunofluorescence was used to screen for positive clones whose supernatants gave filamentous staining patterns on gerbil fibroma cells. Agarose cloning (7) was used to obtain stable monoclones using gerbil fibroma cells as a feeder layer.

Indirect Immunofluorescence. The coverslips were fixed in 3.7% formaldehyde in P_i/NaCl/KCl for 10 min and rinsed first in P_i/NaCl/KCl and then in deionized water. The cells were then made permeable by treatment with acetone at −10°C for 5 min. The coverslips were rinsed again in deionized water and P_i/NaCl/KCl and overlaid with 20 μl of undiluted culture medium from each clone. They were incubated at 37°C in a humidified Petri dish for 30 min. After incubation, they were washed extensively with P_i/NaCl/KCl and stained with 20 μl of fluorescein-conjugated goat anti-mouse IgG (1:25 dilution) in a humidified Petri dish at 37°C for 30 min. After extensive washing in P_i/NaCl/KCl, the coverslips were mounted with gelvatol. To stain myofibrils, the acetone treatment was omitted. The cells or myofibrils were observed and photographed with epifluorescence optics in a Zeiss PMIII microscope as described by Blose (8).

Identification of Antigens. The cell extract was prepared from chicken embryo fibroblasts labeled *in vivo* with [^{35}S]methionine for 15 hr. After washing 3 times with P_i/NaCl/KCl, the culture was lysed in buffer A (15 mM sodium phosphate, pH 7.0/0.5% Triton X-100/1 mM EGTA/1 mM phenylmethylsulfonyl fluoride and passed through a no. 26 needle at least 6 times. The lysates were clearified by centrifugation at 100,000 × *g* for 2 hr.

To the lysates, 1 μl of monoclonal antibody from ascites fluid was added. After a 2-hr incubation at 4°C, 4 μl of rabbit anti-mouse IgM antibody (Litton Bionetics) was added to the mixture; this mixture was then incubated for another hour at 4°C. Finally, the immune complexes were incubated with 80 μl of formalin-fixed *Staphylococcus aureus* for 30 min and then pelleted in an Eppendorf centrifuge. The pellet was washed 3 times with buffer A. Small volumes of sample buffer for gel electrophoresis were added to the pellet to solubilize the labeled antigens, which were analyzed by 10% $NaDodSO_4$/polyacrylamide gel electrophoresis (9). Radioactive proteins in the gel were detected by fluorography (10) on Kodak XR-1 film.

RESULTS

The spleen cells from BALB/c mice immunized with rat skeletal myofibrils were fused with the nonsecreting variant (NS1) of mouse myeloma cells, which are resistant to 8-azoguanine and do not grow in the selective medium. After fusion, the hybrid cells would proliferate in the selective medium. The cultured medium from each hybrid clone was then used to screen for fibrillar patterns on gerbil fibroma cells by indirect immunofluorescence. This screening method detects only those hybrids that produce antibodies reacting with antigenic determinants

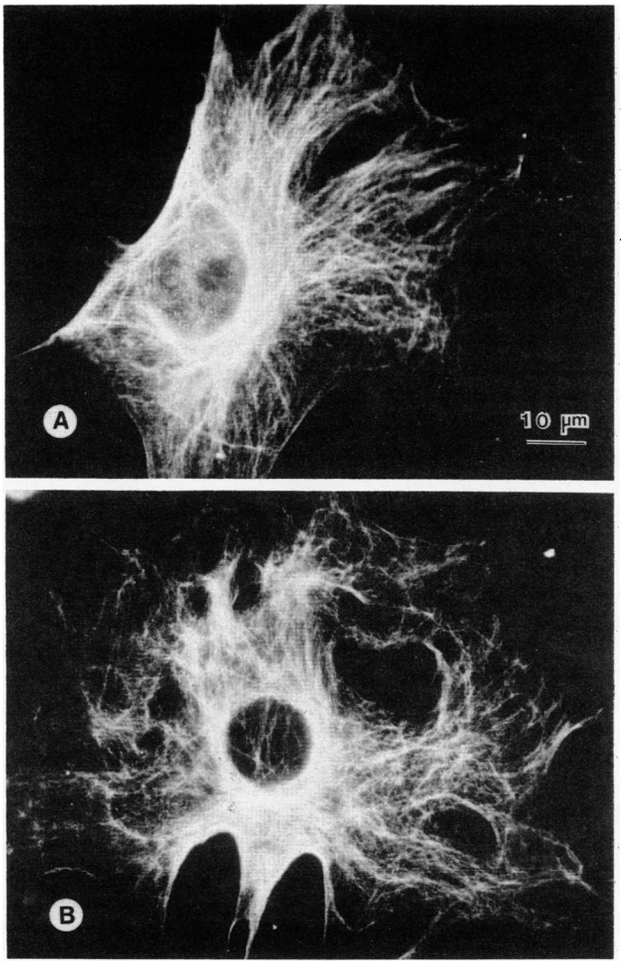

FIG. 1. Immunofluorescence micrographs of gerbil fibroma cells stained with JLB1 antibody (*A*) and JLB7 antibody (*B*).

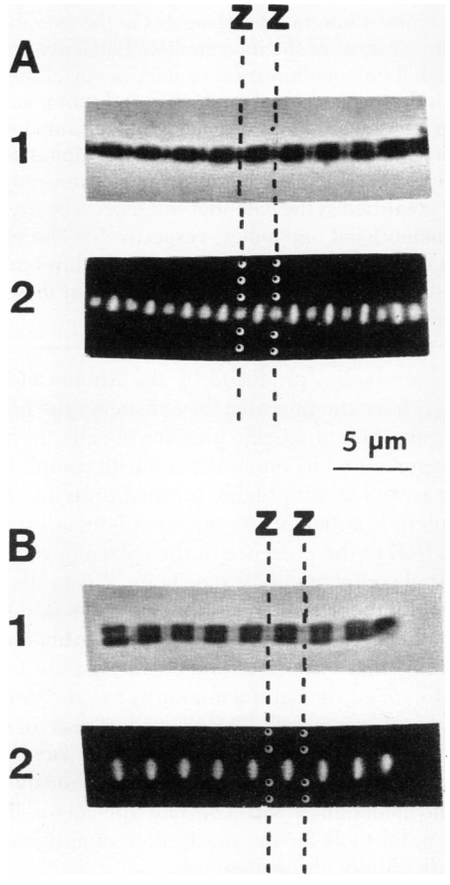

FIG. 2. Indirect immunofluorescence of myofibrils of rat skeletal muscle stained with JLB1 antibody (*A*) and JLB7 antibody (*B*). Lane 1, phase-contrast micrograph; lane 2, immunofluorescence micrograph.

shared by the myofibrils and the fibrillar components of these nonmuscle cells. Two stable clones, JLB1 and JLB7, were obtained, recloned three times, and further characterized. The immunofluorescence patterns obtained on gerbil fibroma cells by JLB1 and JLB7 antibodies are shown in Fig. 1. The fibers stained by both antibodies are concentrated in the perinuclear region; from here the tangled fibers extend into the peripheral cytoplasm. This distribution is similar to the immunofluorescence patterns of intermediate filaments shown by other investigators (3, 11, 12).

Novel staining patterns, however, were obtained on rat skeletal myofibrils (Fig. 2). JLB1 antibody reacts with an antigen distributed in the M-line region and on either side of Z-line myofibrils, while JLB7 antibody only recognizes an antigen located in the M-line region. Similar results were also obtained on rabbit myofibrils stained with both antibodies (data not shown). As both hybridoma cells have been cloned 3 times on agarose (7), it seems unlikely that either contains mixed antibody-producing hybrids.

The capping or coiling of fibers around the nucleus after treatment of cells with antimitotic drugs is a characteristic of the vimentin-type of intermediate filaments (3, 12–17). When gerbil fibroma cells are treated with 1 μM colcemid for 24 hr, the fibers that react with JLB1 and JLB7 coil tightly around the nucleus (Fig. 3 *A* and *B*). This suggests that both JLB1 and JLB7 antibodies recognize components associated with the vimentin-type intermediate filaments. However, under this condition, the microfilaments observed by a monoclonal antibody (JLA20) against actin appear to be not affected (see Fig. 3*C*). After a brief exposure to cytochalasin B (10 μg/ml for 30 min), the cells arborize but continue to show staining of intermediate filaments with both JLB1 and JLB7 (see Fig. 3 *D* and *E*). Under the same conditions, the microfilament bundles are disrupted and give rise to many aggregates visualized by a monoclonal antibody (JLA20) against actin (see Fig. 3*F*).

To further confirm that the filaments decorated by JLB1 and JLB7 monoclonal antibodies are indeed the intermediate filaments, rabbit autoimmune serum (a generous gift of K. Burridge) which has been shown to react with components of intermediate filaments (3), was used together with monoclonal antibodies for indirect double-label immunofluorescence microscopy. As shown in Fig. 4, the individual fibers decorated by rabbit autoimmune serum (see Fig. 4 *C* and *F*) are identical to those stained by JLB1 (see Fig. 4*B*) or JLB7 (see Fig. 4*E*) monoclonal antibody on the same human fibroblast cells. Therefore, I concluded that both JLB1 and JLB7 monoclonal antibodies react with components of the intermediate filaments of cultured cells.

Other cell types including chicken embryo fibroblasts, mouse 3T3 cells, rat L8 myoblasts, neuroblastomas, BSC-1 cells, and PtK-1 cells were tested with the JLB1 and JLB7 antibodies. All gave positive immunofluorescence with both antibodies and colcemid-induced coiling of the fibers.

By using immunoautoradiography on $NaDodSO_4$/poly-

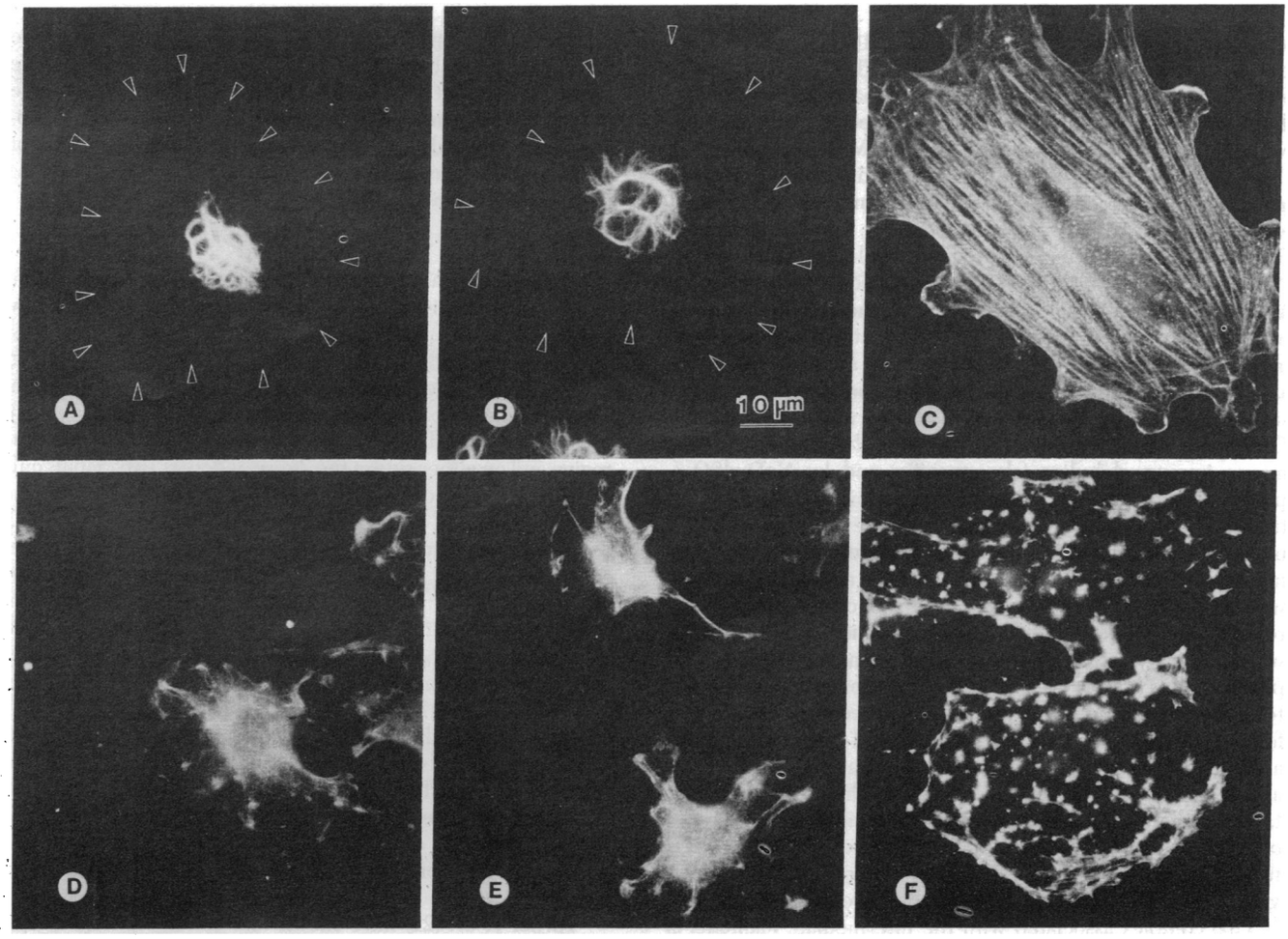

FIG. 3. Immunofluorescence micrographs of gerbil fibroma cells were treated either with colcemid (*A–C*) or with cytochalasin B (*D–F*) before reaction with JLB1 antibody (*A* and *D*), JLB7 antibody (*B* and *E*), or JLA20 antibody (*C* and *F*). Arrowheads in *A* and *B* indicate the cell margin.

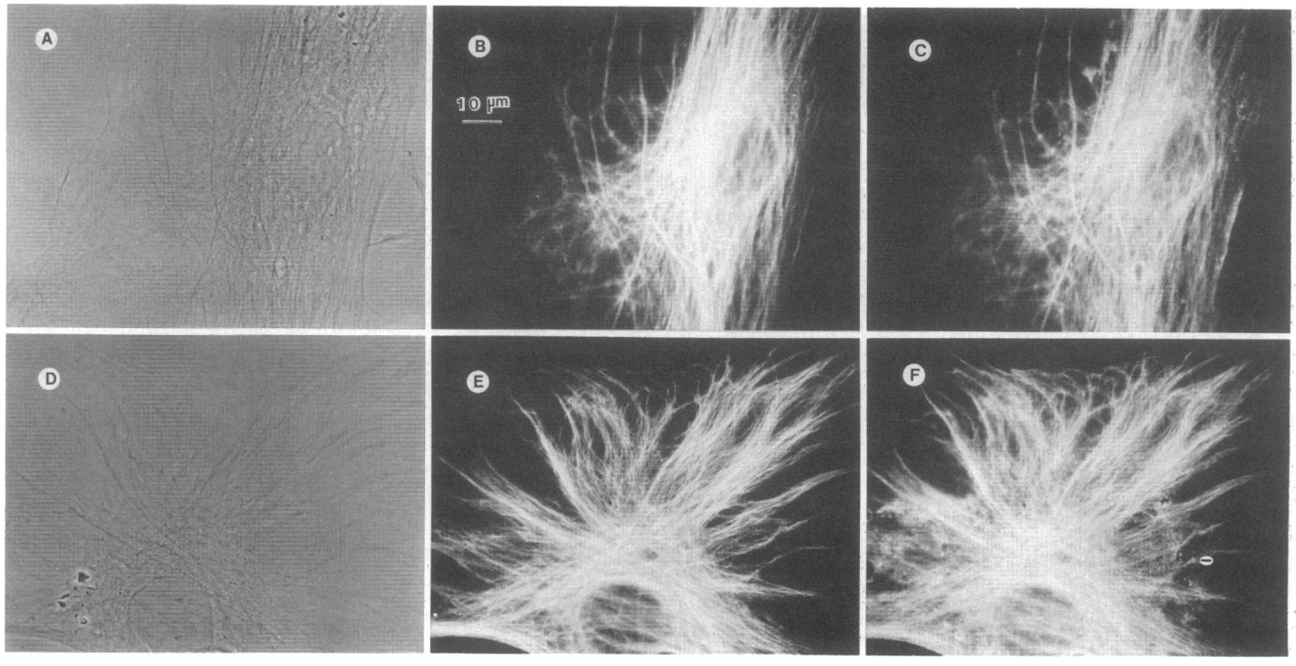

FIG. 4. Double-label immunofluorescence. (*Left*) Phase-contrast micrographs of human skin fibroblasts. (*Middle* and *Right*) fluorescence micrographs of the same cells treated with mouse monoclonal antibody JLB1 (*B*) or JLB7 (*E*) and rabbit autoimmune serum (*C* and *F*). Fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG antiserum were used for the second antibody in the indirect immunofluorescence microscopy.

acrylamide gels as described by Burridge (18), I have failed to identify the antigens reacting with either JLB1 or JLB7 antibody. It may be that the antigenic determinants recognized by these two monoclonal antibodies are especially sensitive to $NaDodSO_4$ or it may be that the larger IgM antibodies (both JLB1 and JLB7) have difficulty in penetrating the polyacrylamide gels. To overcome these problems, immunoprecipitation was chosen to identify the specific antigens. Total cell extracts from chicken embryo fibroblasts labeled *in vivo* with [^{35}S]methionine were prepared as described in *Materials and Methods*. The low ionic strength used in the extraction buffer may, in fact, solubilize some of the vimentin molecules. Immunoprecipitation with JLA20 monoclonal antibody was used as a control for the background; JLA20 monoclonal antibody, similar to JLB1 and JLB7, was IgM as determined both by the Ouchterlony double-diffusion test with rabbit antisera against mouse immunoglobulins (class-specific) and by $NaDodSO_4$/polyacrylamide gel analysis of the [^{35}S]methionine-labeled culture medium of the hybridoma clones. The fluorogram of the immunoprecipitates analyzed by gel electrophoresis is shown in Fig. 5. Specific protein bands at 210,000 and 95,000 daltons were precipitated by JLB1 and JLB7 monoclonal antibodies, respectively, while, under the same conditions, an actin band was specifically detected by JLA20 monoclonal antibody. Other protein bands (two at ≈45,000 daltons and one at ≈34,000 daltons) are considered as nonspecific bands because they are also found in the experiments with JLA20 and other monoclonal antibodies. Occasionally, I have observed that both the 210,000- and the 95,000-dalton proteins coexist in the immunoprecipitate by either JLB1 or JLB7 monoclonal antibody. This is more often seen when immunoaffinity column chromatography is used instead of immunoprecipitation. These observations suggest that the 210,000- and 95,000-dalton proteins may form an *in vivo* complex associated with the intermediate filaments of cultured cells. More experiments are needed to test this possibility.

Immunoprecipitation was also performed on the [^{35}S]methionine-labeled extract from gerbil fibroma cells. The results showed that the 210,000- and 95,000-dalton proteins are the prominent bands precipitated by JLB1 and JLB7 monoclonal antibodies (data not shown). It should be noted that, for an unknown reason, the background in experiments including gerbil cells is always higher.

DISCUSSION

The two monoclonal antibodies described here react with intermediate filaments in a wide variety of cell types of both mammalian and avian origin, including fibroblasts, epithelial cells, myoblasts, and cells of neuronal origin (neuroblastoma), suggesting the highly conservative nature of the corresponding antigens. The distribution of these antigens, however, in myofibrils is unexpected. The antibody JLB1 labels both the M-line region and on either side of the Z disc, whereas JLB7 labels only the M line. Lazarides and Balzer (19) have shown that desmin, the major subunit of the intermediate filaments of muscle, is located at the periphery of the Z disc. More recently, Granger and Lazarides (20) have shown that small amounts of vimentin, the major subunit of most nonmuscle intermediate filaments, is also located in the myofibril Z disc. This suggests that the antigens recognized by JLB1 and JLB7 are neither desmin nor vimentin, but components tightly associated with the intermediate filaments of nonmuscle cells that, in muscle, have a different relative distribution. As anticipated from staining patterns on myofibrils, preabsorption of both JLB1 and JLB7 antibodies with purified vimentin fraction does not decrease the staining of the intermediate filaments. On the other hand, this vimentin fraction can block the staining of intermediate filaments by another monoclonal antibody, LCK16 (data not shown).

Turner *et al.* (21) have shown that creatine phosphokinase (CPK), an enzyme (M_r for monomer, 42,000) involved in ATP formation in muscle, is an M-line protein of myofibrils. Re-

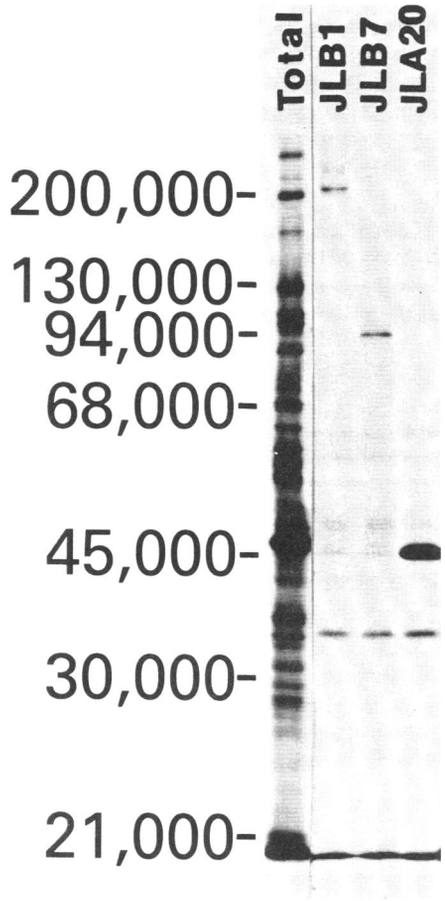

FIG. 5. Fluorograms of immunoprecipitates analyzed by 10% $NaDodSO_4$/polyacrylamide gel electrophoresis. Lanes: Total, total extract (the high-speed supernatant) of chicken embryo fibroblasts labeled *in vivo* with [^{35}S]methionine; JLB1, immunoprecipitate of total extract by JLB1 monoclonal antibody; JLB7, immunoprecipitate of total extract by JLB7 monoclonal antibody; JLA20, immunoprecipitate of total extract by JLA20 monoclonal antibody. Molecular weight markers were run in the same gel; their positions are shown on the left.

cently, it has been reported that antibodies against muscle CPK also decorate the intermediate filaments of PtK-1 and 3T3 cells (22). To test whether either JLB1 or JLB7 was directed against CPK, immunofluorescence was carried out after absorption of the antibodies with CPK (10 μg of commercial CPK from rabbit muscle per μl of antibody). This did not block the staining of the intermediate filaments by either JLB1 or JLB7 on gerbil fibroma cells (data not shown), hinting that the antigens recognized by both antibodies are not CPK.

The antigens recognized by JLB1 and JLB7 do not appear to be any of the M-line proteins reported by Eaton and Pepe (23); extraction of these proteins from myofibrils as described by Kundrat and Pepe (24) did not abolish the staining obtained with these antibodies (data not shown). Incubation of myofibrils with 5 mM Ca^{2+} does cause the disappearance of either side of Z-line staining and the decrease of the M-line staining by JLB1, suggesting that its respective antigen may be sensitive to the Ca^{2+}-activated protease (25) or may be extracted under these conditions.

Immunoprecipitation shows that proteins at 210,000 and 95,000 daltons from chicken embryo fibroblasts are the antigens recognized by JLB1 and JLB7 antibodies, respectively. Similar results but higher backgrounds are also obtained on the immunoprecipitation of total extracts from gerbil fibroma cells by both antibodies. Unfortunately, I have not been able to show the counterparts of myofibrils recognized by these antibodies. Starger *et al.* (26) have found a minor protein component of 250,000–350,000 daltons that copurified with the intermediate filament preparations from BHK-21 cells. Also, Granger and Lazarides (27) have found a 230,000-dalton protein component of the intermediate filaments from smooth muscle. The larger antigen described here may be the functional counterpart to these other proteins.

In experiments in which JLB7 antibodies were preabsorbed by phosphorylase *a* or *b* (M_r 94,000) from rabbit skeletal muscle, the staining of the intermediate filaments was unaffected. This suggests that the smaller antigen recognized by JLB7 antibody is another still to be identified protein associated with the intermediate filaments, rather than phosphorylase.

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