SUBCELLULAR LOCALIZATION OF DNA-BINDING PROTEIN BA BY IMMUNOFLUORESCENCE AND IMMUNOELECTRON MICROSCOPY

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ABSTRACT

Nonhistone protein BA has been shown to decrease in amount in the chromatin of growth-stimulated normal rat liver (Yeoman et al. 1975. *Cancer Res.* **35**:1249–1255) and in mitogen-stimulated normal human lymphocytes (Yeoman et al. 1976. *Exp. Cell Res.* **100**:47–55.). Subsequently, protein BA was purified and was shown to prefer to bind to double-stranded A-T-rich DNAs (Catino et al. 1978. *Biochemistry.* **17**:983–987.).

Immunization of rabbits with highly purified protein BA has resulted in the production of a specific antibody. A specific immunoreactivity for chromosomal protein BA has been demonstrated by immunoelectrophoresis and double antibody immunoprecipitation analysis with rabbit anti-BA immunoglobulin and IgG fractions.

Light microscope examination of normal rat liver cryosections by the indirect immunofluorescence procedure has demonstrated a cytoplasmic as well as a nuclear localization for protein BA with a pronounced perinucleolar fluorescence. Immunoelectron microscopy employing the peroxidase antiperoxidase method of antigen localization has confirmed the immunofluorescence data and has shown a heterochromatin localization for protein BA. The relationship of the localization of protein BA to gene control in quiescent cells or to configurations of heterochromatin as well as the marked reduction in the amounts of protein BA which occur in stimulated growth states remains to be defined.

KEY WORDS nuclear nonhistone protein · immunolocalization · immunofluorescence microscopy · immunoperoxidase electron microscopy

Nonhistone protein BA has been shown to be associated with the chromatin of nongrowing cells (17, 18). Stimulation of rat liver growth by partial hepatectomy resulted in a marked decrease in the quantities of protein BA in the chromatin proteins (19). Stimulation of normal human lymphocytes with PHA[1] rapidly produced a decrease in the amount of chromosomal protein BA (21). Protein

[1] *Abbreviations used in this paper are:* EDTA, ethylenediaminetetraacetic acid; IgG, gamma globulin fraction; i.m., intramuscular; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PMSF, phenylmethanesulfonyl fluoride; s.c., subcutaneous; SDS, sodium dodecyl sulfate; Tris, trihydroxymethylaminomethane.

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BA has recently been purified from normal rat liver and analyzed for its DNA binding properties by the nitrocellulose filter assay (1). It was shown that protein BA bound to DNA and preferred poly dA-dT- and AT-rich DNA (1).

The present study was initiated to determine whether protein BA could be localized *in situ* to the chromatin of nuclei. These experiments describe the production of a specific antibody to purified protein BA and the subcellular localization of protein BA by light and electron microscope techniques.

MATERIALS AND METHODS

Production of Antiserum

Protein BA was extracted from normal rat liver chromatin with 8 M urea, 50 mM sodium phosphate, pH 7.6, and 1% β-mercaptoethanol and purified by preparative acid-urea slab gel electrophoresis as previously described (1). Equal volumes of complete Freund's adjuvant and purified protein BA (150 μg/ml) were mixed, and 1-ml injections were given s.c. and i.m. to a New Zealand white rabbit. Inoculations were given on days 1, 8, and 13 and the first bleeding was taken on day 20. Booster injections containing ~140 μg of protein BA in Freund's incomplete adjuvant were given biweekly starting 1 mo after the first bleeding. The rabbit was bled from the ear 7 d after each booster inoculation (20).

An immunoglobulin fraction was prepared by ammonium sulfate fractionation (9) and dialyzed to 0.15 M NaCl. An IgG fraction was obtained by DEAE-cellulose chromatography as described by Fahey and Terry (4).

Immunoelectrophoresis

Immunoelectrophoretic analysis of various protein mixtures was performed at 100 V for 35 min in 1% agarose (Immunoagaroslides, Worthington Diag., Elk Grove Village, Ill.). After development of precipitin arcs with antibody, the slides were deproteinized in PBS (0.15 M NaCl/0.01 M $NaPO_4$/pH 7.6) for 24 h and stained with Coomassie brilliant blue (2).

Double Antibody Immunoprecipitation

The 70–95% ammonium sulfate fraction from the 0.075 M NaCl, 0.025 M EDTA, pH 8.0, extract (22) of sucrose isolated normal rat liver nuclei was iodinated using Enzymobeads (Bio-Rad Laboratories, Richmond, Calif.), 0.2% β-D-glucose and 1.0 mCi of $Na^{125}I$ (New England Nuclear, Boston, Mass.; >350 mCi/ml) for 25 min at 25°C. The labeled nuclear protein fraction was separated from free iodine by exclusion chromatography on Sephadex G-25 equilibrated with PBS containing 0.25% gelatin (8). Iodinated protein was incubated with immune or preimmune IgG in PBS containing 1% Triton X-100 (3) at 37°C for 30 min followed by a 4°C incubation overnight. Polyethylene glycol 6,000 was added to a final concentration of 2% (16) before addition of goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, Ind.) at a concentration equal to five times the amount of rabbit IgG. The mixture was centrifuged at 7,500 *g* for 2 min, and the pellet was washed twice with PBS (7). The immunoprecipitate was solubilized in Laemmli sample buffer and electrophoresed on SDS containing 10% polyacrylamide gels (10). Gels were sliced in 1-mm sections, dried, and counted by liquid scintillation in a Beckman model LS-230 liquid-scintillation spectrometer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) (5).

Preparation of Nuclei

Nuclei for immunolocalization experiments were prepared from normal rat livers by the Mg^{++}-sucrose method (15). All solutions contained 1 mM PMSF. Nuclei were stored at −80°C before use. Cytosol and nuclear fractions were prepared as described previously (20).

Subcellular Localization

Indirect immunofluorescence as described by Hilgers et al. (6) was performed by the method of Lazarides (11). Fresh rat liver (0.5 cm^3) was washed three times with PBS before freezing in Tissue-Tek II O.C.T. Compound (Lab-Tek Products; Naperville, Ill.). Cryosections (7 μm thick) were cut on a Miles Tissuetek II Cryostat and placed on microscope slides precoated with 5% gelatin. The slides were fixed for 30 min with 0.04% paraformaldehyde in PBS at 25°C according to the method of Lazarides (11). After incubation for 3 min in 1:1 acetone-water (4°C), the slides were incubated in acetone (4°C) for 5 min and again for 3 min in 1:1 acetone-water (4°C). Sections were incubated with anti-BA serum (diluted 1:2 with PBS) for 90 min at 37°C. After three PBS washings (15 min each), the samples were incubated with fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories; Cochranville, Penn.) at 0.8 mg/ml for 60 min at 37°C. Slides were washed three times with PBS (15 min each) and once with deionized water. Moist sections were covered with one drop of 1:1 glycerol-PBS, and a coverslip was sealed over the specimen with clear nail polish. All reagents were filtered through Millex 0.22-μm filters (Millipore Corp., Bedford, Mass.). Samples were examined on a Zeiss fluorescence microscope with epi-illumination, and photographs were taken on Tri-X film (Eastman Kodak Co., Rochester, N. Y.).

The peroxidase-antiperoxidase system for electron microscopic localization of antigen-antibody complexes has been described by Sternberger et al. (13, 14). Nuclei were fixed in 1% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 0.1 M sucrose or in PBS containing 0.1 M sucrose and 0.02% sodium azide. After washing two times with PBS (15 min each), the samples were incubated with anti-BA serum (diluted 1:5 with PBS) for 30 min at 37°C. After washing two times with PBS the nuclei were incubated with goat anti-rabbit antiserum for 30 min at 37°C. Incubated nuclei were washed with PBS and treated with soluble antiperoxidase complex (Miles Labs) for 30 min at 37°C. Peroxidase-labeled samples were washed two times with PBS and two times (15 min each) with 0.05 M Tris-HCl, pH 7.6, before staining with 0.05% 3,3'-diaminobenzidine-4 HCl (Sigma Chemical Co., St. Louis, Mo.) and 0.01% H_2O_2 in 0.05 M Tris-HCl, pH 7.6, for 30 min. Immunochemically stained samples were fixed in 3% glutaraldehyde and postfixed in 2% osmium tetroxide. After dehydrating in a series of graded ethanol solutions, the samples were embedded in Epon-Araldite without counter-staining (12) for examination on a Philips 200 transmission electron microscope.

RESULTS

Antibody Specificity

The rabbit antibody obtained to protein BA was

analyzed by several techniques. Fig. 1 shows an immunoelectrophoretic analysis of four normal rat liver cell fractions with the immunoglobulin fraction from the anti-BA serum. The cytosol, the saline-EDTA wash, and the whole nuclear homogenate each displayed one precipitin arc with the anti-BA serum. Only the 0.01 M Tris nuclear fraction did not show any immunoreactivity. Preimmune immunoglobulin fraction did not react with any of the fractions.

The reactivity of the antibody was also tested by precipitation of protein BA from an iodinated protein mixture. The 70–95% ammonium sulfate fraction from the saline-EDTA extract of normal rat liver was previously shown to contain protein BA (1). This extract was iodinated by lactoperoxidase (8) and separated from free iodine on a Sephadex G-25 column. The void fraction which contained the labeled proteins was incubated with immune or preimmune IgG in the presence of 1% Triton X-100. The optimal ratio of goat anti-rabbit IgG required to precipitate the rabbit IgG was determined to be 5:1. After equilibration of the rabbit IgG with the iodinated protein mixture, soluble immune complexes were precipitated by the addition of second antibody (Fig. 2). The immune precipitates obtained by this method were dissociated by heating in Laemmli SDS sample buffer and analyzed on 10% Laemmli denaturing gels for the presence of iodinated proteins (10). Fig. 3 shows the radioactive profiles of the total iodinated mixture, the material precipitated with anti-BA IgG, and the material treated with preimmune IgG. One component was precipitated by the anti-BA IgG. This iodinated component had a molecular weight of 31,000 and comigrated with purified nonhistone protein BA.

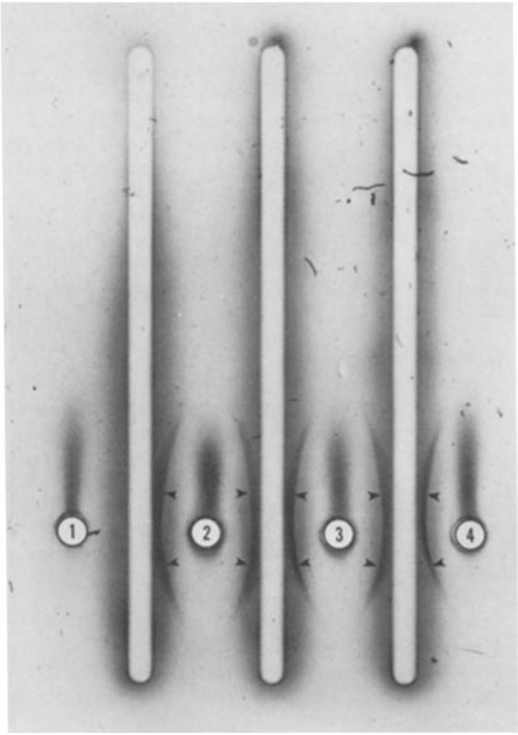

FIGURE 1 Immunoelectrophoretic analysis of normal rat liver nuclear and cytoplasmic cell fractions. All antibody troughs contained anti-BA immunoglobulin fraction at 140 mg/ml. The antigen wells contained: (*1*) 0.01 M Tris-HCl, pH 8.0, extract of saline-EDTA washed nuclei; (*2*) 0.075 M NaCl, 0.025 M EDTA, pH 8.0, extract of whole nuclei; (*3*) cytosol; (*4*) whole nuclear homogenate (10 mg/ml).

Subcellular Localization

Analysis of normal rat liver cryosections for the subcellular localization of protein BA by the indirect immunofluorescence technique is shown in Fig. 4. Fluorescence was observed both in the nuclei and in the cytoplasm (Fig. 4*A*). The cytoplasm appeared to be collapsed against the plasma membrane and provided a fluorescent outline of the individual cells (black and white arrows). The nucleolar fluorescence (black arrows) was sufficiently bright to be detected over the nuclear fluorescence. Immunofluorescent staining of normal rat liver cryosections with preimmune serum did not reveal nuclei or cell membranes (Fig. 4*C*).

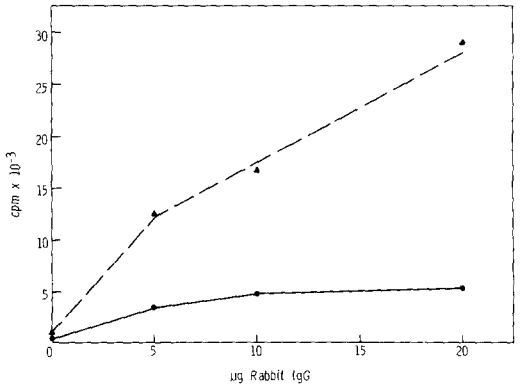

FIGURE 2 Double antibody precipitation of an immune complex. Samples were prepared as described under Materials and Methods. Precipitation of an iodinated immune complex with (▲—▲) anti-BA IgG, and (●—●) preimmune IgG is shown.

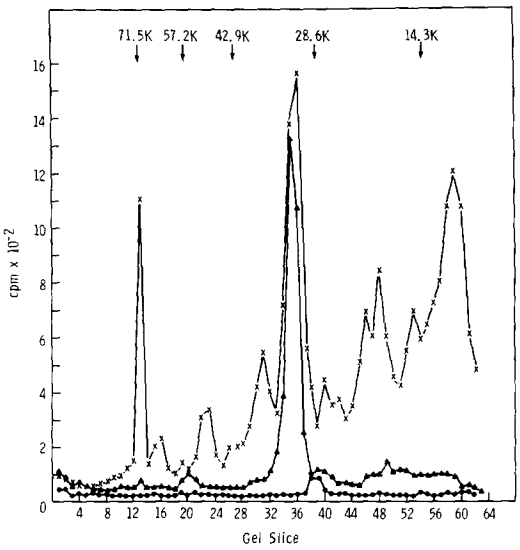

FIGURE 3 Polyacrylamide gel analysis of precipitated immune complex. The immunoprecipitated material obtained from the double antibody-protein incubation was dissociated in SDS and subjected to 10% Laemmli polyacrylamide gel electrophoresis. The radioactive electrophoretic profiles of: anti-BA IgG precipitated material (▲—▲); preimmune IgG precipitated material (●—●); total iodinated protein mixture (×—×); and molecular weight markers are shown (arrows).

Instead, a low intensity and nonspecific fluorescence was observed.

Electronmicroscopic localization of protein BA was determined by the immunoenzymatic technique of Sternberger et al. (13, 14). Fig. 5 shows the reaction of normal rat liver nuclei with anti-BA serum (Fig. 5 *A*) and with preimmune serum (Fig. 5 *B*). The increased electron density observed in samples treated with anti-BA serum in specific regions of condensed chromatin represents immunochemically stained sites. In general, the antigenic sites appeared to be localized to heterochromatin, namely chromatin adjacent to the nuclear membrane and in perinucleolar chromatin (Fig. 5 *A*, pointers). An immunochemical reaction was also noted for chromocenters within the nucleoplasm of nuclei treated with anti-BA serum (Fig. 5 *A*, arrows). The nucleoli of nuclei treated with anti-BA serum appeared to lack resolution of their nucleolar morphology and were devoid of vacuoles or fibrillar clusters when compared to nucleoli of cells treated with preimmune serum.

DISCUSSION

The association of protein BA with isolated chromatin of non-growing cells (17, 18, 19, 21) and the DNA binding properties of protein BA (1) have suggested that protein BA may aid in the regulation of growth. A rabbit antibody was developed for purified protein BA (1), as a specific and sensitive probe for the *in situ* localization of protein BA. The specificity of the antibody was demonstrated by two immunoprecipitation techniques. Immunoelectrophoretic analysis demonstrated a single precipitin arc in extracts known to contain protein BA, i.e., cytosol and the saline-EDTA extract of normal rat liver nuclei (1).

Additional evidence for antibody specificity was obtained by double antibody immunoprecipitation. A single component of mol wt 31,000 was precipitated from a mixture of iodinated proteins by the anti-BA IgG. The labeled component was shown to comigrate with purified protein BA, lending further support to the specificity of the antibody.

The indirect immunofluorescence investigation of rat liver cryosections indicated that the antibodies to protein BA produced both a cytoplasmic cell membrane fluorescence and a nucleoplasmic fluorescence (Fig. 4). Within the nucleoplasm, the nucleoli were observed to be slightly more fluorescent than the nucleoplasm. It could not be determined by this technique whether the nucleolar fluorescence was localized to the nucleolar surface or was generally distributed throughout the nucleolus. With the peroxidase-labeling technique, electronmicroscopic analysis of the enzymatically labeled sample showed that the nucleolar fluorescence resulted from the localization of protein BA in the perinucleolar heterochromatin. In addition, protein BA was shown to be present with the heterochromatic regions contiguous to the inner layer of the nuclear envelope as well as in small chromocenters throughout the nucleoplasm. The general heterochromatic localization was thought to be responsible for the general nuclear fluorescence observed in Fig. 4 *A*.

The role of protein BA in heterochromatin is not known at this time. It may function more as part of a general repression mechanism rather than acting upon specific template sequences necessary for the synthesis of growth-related RNA. It seems more likely that protein BA will be found to serve a role in stabilizing heterochromatin packaging. Reduction in the amount of protein BA in chromatin may result in a destabilization of structures which must be disrupted before cell proliferation. The amounts of BA (up to 3.0% of the nuclear

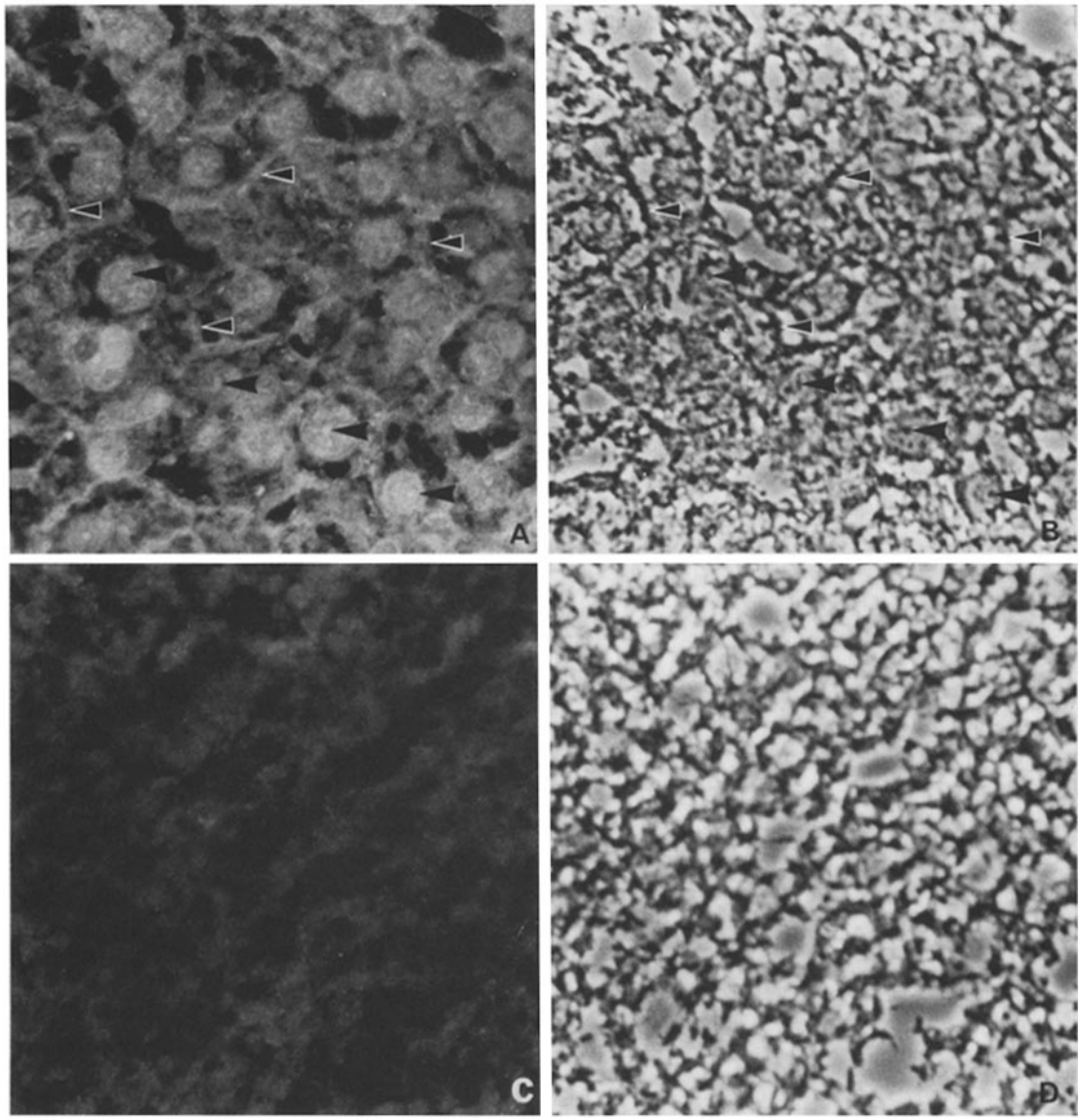

FIGURE 4 Immunofluorescence localization of protein BA in normal rat liver cells. Cryosections were prepared and analyzed by the indirect immunofluorescence technique as described in Materials and Methods. *A* shows the immunofluorescence of cryosections incubated with anti-BA serum (1:2). *B* shows a phase contrast micrograph of the field in *A*. *C* shows the minimal background fluorescence observed in cryosections treated with preimmune serum (1:2). *D* is a phase contrast micrograph of the field shown in *C*. The black arrows point to examples of nucleolar fluorescence within fluorescent nuclei. The black and white arrows point to a cytoplasmic cell membrane fluorescence. × 1,300.

nonhistone protein) favor this hypothesis (1).

The identification of a BA immunoreactivity in the cytosol (Fig. 1) and at the cell membrane (Fig. 4*A*) would indicate that amounts of protein BA are in the cytoplasm. It is not known whether protein BA has a cytosol function or whether this merely represents cytoplasmic protein synthesis of protein BA.

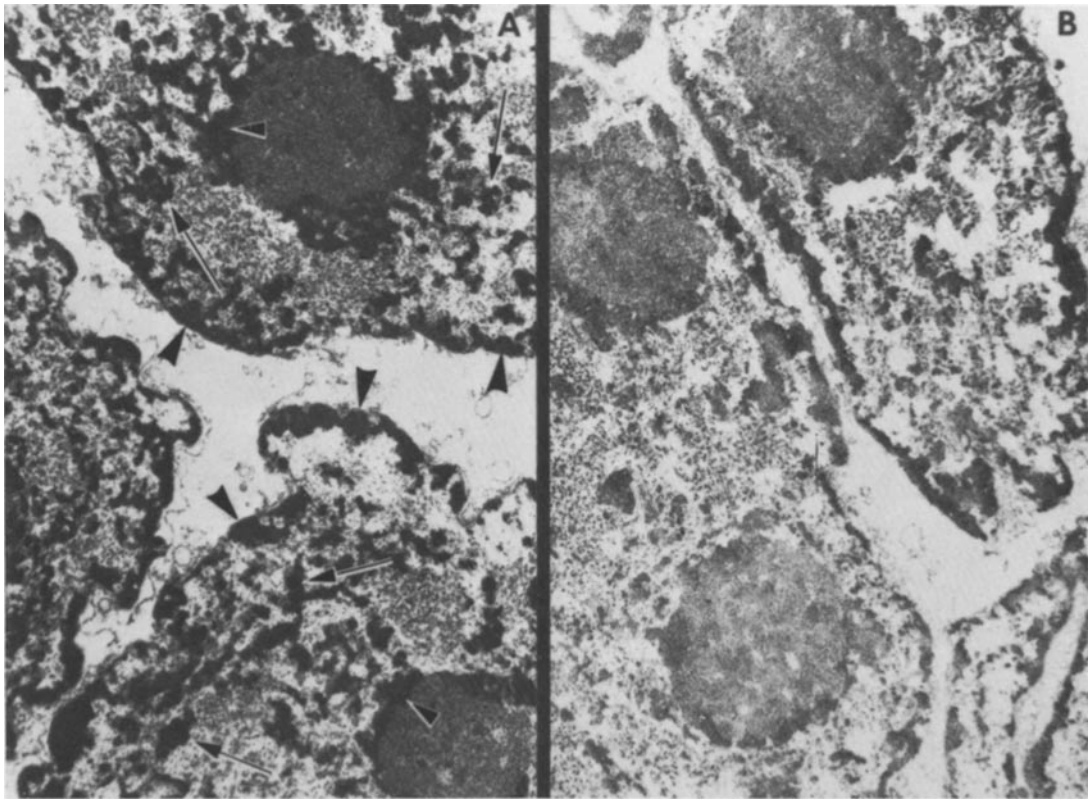

FIGURE 5 Immunoenzymatic localization of protein BA in normal rat liver nuclei. An electronmicrograph of normal rat liver nuclei was analyzed by the unlabeled antibody enzyme method utilizing peroxidase enzyme as described in Materials and Methods. *A* shows the immunoenzymatic staining with anti-BA serum, and *B* shows analysis with preimmune serum. × 15,000.

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