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Nonhistone Protein BA Is a Glutathione S-Transferase Localized to Interchromatinic Regions of the Cell Nucleus

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Abstract. A DNA-binding nonhistone protein, protein BA, was previously demonstrated to co-localize with U-snRNPs within discrete nuclear domains (Bennett, F. C., and L. C. Yeoman, 1985, *Exp. Cell Res.*, 157:379–386). To further define the association of protein BA and U-snRNPs within these discrete nuclear domains, cells were fractionated in situ and the localization of the antigens determined by double-labeled immunofluorescence. Protein BA was extracted from the nucleus with the 2.0 M NaCl soluble chromatin fraction, while U-snRNPs were only partially extracted from the 2.0 M NaCl-resistant nuclear structures. U-snRNPs were extracted from the residual nuclear material by combined DNase I/RNase A digestions. Using an indirect immunoperoxidase technique and electron microscopy, protein BA was localized to interchromatinic regions of the cell nucleus.

Protein BA was noted to share a number of chemical and physical properties with a family of cytoplasmic enzymes, the glutathione S-transferases. Comparison of the published amino acid composition of protein BA and glutathione S-transferases showed marked similarities. Nonhistone protein BA isolated from saline-EDTA nuclear extracts exhibited glutathione S-transferase activity with a variety of substrates. Substrate specificity and subunit analysis by SDS polyacrylamide gel electrophoresis revealed that it was a mixture of several glutathione S-transferase isoenzymes. Protein BA isolated from rat liver chromatin was shown by immunoblotting and peptide mapping techniques to be two glutathione S-transferase isoenzymes composed of the Yb and Yb′ subunits.

Glutathione S-transferase Yb subunits were demonstrated to be both nuclear and cytoplasmic proteins by indirect immunolocalization on rat liver cryosections. The identification of protein BA as glutathione S-transferase suggests that this family of multifunctional enzymes may play an important role in those nuclear domains containing U-snRNPs.

NONHISTONE protein BA was first identified in studies comparing chromatin-associated proteins isolated from normal and transformed tissues. It was noted that protein BA was present in chromatin isolated from normal liver and lymphocytes, but was markedly reduced or undetectable in chromatin isolated from various neoplastic cells (47–49). Protein BA was purified to homogeneity from rat liver chromatin and partially characterized chemically (9). Recently, we have described a second purification procedure in which protein BA was purified from the initial saline–EDTA extracts of rat liver nuclei (3). Comparison of the amino acid compositions, isoelectric points, molecular weights, as well as amino and carboxy terminal amino acids suggested that these protein BA populations were related (3). However, they do differ in that protein BA isolated from chromatin, as previously described (9), exhibits DNA binding activity, whereas protein BA isolated from saline–EDTA nuclear extract fails to bind DNA. Examination of circular dichroism spectra for both forms of protein BA revealed marked differences in secondary structure that may partially explain the differences in DNA binding observed (3). Because of the differences in their extractability from the cell nucleus, we have termed protein BA isolated from chromatin as protein BA_{bound} and the form isolated from saline–EDTA nuclear extracts as protein BA_{free}.

We have recently shown by indirect immunofluorescence that protein BA co-localizes with U-snRNPs in discrete regions of the cell nucleus (2). Protein BA is not a component of U-snRNPs but appears to be concentrated within the same regions of the cell nucleus that contain U-snRNPs. The U-snRNAs are a family of small nuclear RNAs, rich in uridylic acid (36), which associate with proteins to form particles (U-snRNPs). The U-snRNPs are proposed to play a critical role in the maturation of hnRNA to mRNA (reviewed in reference 36). Therefore, protein BA may function in those regions of the cell nucleus where processing of hnRNA takes place.

In this report, the association of protein BA with U-snRNPs in the cell nucleus is further characterized. Protein BA is

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identified as glutathione S-transferase, a family of enzymes generally believed to be involved in xenobiotic detoxification (20).

Materials and Methods

Materials

1-Chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene were purchased from Eastman Kodak Co. (Rochester, NY). 1-Chloro-2,4-dinitrobenzene was further purified by recrystallization from ethanol. 1,2-Epoxy-3-(*p*-nitrophenoxy)-propane, *p*-nitrophenyl acetate, cumene hydroperoxide, ethacrynic acid, *p*-nitrophenyl chloride, and *N*-chlorosuccinimide were purchased from Sigma Chemical Co. (St. Louis, MO). ^{125}NaI was purchased from Amersham Corp. (Arlington Heights, IL). *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin was purchased from Worthington Biochemical Corp. (Freehold, NJ).

Isolation of Proteins

Protein BA_{bound} was isolated from rat liver chromatin as described by Catino et al. (9). Protein BA_{free} was isolated from 0.075 M NaCl/0.025 M EDTA, pH 8.0 extracts of rat liver nuclei as previously described (3). Individual glutathione S-transferase isoenzymes were prepared from rat liver cytoplasm as described by Habig et al. (17). Phenylmethylsulfonyl fluoride (1.0 mM) and leupeptin (0.1 mM) were added to all cellular extracts to inhibit proteolysis. Total rat liver glutathione S-transferases were purchased from Sigma Chemical Co. Glutathione S-transferase activity towards various substrates was determined by spectrophotometric assays (16). Protein concentrations were determined by the method of Lowry et al. (27).

Immunoassay

Antibodies to protein BA were prepared by immunizing a New Zealand White male rabbit with 50 μg of protein BA_{free} as previously described (2). Immunoblots (44) were performed using primary antibody dilutions of 1:250. Immunoreactivity was detected with ^{125}I-protein A, followed by autoradiography (32). Hybridoma cells secreting monoclonal Sm antibody (24) were a gift from Dr. Joan A. Steitz.

Peptide Mapping

Partial *N*-chlorosuccinimide peptide maps were performed using the method of Lischwe and Ochs (25) with 5.0 μg of gel purified proteins. Peptides were detected by silver staining (46).

For tryptic digests, purified protein BA_{free} and protein BA_{bound} (20–50 μg) were labeled with 1 mCi of ^{125}I by the chloramine-T procedure to an approximate specific activity of 5×10^6 cpm/μg protein (19). Individual subunits were separated on a 10% SDS polyacrylamide gel (23), visualized by brief staining with 0.25% Coomassie Brilliant Blue R, and extracted from a gel slice with 0.1% SDS/0.025 M Tris/0.19 M glycine/1% β-mercaptoethanol, pH 8.3. Extracted protein was precipitated with acetone, re-dissolved in 200 μl of 0.5 M Tris HCl/2 mM EDTA/8 M urea/8 mM β-mercaptoethanol, pH 8.5 and carboxymethylated according to the method of Gracy (15). Protein was dialyzed against 200 mM $(NH_4)HCO_3$, pH 8.0 and digested with three separate additions of 1 μg of *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin at 0, 3, and 6 h. At 24 h the digestion was terminated by freezing and lyophilization. Labeled peptides were dissolved in 0.05 ml of 20% acetic acid and injected onto a Waters C18 μBondapak column (Waters Associates, Millipore Corp., Milford, MA). A linear gradient of 0.1% trifluoroacetic acid to 60% acetonitrile containing 0.05% trifluoroacetic acid was developed over 2 h at a flow rate of 2.0 ml/min (14, 29) on a Varian Model 5000 Liquid Chromatograph (Varian Associates, Inc., Palo Alto, CA). Trial runs had shown that no peptides eluted after 60% acetonitrile. Fractions of 2.0 ml were collected and counted in a Beckman gamma 4000 counter (Beckman Instruments, Inc., Palo Alto, CA).

Immunofluorescence

Fresh rat liver cryosections (10 μM) were fixed in 2.0% paraformaldehyde diluted in 10 mM sodium phosphate/150 mM NaCl, pH 7.4 (PBS) for 10 min at 25°C. Tissue sections were washed in PBS, permeabilized in acetone for 4 min at −20°C, washed again in PBS for 45 min, then in PBS containing 10% goat serum (Gibco, Grand Island, NY) for 30 min at 37°C. Primary antibodies were diluted in 10% goat serum and incubated with the samples for 1 h at 37°C in a humidified chamber. Samples were washed in several changes of PBS for 1 h before incubation for 45 min at 37°C with 5.0 μg/ml biotinylated goat anti-rabbit IgG diluted in 10% goat serum. Cryosections were again washed in several changes of PBS for 1 h, then incubated with 5.0 μg/ml fluorescein isothiocyanate-conjugated streptavidin (Bethesda Research Laboratories, Gaithersburg, MD) for 45 min at 37°C. Samples were washed four times with PBS, 15 min each, at 25°C, then overnight at 4°C. Samples were mounted in glycerol/PBS and viewed and photographed on a Zeiss epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a Nikon UFX automatic camera (Nikon Inc., Garden City, NY).

Normal rat liver cells, clone 9, were purchased from the American Type Culture Collection (Rockville, MD). Cells were grown on 22 × 22-mm coverslips in William's medium E (Gibco) supplemented with 10% fetal calf serum and 5 U insulin/liter. Indirect immunofluorescence was performed as previously described (2, 32); polyclonal rabbit antibody against protein BA was detected using a 1:100 dilution of fluorescein-conjugated goat anti-rabbit IgG; monoclonal antibody Y12 against Sm antigen (a gift from Dr. Joan A. Steitz) was detected using a 1:50 dilution of rhodamine-conjugated goat anti-mouse Igs (Cappel Laboratories, Cochranville, PA). In situ nuclear matrices were prepared using the procedure of Staufenbiel and Deppert (42). After each extraction cells were fixed in 2.0% paraformaldehyde and processed for indirect immunofluorescence.

Immunoelectron Microscopy

Immunoelectron microscopy (41) was performed on the normal rat liver cells grown in 35-mm petri dishes (Permanox tissue culture dish; Lab-Tek Division, Miles Laboratories, Inc., Naperville, IL). Samples were processed for immunoelectron microscopy according to the following procedure. Cells were fixed in 2% paraformaldehyde in PBS/pH 7.3, made fresh before use, for 1 h at 4°C. Cells were washed in four changes, 15 min each, of PBS/pH 7.3 and incubated in PBS/0.2% Triton X-100/0.5% normal goat serum for 5 min at 4°C. Samples were washed for 30 min in several changes of PBS/pH 7.3, containing 0.5% normal goat serum and incubated with antibody (1:20) or preimmune sera (1:20) for 1 h at 37°C. Cells were washed in several changes of PBS/pH 7.3, the last wash extended to 16 h.

Samples were incubated in peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories) at a dilution of 1:20 for 1 h at 37°C and washed for an additional hour in PBS/pH 7.3. Cells were fixed in 2% glutaraldehyde in PBS/pH 7.3 for 30 min, washed in 0.3 M glycine for 15 min, PBS/pH 7.3 for 15 min, and 0.5 M Tris-HCl for 30 min at 4°C. Samples were incubated in 0.05% 3,3′-diaminobenzidine in 0.05 M Tris-HCl/pH 7.6 for 25 min at room temperature and suspended in 0.05% 3,3′-diaminobenzidine supplemented with 0.01% H_2O_2 for 4 min. Cells were washed in 0.05 M Tris-HCl, pH 7.6 for 30 min and fixed in 2% OsO_4 in 0.1 M sodium cacodylate buffer pH 7.3 for 1 h. Samples were rinsed in distilled water, dehydrated in a graded ethanol series followed by 100% propylene oxide, infiltrated, and embedded in Epon 812 (41). Polymerization was carried out at 60°C for 48 h.

Sections were cut on a Sorvall MT-2 ultramicrotome using a SAG International diamond knife and were examined in a JEOL 100 transmission electron microscope (JEOL USA, Peabody, MA) operated at 60 kV.

Results

Co-localization of Protein BA with U-snRNPs

It was demonstrated in previous studies that protein BA co-localizes with U-snRNPs within the same nuclear domains (2). Isolation of nuclei, under differing conditions, suggested that protein BA and the U-snRNPs are associated in these nuclear domains via different types of molecular interactions (2). To further define the associations of protein BA and U-snRNPs to these nuclear domains, cells were fractionated in situ (42), and the distributions of the respective antigens determined by double-label immunofluorescence using a rabbit anti-protein BA antibody (2) that recognizes both the bound and free forms of protein BA, and a mouse monoclonal Sm antibody (24) that immunoprecipitates U1, U2, U4, U5, and U6 snRNAs. Protein BA antibodies produced both a nuclear and a cytoplasmic fluorescence in a normal rat liver cell line fixed with paraformaldehyde and permeabilized with acetone (Fig. 1 *a*). The nuclear staining was inhibited by preabsorption of the antibodies with highly purified protein BA (2). In contrast, the Sm antigens were localized exclusively within the nucleus (Fig. 1 *b*) as previously demonstrated (2, 24, 31, 41). By using double label immunofluorescence it was possible to determine that protein BA and U-snRNPs were

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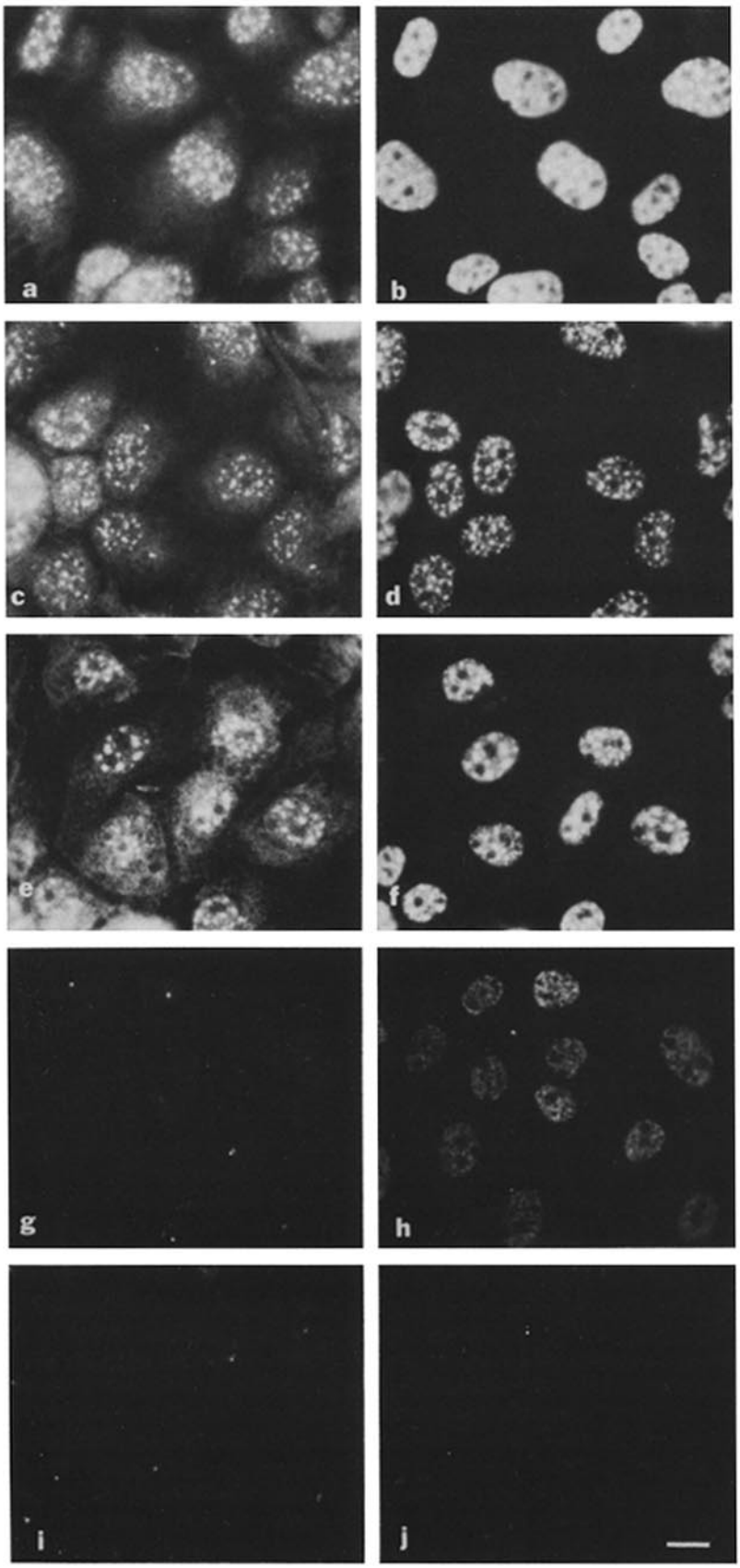

Figure 1. Co-localization of protein BA and U-snRNPs during cellular fractionation. Normal rat liver cells were grown on coverslips, sequentially fractionated, and fixed for immunofluorescence as described in Materials and Methods. Cells were double-labeled with polyclonal protein BA rabbit antibody (*a, c, e, g,* and *i*) and monoclonal Sm antibody (*b, d, f, h,* and *j*). Rabbit antibody against protein BA was detected with fluorescein-conjugated goat anti-rabbit IgG; monoclonal antibody Y12 against Sm antigen was detected with rhodamine-conjugated goat anti-mouse Igs. (*a* and *b*) Control cells; (*c* and *d*) cells extracted with NP-40; (*e* and *f*) cells digested with 50 μg/ml DNase I for 15 min; (*g* and *h*) cells extracted with 2.0 M NaCl; (*i* and *j*) cells digested with DNase I and RNase A. Bar, 10 μm.

both concentrated in the same nuclear regions, corresponding to the speckled immunostaining domains (Fig. 1, *a* and *b*). Extraction of the cells with 1% Nonidet P-40 before fixation did not result in a change in the number or the location of those regions that contain protein BA (Fig. 1 *c*) or the Sm antigen (Fig. 1 *d*). Some reduction in fluorescence intensity was observed with the Sm antibody, while only a slight reduction in fluorescence intensity was detected with anti-BA

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antibody. Extraction of the cells with 1% Nonidet P-40 followed by digestion with DNase I also failed to produce a change in the distribution or the fluorescence intensity resulting from each of the antigens (Fig. 1, *e* and *f*). However, protein BA was extracted from the nucleus and cytoplasm with the 2.0 M NaCl soluble chromatin fraction (Fig. 1*g*), whereas a portion of the Sm antigen remained associated with the high salt resistant nuclear residue (Fig. 1*h*) as previously demonstrated (30, 41, 50). The Sm antigen was extracted from the high salt resistant nuclear structures by combined DNase I/RNase A digestion (Fig. 1*j*). Protein BA antibodies stained a small cytoplasmic structure in cells extracted with 2.0 M NaCl (Fig. 1*g*) and in cells digested with DNase I and RNase A (Fig. 1*i*), which may correspond to a microtubule organizing center. These results demonstrate that protein BA and U-snRNPs (detectable with the Sm antibody) share common areas of localization in the cell nucleus, but their association with these nuclear domains is mediated by different types of molecular interactions. Protein BA was extracted in the 2.0 M NaCl soluble chromatin fraction, while at least a portion of the U-snRNPs was more tightly associated with the nuclear residue fraction.

Immunoelectron Microscopy

Immunoelectron microscopy was used to sublocalize protein BA within the nucleus of the normal rat liver cell line. Immunoreactivity was detected with peroxidase-labeled goat anti-rabbit antibodies. Protein BA antibodies stained discrete domains within the nucleus, which corresponded to interchromatinic regions (Fig. 2*a*). Regions of condensed chromatin (*c*) were not immunostained with protein BA antibodies (Fig. 2*a*). There was no specific immunoreaction product observed in normal rat liver cells labeled with preimmune immunoglobulins (Fig. 2*b*). Previous studies have demonstrated that the Sm antibody labels interchromatinic regions of the nucleus (11, 41), thus both protein BA and U-snRNPs are concentrated within interchromatinic nuclear domains.

Identification of Protein BA as Glutathione S-Transferase

Comparison of the physical and chemical properties of protein BA with other proteins reported in the literature revealed a marked similarity between protein BA and the glutathione S-transferases (EC2.51.18). The glutathione S-transferases are a family of enzymes, which in addition to their transferase activity, exhibit selenium-independent peroxidase activity (35), steroid isomerase activity (4), and the ability to bind various ligands (20, 26). All glutathione S-transferase isoenzymes are dimeric proteins, forming either homo- or heterodimers from at least six different subunits that range in molecular weight from 25,000 to 29,000 (21). The major glutathione S-transferase isoenzymes found in rat liver are

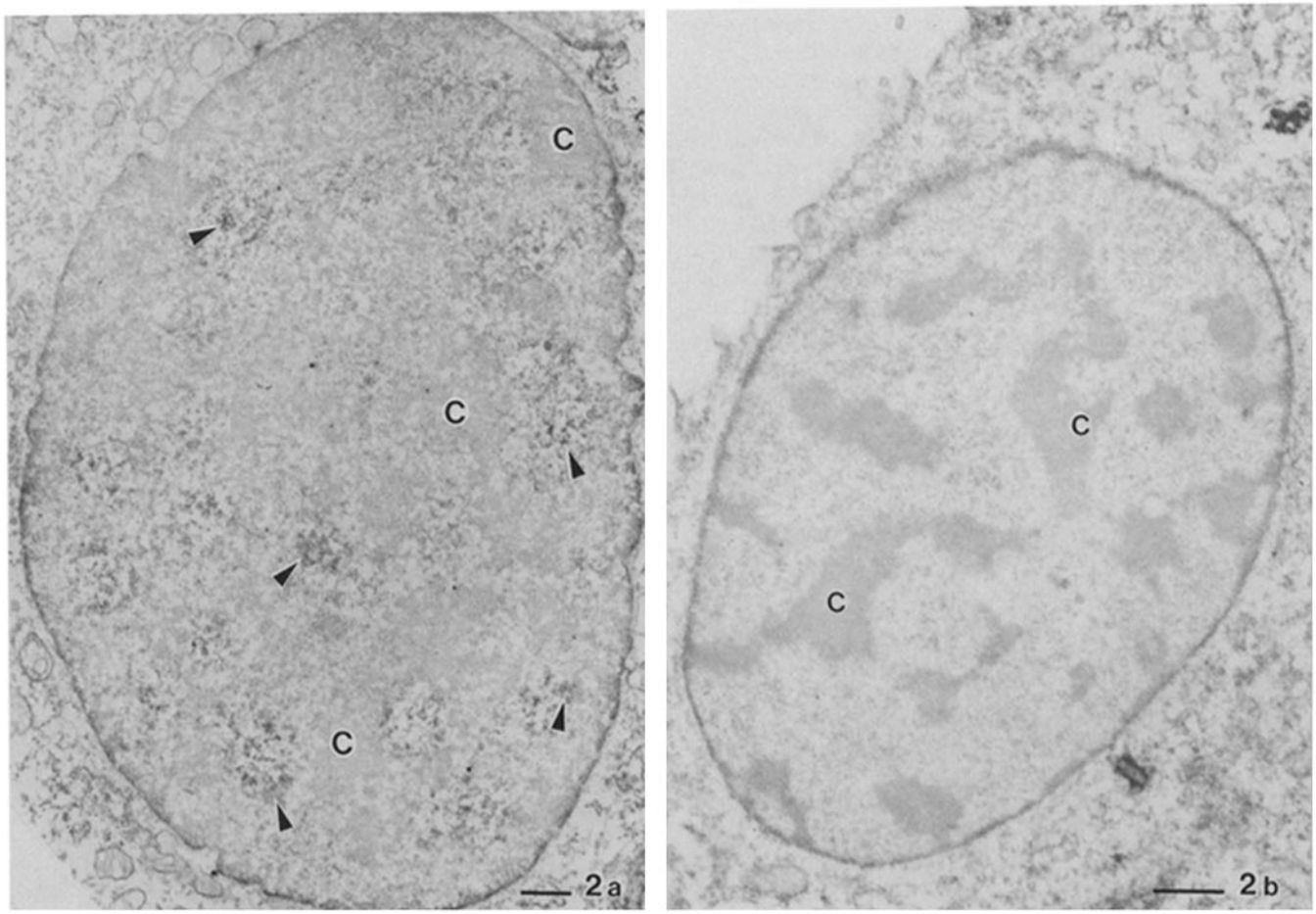

Figure 2. Electron microscopic immunolocalization of protein BA. Normal rat liver cells were incubated with protein BA antibodies (*a*) or preimmune immunoglobulins (*b*) followed by peroxidase-conjugated goat anti-rabbit antibodies. Numerous interchromatinic regions appear immunostained (Fig. 2*a*, arrowheads); however, regions containing condensed chromatin (*C* in Fig. 2*a*) are not immunostained with protein BA antibodies. No post-staining. Bars, 1 μm.

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Table I. Amino Acid Composition of Protein BA and Glutathione S-Transferase

Amino acid	Mole percent				
	BA_{bound}*	BA_{free}*	Transferase-A‡	Transferase-B‡	Transferase-C‡
Lys	8.2	9.7	8.8	9.2	9.2
His	2.8	2.2	1.6	1.5	1.6
Arg	6.5	5.8	5.5	5.6	5.5
Asp	9.5	11.2	11.7	9.4	11.6
Thr	4.3	3.9	3.4	2.8	3.2
Ser	6.3	5.0	5.2	4.6	4.5
Glu	11.4	12.2	10.9	11.7	10.8
Pro	6.0	5.4	5.7	5.1	5.8
Gly	8.8	6.0	4.9	5.4	5.5
Ala	5.4	7.1	5.2	7.9	5.3
Cys	ND	ND	1.6	1.0	1.6
Val	6.5	6.0	2.9	6.4	3.2
Met	2.2	1.5	2.6	2.0	2.6
Ile	5.0	5.2	5.7	4.6	5.0
Leu	10.0	11.6	11.7	12.8	11.8
Tyr	3.4	3.5	6.0	3.3	5.8
Phe	4.1	3.9	5.2	4.3	5.8
Trp	0.5	ND	1.6	2.3	1.6

ND, not determined.
* Values from reference 3.
‡ Values from reference 17.

transferase AA (Yc homodimer), transferase A (Yb homodimer), transferase B (Ya, Yc heterodimer), transferase C (Yb,Yb′ heterodimer), and transferase D (Yb′ homodimer).[1]

The published amino acid composition of both the bound and the free forms of protein BA (3) and glutathione S-transferase isoenzymes A, B, and C (17) are presented in Table I. There was a marked similarity between the reported amino acid compositions of protein BA_{free} and glutathione S-transferase B. A statistical analysis of the values (28) suggested that all of the proteins in Table I were closely related, i.e., S ΔQ values comparing the compositions of BA_{bound} with transferases A, B, and C were 51, 39, and 48, respectively. Values comparing the compositions of BA_{free} with transferases A, B, and C were 27, 9, and 25, respectively. Values lower than 100 indicate relatedness, whereas a value of unity indicates identity (28).

To ascertain whether protein BA was in fact a glutathione S-transferase, the reactivity of protein BA_{free} towards various transferase substrates was determined (Table II). The specific activity of protein BA_{free} towards these substrates was close to values reported in the literature for glutathione S-transferases (16), thus ruling out the possibility that the enzyme activity was due to contaminating glutathione S-transferase. Furthermore, protein BA_{free} was selectively isolated from saline–EDTA nuclear extracts using a glutathione-affinity column (unpublished data). Simons and Vander Jagt (40) have previously reported that glutathione S-transferases selectively bind to glutathione-affinity columns, while other enzymes involved in glutathione metabolism do not bind. These data strongly implicate protein BA_{free} as being glutathione S-transferase. Analysis of the substrate specificity of protein BA_{free} suggested that it was actually a mixture of several glutathione S-transferase isoenzymes. This was further substantiated by SDS polyacrylamide gel electrophoresis analyses. Protein BA_{free} resolved into three distinct polypeptides corresponding to BA^{a}_{free}, BA^{b}_{free}, and BA^{c}_{free} subunits that co-migrate with glutathione S-transferase subunits Ya, Yb, and Yc, respectively (Fig. 3*a*, lane *D*).

Protein BA isolated from chromatin, protein BA_{bound}, does not exhibit enzymatic activity. This was anticipated after considering the rather harsh conditions required to isolate protein BA_{bound} from chromatin, which include extraction of chromatin with 0.4 N H_2SO_4, denaturation in urea, and preparative gel electrophoresis (9).

Immunological Analysis

To identify which glutathione S-transferase subunits correspond to protein BA_{bound}, immunoblots were performed. An antibody produced against protein BA_{free}, which was known to also recognize protein BA_{bound} (Fig. 3*b*, lane *C*), was used to probe the individual glutathione S-transferase subunits. Protein BA_{bound}, as isolated from chromatin, migrates on SDS polyacrylamide gels as a single band between the Yb and Yc subunits (Fig. 3, *a* and *b*, lane *C*). Total liver proteins (Fig. 3*a*, lane *A*), probed with the antibodies that recognize protein BA_{bound}, produced an immunoreaction product with a 27,000-mol-wt protein (Fig. 3*b*, lane *A*). The antibody also reacted with a 27,000-mol-wt polypeptide in protein BA_{free} preparations and weakly with a 25,500-mol-wt polypeptide (Fig. 3*b*, lane *D*). It was determined by excising the individual protein BA_{free} subunits from a polyacrylamide gel and re-electrophoresis on an SDS polyacrylamide gel (Fig. 3*a*, lanes *E–G*), followed by immunoblotting, that the major immunoreactive polypeptide corresponded in migration to the BA^{b}_{free} subunits (Fig. 3*b*, lane *F*). This was further supported by the strong immunoreaction product obtained with glutathione S-transferase A purified from rat liver cytosol, which is a homodimer of Yb subunits (Fig. 3*b*, lane *B*). The BA^{a}_{free} subunit produced a very weak immunoreaction product (Fig. 3*b*, lane *G*).

The greater molecular weight observed for protein BA_{bound} when compared to the Yb subunits was probably caused by the purification procedure, since total liver proteins probed with the antibody produced only one band on immunoblots. This band corresponded in migration to the Yb subunits (Fig. 3*b*, lane *A*). The antibody reacts equally well with glutathione S-transferase Yb subunits purified either from nuclei or cytoplasm (Fig. 3*b*, lanes *B* and *D*). These data suggest that protein BA_{bound} corresponds to the Yb subunits of glutathione S-transferase, and that Yb subunits isolated from rat liver cytosol are immunologically related to BA^{b}_{free} subunits isolated from nuclei.

Table II. Substrate Specificity of Protein BA_{free}

Substrate	Specific activity
	μmol/min per mg
1-Chloro-2,4-dinitrobenzene	11.05
1,2-Dichloro-4-nitrobenzene	0.64
1,2-Epoxy-3-(*p*-nitrophenoxy)-propane	ND
p-Nitrophenyl acetate	0.26
Cumene hydroperoxide	4.13
Ethacrynic acid	0.55
p-Nitrobenzyl chloride	0.86

Substrate specificity of nuclear glutathione S-transferase. The specific activity of glutathione S-transferase isolated from saline–EDTA rat liver nuclear extracts was determined as described in Materials and Methods. ND, no detectable activity under conditions used.

[1] It has recently been suggested that the nomenclature of the rat glutathione S-transferases be changed to reflect their subunit composition (21). Following this proposed nomenclature the Ya subunit corresponds to subunit 1, Yb corresponds to subunit 3, Yb′ corresponds to subunit 4, and Yc corresponds to subunit 2 (21).

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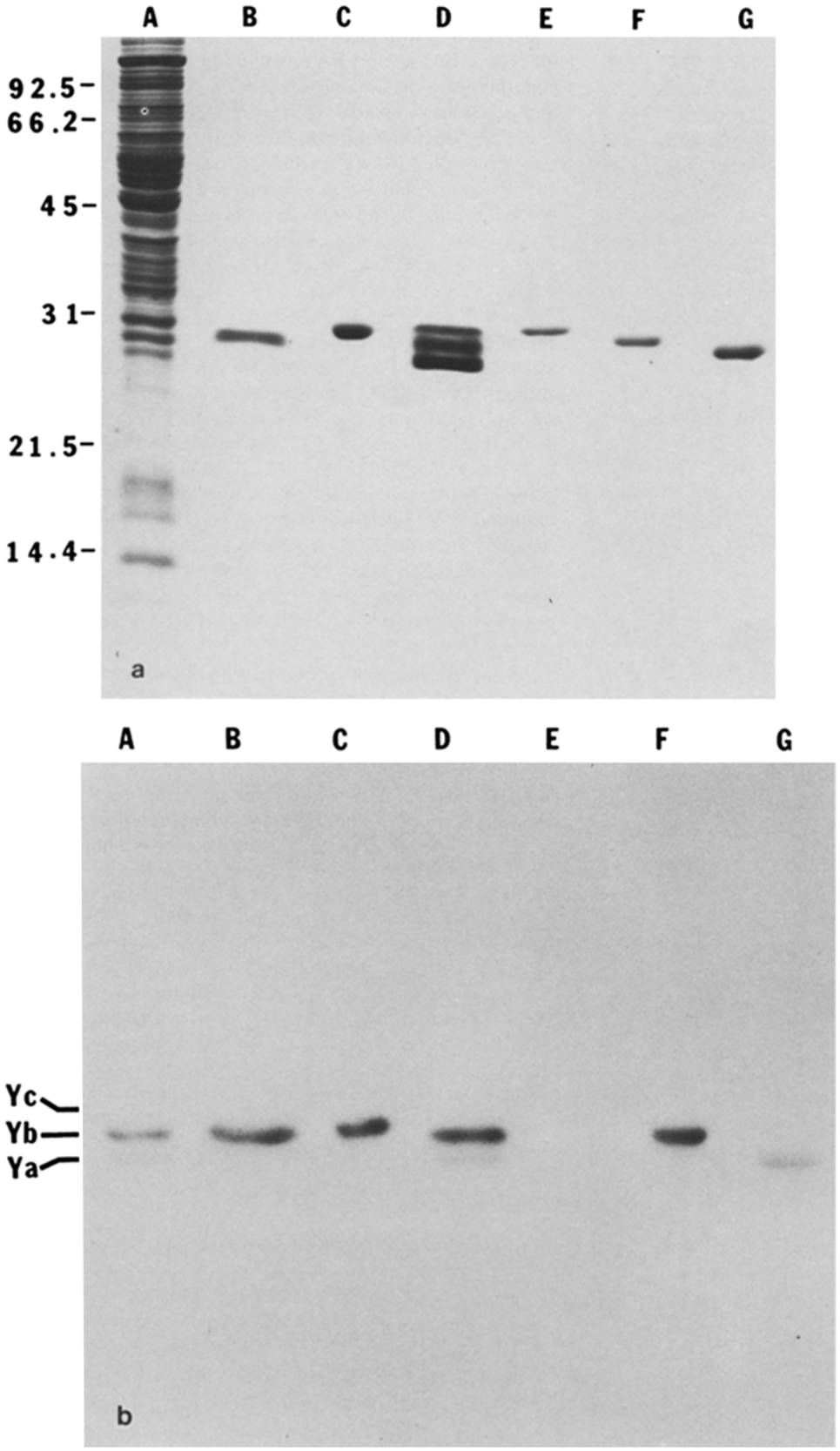

Figure 3. Immunoblot analysis of glutathione S-transferase subunits. Proteins were separated on a 12.5% SDS polyacrylamide gel (23) and either stained with Coomassie Brilliant Blue R (*a*) or transferred to nitrocellulose paper (44) and reacted with antibodies directed against protein BA (*b*). Lanes: (*A*) 150 μg total rat liver proteins; (*B*) 5 μg glutathione S-transferase A (Yb homodimer); (*C*) 5 μg protein BA_{bound}, (*D*) 10 μg protein BA_{free}; (*E*) BA^{c}_{free} subunits; (*F*) BA^{b}_{free} subunits; (*G*) BA^{a}_{free} subunits.

Peptide Mapping

To further show that protein BA_{bound} was homologous to the Yb subunits of glutathione S-transferase, peptide fragments provided by two different methods were compared. Partial peptide maps of cytosolic Yb subunits (Fig. 4, lane *A*), protein BA^{b}_{free} subunits (Fig. 4, lane *B*), glutathione S-transferase A (Fig. 4, lane *C*), and protein BA_{bound} (Fig. 4, lane *D*) were obtained using the *N*-chlorosuccinimide procedure (25), which cleaves tryptophanyl peptide bonds (39). The Yb sub-

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units were isolated from cytoplasmic glutathione S-transferase (Fig. 4, lane *A*) and protein BA_{free} (Fig. 4, lane *B*) by excising the bands from an SDS polyacrylamide gel (23). BA^{b}_{free} subunits isolated from nuclei and Yb subunits isolated from cytoplasm gave identical peptide fragments (Fig. 4, lanes *A* and *B*). *N*-Chlorosuccinimide cleavage of glutathione S-transferase A (Yb homodimer) yielded four peptides with molecular weights of 21,000, 17,000, 11,000, and 6,300. These data suggest that there are at least two tryptophan residues in the Yb subunits. Protein BA_{bound} gave a very similar partial peptide fragment pattern to that obtained for glutathione S-transferase A. The BA^{b}_{free} subunits from nuclear and Yb subunits from cytoplasmic glutathione S-transferases produced very similar partial peptide patterns to those obtained for glutathione S-transferase A and protein BA_{bound}. The peptides near uncleaved Yb and BA^{b}_{free} subunits (Fig. 4, lanes *A* and *B*) represent contamination by BA^{a}_{free} subunits when the BA^{b}_{free} subunits were excised from the gel.

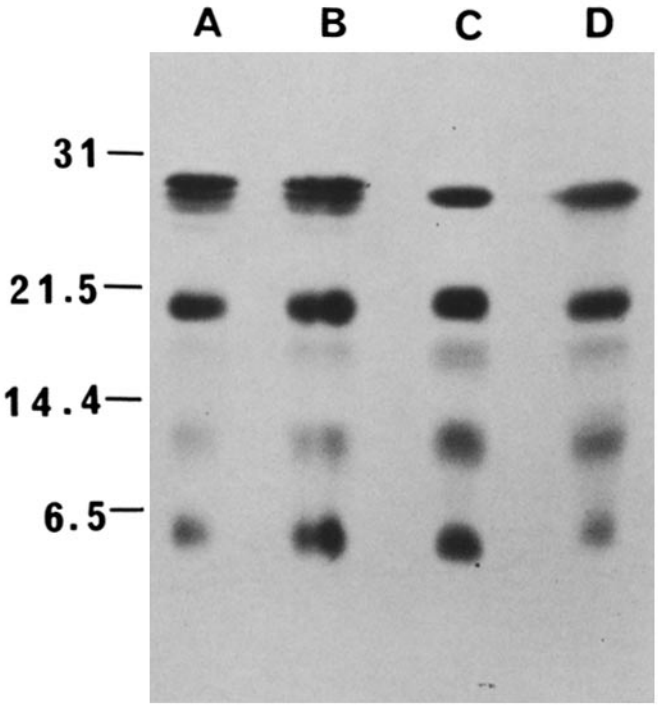

Figure 4. *N*-Chlorosuccinimide partial peptide cleavage of Yb subunits. The Yb subunits of cytoplasmic rat liver glutathione S-transferase (a mixture of AA, A, B, C, D, and E) (*A*), protein BA_{free} (*B*), glutathione S-transferase A (*C*), and protein BA_{bound} (*D*) were excised from a gel and treated with *N*-chlorosuccinimide as described in Materials and Methods. Peptides were detected by silver staining (46).

Further demonstration that protein BA_{bound} corresponded to the Yb subunits of glutathione S-transferase was provided by high performance liquid chromatography analysis of ^{125}I-tryptic peptides. The individual subunits of protein BA_{free} (BA^{a}_{free}, BA^{b}_{free}, and BA^{c}_{free}) share some homologous peptides (Fig. 5 *b, c,* and *d*); however, upon close examination it was evident that there are differences between them. These data agree with other reports suggesting that the individual glutathione S-transferase subunits are related but distinct polypeptides (5, 13, 45). The ^{125}I-tryptic peptide pattern obtained for protein BA_{bound} (Fig. 5*A*) was very similar to that obtained for BA^{b}_{free} subunits (Fig. 5*C*) and distinct from BA^{a}_{free} subunits (Fig. 5*B*) and BA^{c}_{free} subunits (Fig. 5*D*), providing further evidence that protein BA_{bound} isolated from chromatin is most similar in peptide composition to the Yb subunits of glutathione S-transferase. The ^{125}I-tryptic peptide patterns obtained for subunits BA^{a}_{free} and BA^{c}_{free} isolated from nuclei were virtually identical to the patterns obtained for cytoplasmic glutathione S-transferase subunits Ya and Yc (data not shown).

Immunolocalization of Glutathione S-Transferase Yb subunits

The subcellular localization of the glutathione S-transferases has been controversial (1, 6, 12, 37). The differences in results may be due to a different subcellular localization for each isoenzyme, or subunit (37). To confirm that glutathione S-transferase Yb subunits (protein BA^{b}_{free} and BA_{bound}) were in fact nuclear proteins in rat liver and not contaminants produced by the nuclear isolation procedure or growth of cells in culture, we localized the Yb subunits in rat liver cryosections. Antibodies reactive against protein BA^{b}_{free} and BA_{bound} (Fig. 3*b*, lanes *C* and *F*) produced both a mottled nuclear fluorescence and a generalized cytoplasmic fluorescence (Fig. 6, *a* and *b*). Preimmune immunoglobulins produced a weak cytoplasmic fluorescence (Fig. 6, *c* and *d*). Thus, glutathione S-transferases are found both in the nucleus and in the cytoplasm of rat liver and in an established rat liver cell line. The more intense and generalized fluorescence is thought to result

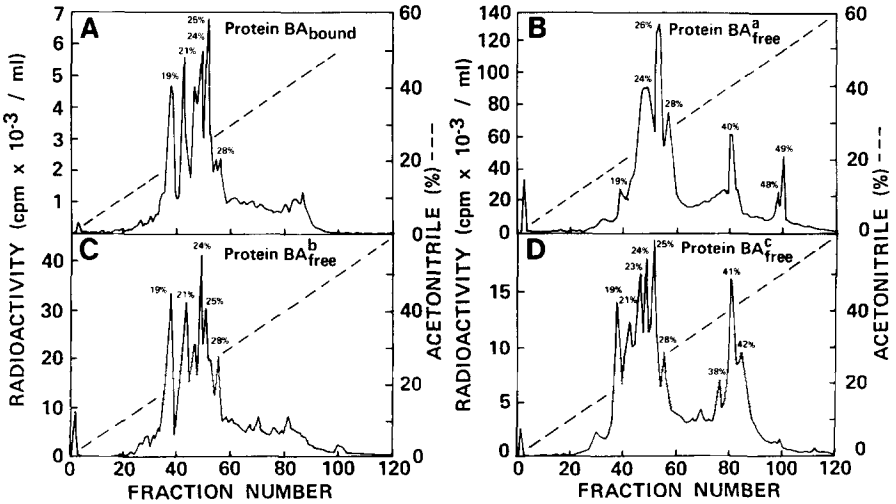

Figure 5. ^{125}I-Tryptic peptides of nuclear glutathione S-transferase subunits. Proteins were iodinated, separated by SDS polyacrylamide gel electrophoresis (23), eluted from gel slices, and digested with trypsin as described in Materials and Methods. ^{125}I-Tryptic peptides were separated by reverse phase high performance liquid chromatography on a C18 μBondapak column. (*A*) Protein BA_{bound}; (*B*) BA^{a}_{free} subunits; (*C*) BA^{b}_{free} subunits; (*D*) BA^{c}_{free} subunits.

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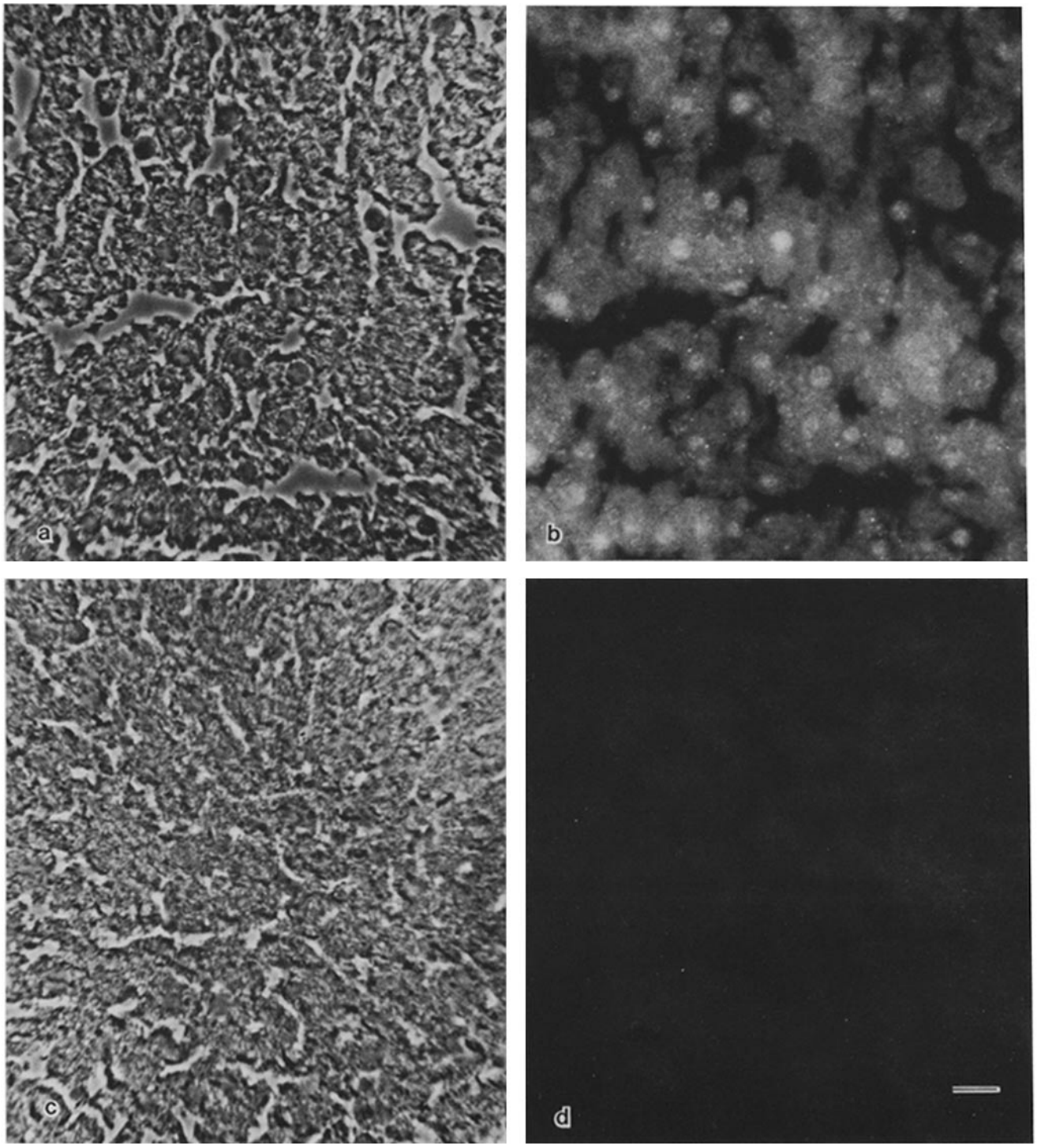

Figure 6. Immunolocalization of glutathione S-transferase Yb subunits. Rat liver cryosections were reacted with Yb subunit antibodies (*a* and *b*) or with preimmune immunoglobulins (*c* and *d*), followed by biotinylated-goat anti-rabbit and fluorescein isothiocyanate-conjugated streptavidin as described in Materials and Methods. Phase contrast (*a* and *c*) and immunofluorescent images (*b* and *d*). Bar, 20 μm.

largely from the 40-fold greater amount of transferase in liver than in cultured hepatocytes (unpublished data).

Discussion

There have been numerous nonhistone proteins that have been identified in the literature (reviewed in reference 7). However, relatively few of these proteins, including such widely studied proteins as the HMG proteins, have been identified with an enzyme activity or assigned a specific function. In this report we have identified nonhistone protein BA (3, 9) as glutathione S-transferase. This conclusion was based on homologies in amino acid composition, immunological identification, peptide maps, and enzymatic activity. Using the criterion mentioned above, we have determined that protein BA isolated from the saline–EDTA nuclear extract (protein BA_{free}) was a mixture of several glutathione S-transferase isoenzymes, composed of BA^{a}_{free}, BA^{b}_{free}, and

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BA^{c}_{free} subunits, that co-migrate with cytoplasmic Ya, Yb, and Yc subunits, respectively. Protein BA isolated from rat liver chromatin (protein BA_{bound}) was determined to be composed of glutathione S-transferase Yb subunits. Two-dimensional, non-equilibrium, isoelectric focusing/SDS polyacrylamide gel analysis has demonstrated that protein BA_{bound} exhibits two major pI variants, with the majority of the protein present in the more basic form (3). Thus, protein BA_{bound} is probably a mixture of subunits corresponding to Yb and Yb′ cytoplasmic glutathione S-transferase subunits, with the more basic Yb subunit (13) comprising the majority of the protein.

Antibodies that recognize glutathione S-transferase Yb subunits stained discrete regions of the cell nucleus that were also shown to contain U-snRNPs (2), which have been implicated in RNA splicing (33, reviewed in reference 36). Thus, we have identified an enzyme generally thought to be involved in detoxification (20) in the same interchromatinic domains where RNA processing occurs. The associations of BA^{b} subunits and U-snRNPs to these regions are mediated by different types of molecular interactions. The BA^{b} subunits were associated with the 2.0 M NaCl soluble chromatin fraction, while a portion of the U-snRNPs remained associated with the nuclear residue.

The nuclear domains that contain both the BA^{b}_{free} subunits, BA_{bound}, and U-snRNPs (11, 41) were shown by immunoelectron microscopy to be the interchromatinic regions of the nucleus. Previous studies demonstrated that protein BA_{bound} was localized to areas of condensed chromatin in isolated rat liver nuclei (8). This variance is probably the result of antigen redistribution during nuclear isolation or differences in epitope specificity between this antibody and the one used in previous studies. Our studies have shown that when rat liver is homogenized in aqueous buffers (pH 7–8) most, but not all, of the immunological and enzymatic activity are recovered in the soluble fraction (2, unpublished results). Thus, amounts of protein BA_{free} and BA_{bound} are thought to diffuse out of the nucleus during cellular fractionation.

The precise function or functions of the glutathione S-transferases in the cell nucleus are not known. The possibility that would seem to be the most obvious is the biotransformation of electrophilic compounds that have escaped detoxification in the cytoplasm thus preventing their interaction with DNA or other sensitive macromolecules. Many chemical carcinogens are known to form conjugates with glutathione, and in many cases, this reaction is catalyzed by the glutathione S-transferases (10). Glutathione S-transferases inhibit the formation of covalent adducts between benzo(a)pyrene and DNA in vitro and in vivo (18, 22). Furthermore, administration of anticarcinogenic antioxidants results in an increased synthesis of glutathione S-transferases, which is one proposed mechanism for their protective action (34).

A second possibility is that they may play a regulatory role in nuclear function. The discrete subnuclear localization of the Yb subunits to areas that contain U-snRNPs (2) suggests that these subunits function in or adjacent to regions of the nucleus where transcription or processing of RNA takes place. The Yb subunits have also been shown to associate with DNA in vitro (3, 9) and with DNA-containing structures in vivo (2, and Fig. 1). Granted, the amounts of glutathione S-transferase in the cell (0.1 mM in rat liver, reference 43) would seem to argue against a role involving specific gene regulation, however, this does not rule out the possibility that glutathione S-transferases play some, as yet undefined, role in modulating gene expression. It has recently been reported that actin, a major cellular protein, is involved in the transcription of chromosome loops in Pleurodeles oocytes (38), suggesting that abundant cellular proteins may play a role in the transcriptional process.

Although a specific role for the glutathione S-transferases in modulating gene function has not been demonstrated, there is some evidence to suggest that they may do so. Further experiments addressing this question are needed. The identification of nonhistone protein BA as glutathione S-transferase indicates an enzymatic activity for a previously described nonhistone protein, while the identification of glutathione S-transferase as a DNA-binding nonhistone protein opens up the possibility of nuclear interactions for this group of multifunctional enzymes.

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