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Immunodetection of Poly(A) Binding Protein II in the Cell Nucleus

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During the polyadenylation of pre-mRNA *in vitro*, poly(A) binding protein II (PAB II) binds to the growing poly(A) tail, stimulating its extension. The subcellular localization of PAB II was investigated with an antibody affinity-purified from rabbit serum raised against the purified protein. Immunofluorescence microscopy detected PAB II exclusively in the cell nucleus, both in a widespread staining and in more intensely stained "speckles." PAB II was excluded from the nucleoli. By electron microscopy, PAB II was also found almost exclusively in the nucleus, predominantly in clusters of interchromatin granules, likely corresponding to the speckles observed by immunofluorescence microscopy, and in perichromatin fibrils, which represent nascent transcripts and probably the sites of pre-mRNA processing. In addition, electron microscopy also detected PAB II in nucleoli. The distribution corresponds largely to that of other factors involved in the processing of pre-mRNA and is thus in agreement with the proposed role of the protein in polyadenylation.

INTRODUCTION

Two proteins have been described that can bind specifically to the poly(A) tails of mRNA. The highly conserved 68-kDa poly(A) binding protein I (PAB I) has been cloned from several species from yeast to man [reviewed in 1]. Biochemical and genetic analysis in yeast indicates that this protein is essential for translation and also involved in poly(A) tail shortening and, probably, the regulation of mRNA stability [2, 3]. A second poly(A) binding protein, PAB II, has a molecular weight of 49 kDa and is distinct from PAB I by physical and functional criteria [4, 5]. PAB II was identified by its role in the 3′-end processing of pre-mRNA *in vitro*. In this reaction, the RNA is first cleaved endonucleolytically at a particular position downstream of the coding sequence. The upstream cleavage fragment then receives a poly(A) tail. PAB II binds the growing poly(A) tail and strongly stimulates its extension. The protein is also involved in the mechanism that limits the length of poly(A) tails to 200–250 nucleotides [4, 6].

The proposed role of PAB II in mRNA polyadenylation predicts that PAB II is a nuclear protein. To test this, a polyclonal antiserum was raised against purified bovine PAB II. Antibodies were affinity-purified from the crude serum and used for *in situ* immunodetection of PAB II in HeLa cells. The experiments showed the anticipated nuclear localization of the protein and an intranuclear distribution common to many factors involved in pre-mRNA metabolism.

MATERIALS AND METHODS

Cell culture. HeLa cells were grown on glass coverslips in Joklik's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (Seromed, Biochrom KG, FRG). Cells were used at 60–80% confluency. Cells were treated with drugs as described [7]. For cordycepin treatment (3′-deoxyadenosine, Sigma) the cells were placed in fresh medium supplemented with cordycepin (10–500 μg/ml). Incubation was continued for 2 h prior to fixation. HeLa cell nuclear extract was prepared according to [8].

Antibodies. A New Zealand White rabbit was primed with 100 μg highly purified poly(A) binding protein from calf thymus [5] emulsified with complete Freund's adjuvans (Difco) and Arlacel A (Sigma). Boosts were given 4, 8, and 11 weeks after the initial injection with the same amount of protein mixed with incomplete Freund's adjuvans (Difco). Twelve days after each boost, the antibody titer was tested on Western blots. For affinity purification of PAB II-specific antibodies, 10 μg of purified PAB II was loaded onto a SDS–polyacrylamide gel and transferred to nitrocellulose. The blot was stained with Ponceau S, and the band of PAB II was cut out and used for affinity purification according to [9]. Other antibodies used in this study were a monoclonal antibody (3A7) directed against the 64-kDa subunit of the human cleavage stimulation factor [10], a monoclonal antibody against the splicing factor SC-35 [11], p80 coilin-specific human autoimmune serum (a gift from Dr. A. Lamond), anti-Sm human autoimmune serum (Küng) [12], and a monoclonal antibody (10E10) against human PAB I [13].

Western blotting. Proteins were separated on 10% polyacrylamide–SDS gels [14] and blotted to nitrocellulose membranes by the semidry procedure [15]. Blots were blocked in 20 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 0.05% Tween 20, 5% nonfat dry milk. Incubation with antibodies and washing were done in the same buffer lacking dry milk. Proteins were detected by peroxidase-conjugated swine antibodies directed against rabbit immunoglobulins (DAKO, Denmark) and chemiluminescence (ECL kit, Amersham).

Immunofluorescence staining of cells. Immunolabeling was performed as described [16]. The specimens were examined in a fluores-

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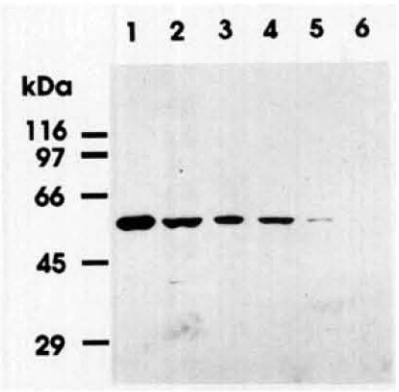


FIG. 1. Western blot detection of PAB II. A dilution series of HeLa cell nuclear extract, corresponding to 4, 2, 1, 0.5, and 0.25 μl was separated on a 10% SDS-polyacrylamide gel (lanes 2–6). 240 ng of purified PAB II were run in parallel (lane 1). The gel was blotted to nitrocellulose, and PAB II was detected with affinity-purified antibody.

cence microscope (IM35 or Axiophot, Carl Zeiss, FRG) equipped with epifluorescence. Photographs were taken on Fujichrome 100 film. Alternatively, the modular confocal microscope at the EMBL [17] was used as described [16].

Electron microscopy. Monolayers of exponentially growing HeLa cells or fragments of mouse liver were fixed with 4% paraformaldehyde in Sörensen phosphate buffer, pH 7.4, for 2 h on ice. The specimens were then dehydrated in ethanol and embedded in LR White resin following the manufacturer's recommendations. Some liver specimens were cryofixed, cryosubstituted with acetone, and embedded in the same resin [18]. Ultrathin sections were incubated with affinity-purified anti-PAB II antibody according to a standard protocol [19], in most cases in such a way that both sides of the section were labeled. Goat anti-rabbit IgG coupled with 15-nm colloidal gold particles (Aurion) was used as a marker. Control sections were incubated in the absence of the primary antibody. The sections of paraformaldehyde-fixed specimens were stained with the EDTA method preferential for nuclear ribonucleoproteins [20], whereas cryofixed and cryosubstituted material exhibited such contrast already after staining with uranyl acetate and lead citrate. The preparations were observed in a Philips CM10 electron microscope at 80 kV using a 30-40-μm objective aperture.

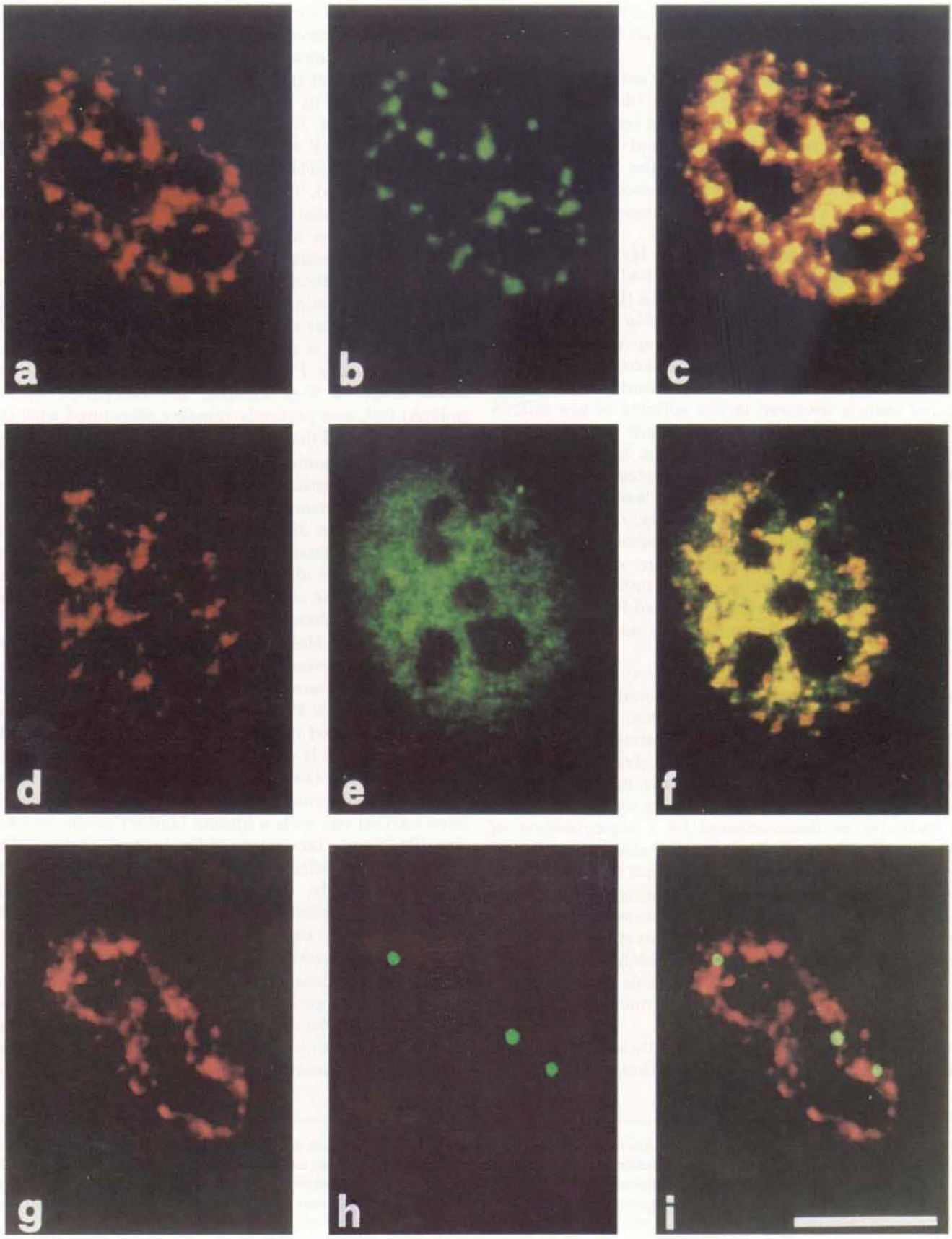
For quantitative estimates of the distribution of label, gold particles were counted over the interchromatin granule clusters, nucleoli, nucleoplasm (corresponding to the nuclear area with the exclusion of clusters of interchromatin granules and nucleoli) and cytoplasm. For this purpose, 15 randomly chosen cells of each group (paraformaldehyde-fixed HeLa cells and liver and cryofixed and cryosubstituted liver) were photographed and printed at the same magnification (×28,800). Labeling densities (number of gold grains per square micrometer) were determined for each cell compartment. The significance of the labeling density differences between the different cell compartments within each group was evaluated using the Wilcoxon signed rank test.

RESULTS AND DISCUSSION

Antiserum against PAB II was produced by repeated injections of the purified protein into a rabbit. On Western blots, a protein comigrating with purified PAB II was the major protein recognized by the crude serum in cell extracts (data not shown). PAB II purified to near homogeneity [5] was run on a preparative SDS-polyacrylamide gel and transferred to nitrocellulose. After staining, the band of PAB II was cut out and used for affinity purification of the antibody. Antibody obtained in this way reacted with purified PAB II. A single prominent band comigrating with purified PAB II was detected in HeLa cell nuclear extract (Fig. 1). A weak signal at 40 kDa was also seen, which was estimated by serial dilution of the extract to be about 10-fold weaker than that of PAB II (Fig. 1). In a purification of PAB II we have observed a protein of this molecular weight that had the same activities as PAB II, poly(A) binding and stimulation of polyadenylation, but was separated from it during ion-exchange chromatography. This protein also reacted with affinity-purified antibody (E. Wahle and S. Krause, unpublished data). Thus, the second strongest signal detected by the affinity-purified antibody very likely represents a protein closely related to PAB II, possibly a degradation product. Additional signals were even weaker and could only be seen with long exposures of the Western blot.

A crude estimate of the abundance of PAB II was obtained by Western blot analysis of aliquots from a total SDS lysate of a known number of HeLa cells along with known quantities of purified PAB II. Under the assumption that the HeLa cell protein reacts with the antibody in a manner similar to the bovine protein, two experiments with independent lysates detected approximately 2×10^6 and 3.4×10^6 molecules of PAB II per cell. The amount of hnRNA per nucleus in mouse L cells has been determined as 1.8 pg [21]. If the average molecular weight of hnRNA is 5×10^6, and 20% of the molecules carry poly(A) tails [22], nearly 40,000 poly(A) tails are estimated to be present in one nucleus. With a poly(A) tail length of 250 nucleotides and a packing density of one PAB II molecule for every 25 nucleotides (5), 400,000 molecules of the protein would be required to coat this amount of poly(A). Thus, PAB II appears to be present in sufficient quantities to coat all nuclear poly(A), and possibly in excess. Its intranuclear concentration is in the micromolar range, three orders of magnitude above the K_D for specific binding to an isolated

FIG. 2. Immunofluorescence staining of HeLa cells. HeLa cells were fluorescence-labeled with affinity-purified anti-PAB II (red, a) and anti-SC-35 (green, b) simultaneously. The secondary antibodies were labeled with Texas Red (PAB II) and FITC (SC-35). The overlay (c) reveals colocalization of PAB II and SC-35 in speckles (yellow). Double labeling of HeLa cells with anti-PAB II (red, d) and a monoclonal antibody against CStF (green, e), and false color overlay (yellow, f). Double labeling of cells with anti-PAB II (red, g) and with anti-p80 coilin antibodies (green, h) reveals no colocalization in the overlay (i). Bar, 10 μm.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oligo(A) binding site [5]. At this concentration, binding to sequences other than poly(A) would be expected [5] (E. Wahle, unpublished data).

The PAB II signal in cytoplasmic extract was much weaker than that in nuclear extract (data not shown) and may result at least partially from leakage or breakage of nuclei during isolation. No signal was observed in nuclear or cytoplasmic extract in the 70 kDa region where PAB I would be expected. A monoclonal antibody directed against PAB I also did not crossreact with PAB II (data not shown).

Immunofluorescence staining of HeLa cell monolayers with the affinity-purified PAB II antibody showed an exclusively nuclear reaction (Figs. 2a, 2d, and 2g). Little or no staining was detected in the nucleoli. In the nucleoplasm, there was a wide-spread staining in addition to an intense spot-like pattern reminiscent of the "speckled" pattern described for antibodies directed against factors involved in the splicing of pre-mRNA [11, 16]. The speckled nuclear pattern was evident in growing as well as in confluent cells. In mitotic cells, PAB II was present throughout the cytoplasm (data not shown). When the labeling reaction was carried out in the absence of the primary antibody, no fluorescence was detectable. With preimmune serum, only a weak general staining was seen without any specific nuclear reaction. When the affinity-purified antibody was used in the presence of an excess of purified PAB II, all fluorescence was uniformly reduced to near background level (data not shown).

The PAB II staining pattern was compared to the staining patterns of other nuclear proteins. Monoclonal antibodies against the SC-35 protein, a non-snRNP splicing factor, show a speckled pattern [11]. These speckles obviously correspond to clusters of interchromatin granules visible in the electron microscope [23]. PAB II and SC-35 were colocalized in the interchromatin granules as demonstrated by a superposition of pseudo-colored confocal images of HeLa cell nuclei immunolabeled with anti-PAB II and anti-SC-35 simultaneously. Outside the granules, staining was predominantly by PAB II (Figs. 2a–2c). The same speckles that were stained with anti-PAB II also reacted with a human anti-Sm autoimmune serum, which recognizes the snRNPs involved in splicing [24]. In this case, the staining also overlapped throughout the nucleoplasm (data not shown).

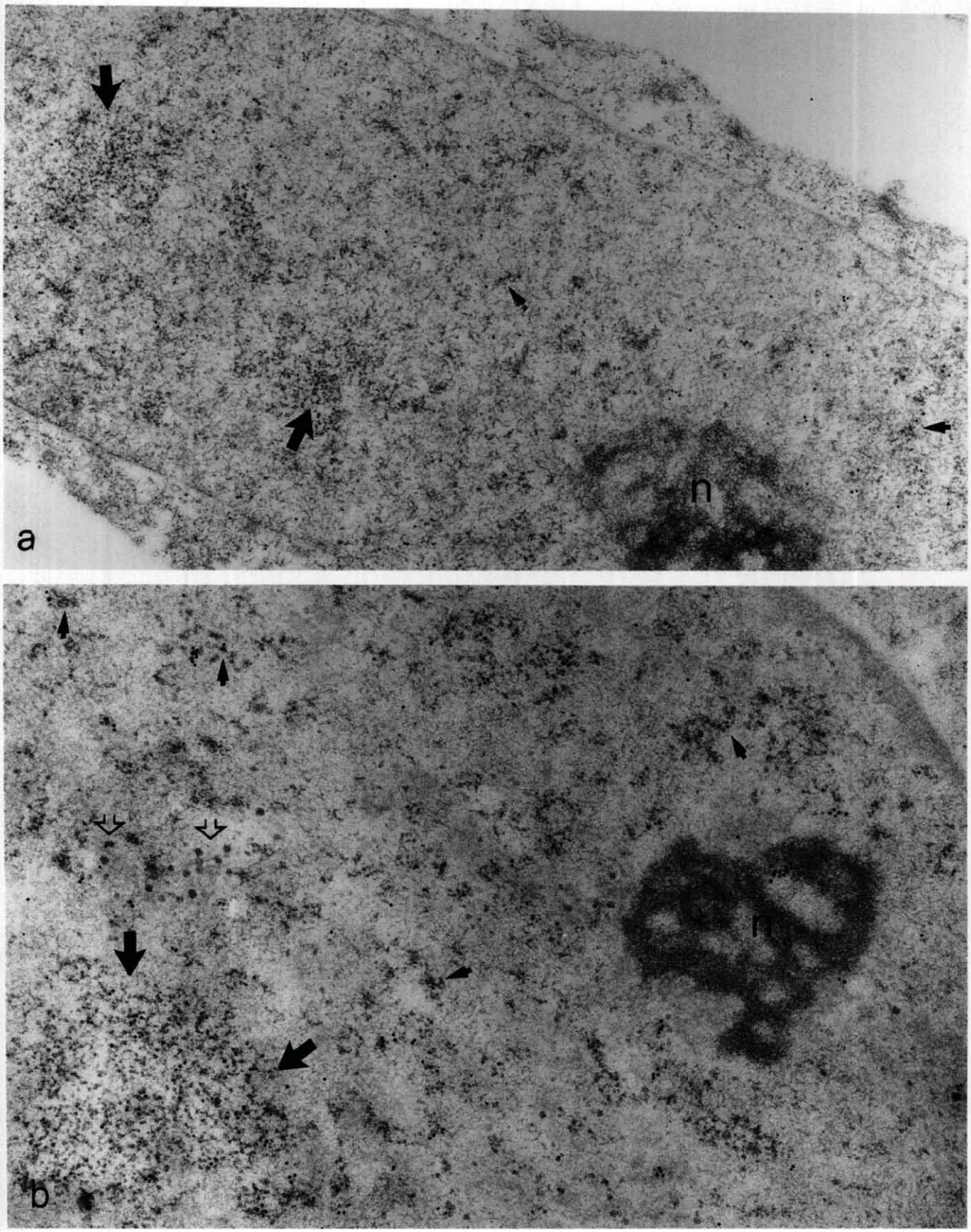
CStF, a factor involved in the 3′-cleavage of pre-mRNA that precedes polyadenylation, has been reported to be widely distributed throughout the nucleoplasm [10]. We have confirmed this wide-spread distribution. More intensely labeled regions were also visible. Although some of these appeared to correspond to the speckles stained by anti-PAB II, the colocalization was not perfect (Figs. 2d–2f). Upon treatment of cells with α-amanitin, CStF was almost exclusively localized in the same speckles that were recognized by anti-PAB II (data not shown). Treatment with α-amanitin also increases the association of snRNPs with interchromatin granules [7]. The only partial overlap of PAB II and CStF may be related to their functions: CStF (also called CF1) is thought to interact with an RNA sequence downstream of the cleavage site [25] and is probably released after the pre-mRNA has been cleaved. *In vitro,* the factor is not required for the second step of 3′-end processing. PAB II, in contrast, is involved in the latest stage of 3′-processing, the elongation of the poly(A) tail, and probably remains associated with the poly(A) tail until the RNA is exported from the nucleus.

Several antibodies and antisense probes directed against components of the splicing machinery recognize not only interchromatin granules but also "coiled bodies" [reviewed in 26]. Figures 2g–2i demonstrate that the antibody against PAB II does not decorate the coiled bodies, which are identified by their reaction with a human autoimmune serum against coilin, an 80-kDa protein present in these structures [27, 28].

Treatment of HeLa cells with drugs interfering with transcription, actinomycin D, α-amanitin, or cordycepin (3′-deoxyadenosine), did not result in cytoplasmic staining with anti-PAB II. Exclusively nuclear staining was also observed in other cell lines (HepG2, 3T3). In BHK cells, PAB II was also predominantly localized in the nucleus, but there was a higher cytoplasmic signal. Immunofluorescence experiments have independently been carried out with a human bladder carcinoma cell line, T24, in the laboratory of Dr. Luitzen de Jong. The experiments confirmed the high specificity of affinity-purified antibody, the localization pattern described above, and the colocalization of PAB II and SC-35 in the clusters of interchromatin granules (L. de Jong, personal communication).

The intranuclear distribution of PAB II described above is in very good agreement with light microscopic observations of the distribution of poly(A): After hybridization with biotinylated poly(dT), labeling is observed both in a widespread nucleoplasmic distribution and in

FIG. 3. Localization of PAB II by electron microscopy. (a) Immunogold-labeled section of a paraformaldehyde-fixed HeLa cell. The antibody is mainly associated with perichromatin fibrils (some indicated with small arrows) and with clusters on interchromatin granules (large arrows). Few gold grains are seen on the nucleolus (n). ×28,800. (b) Immunogold-labeled section of paraformaldehyde-fixed mouse liver. Labeling occurs mostly in association with perichromatin fibrils (small arrows) and a cluster of interchromatin granules (large arrows). Perichromatin granules (open arrows) are rarely labeled. Some gold grains can be observed on the nucleolus (n). ×38,800.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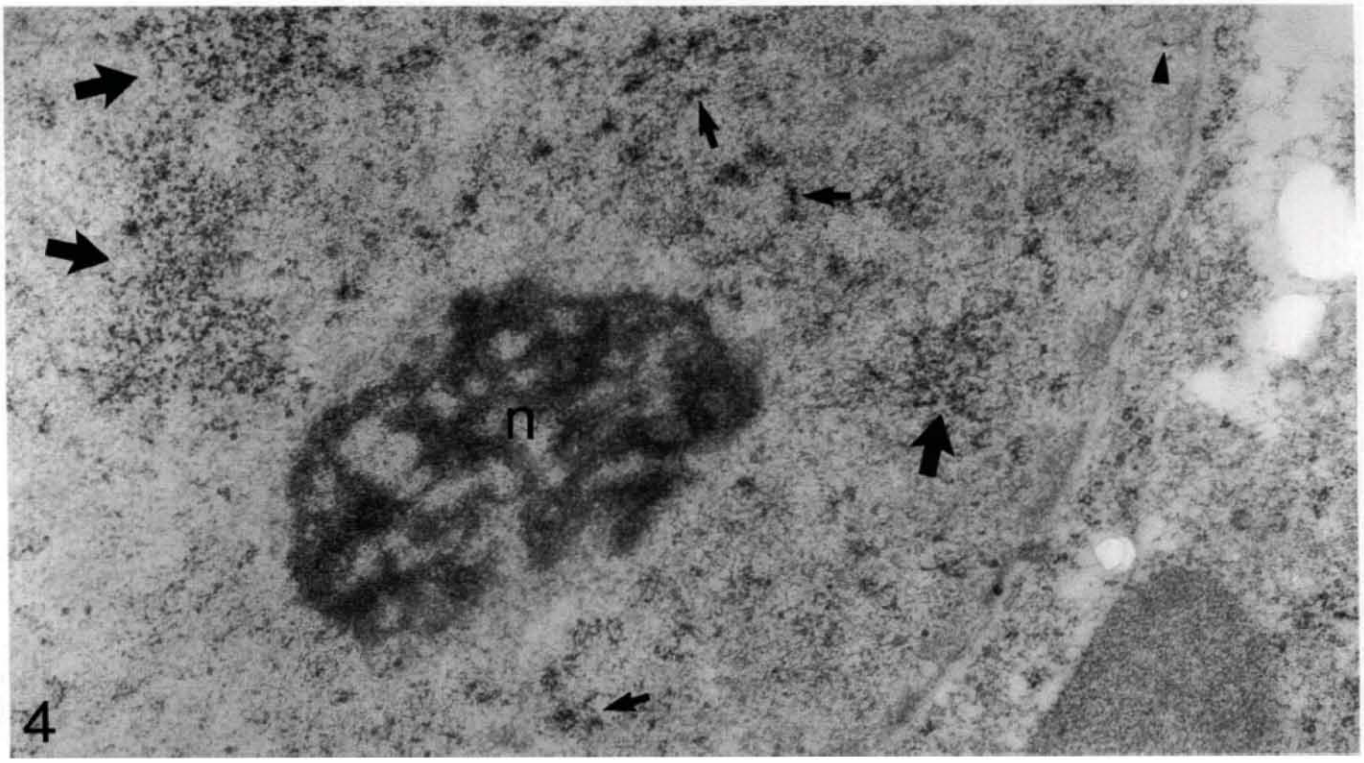

FIG. 4. Section of a paraformaldehyde-fixed mouse hepatocyte incubated under control conditions, in the absence of the anti-PAB II antibody. Symbols as for Fig. 3. The arrowhead points to a gold grain. ×28,800.

the same speckles that are strongly labeled with anti-Sm antibodies [29, 30].

The intracellular localization of PAB II was also investigated at the ultrastructural level with affinity-purified primary and gold-labeled secondary antibody. In all specimens, the majority of label was nuclear and associated with perichromatin fibrils and clusters of interchromatin granules (Figs. 3a and 3b). Perichromatin granules showed only occasional labeling (Fig. 3b). Labeling of control sections incubated without primary antibody was negligible (Fig. 4). A quantitative estimate of the labeling densities in HeLa and liver cells (Table 1) confirmed a significant difference between the predominant nuclear and the weak cytoplasmic labeling. The analysis shows a highly significant difference in labeling densities between interchromatin granules and other compartments ($P \leq 0.001$). It thus confirms interchromatin granules as the structural component of the nucleus containing the most significant concentration of PAB II. The labeling density in the nucleoplasmic area outside the interchromatin granule clusters appears relatively low in comparison. However, perichromatin fibrils are the major structural component strongly labeled with anti-PAB II antibody in such nucleoplasmic regions. Since the surface occupied by the fibrils is rather limited and virtually impossible to evaluate, the overall labeling density in the nucleoplasm certainly underestimates the association of PAB II with perichromatin fibrils. In contrast to the immunofluorescence experiments, nucleolar labeling was clearly detectable in the electron microscope.

TABLE 1

Values for Gold Grain Densities (Grain Number per Square Micrometer ± SEM) in Different Cell Compartments after Labeling with Anti-PAB II Antibody

	Nucleoplasm	IG	Nucleolus	Cytoplasm
HeLa	7.42 ± 0.25	27.56 ± 1.91	8.75 ± 0.68	1.87 ± 0.23
Liver pf	6.44 ± 0.43	21.93 ± 2.04	7.27 ± 1.17	1.84 ± 0.20
Liver cryo	8.46 ± 0.27	40.00 ± 3.19	13.92 ± 1.48	3.14 ± 0.25

Note. IG, interchromatin granule clusters; pf, paraformaldehyde fixation; cryo, cryofixation and cryosubstitution.

As in the immunofluorescence experiments, the distribution of PAB II revealed in the electron microscope was in good agreement with previous studies on the intranuclear distribution of poly(A), in that the major labeled structures were clusters of interchromatin granules and perichromatin fibers, while the perichromatin granules were less strongly labeled [30]. The same report [30] also found coiled bodies to be devoid of poly(A), in agreement with the immunofluorescence re-

sults presented above. The major discrepancy concerns the presence of PAB II in the nucleolus, in which poly(A) has not been found [30]. Other nuclear RNA binding proteins involved in mRNA metabolism have been detected in the nucleolus as well, even though no nucleolar function of these proteins is known [31, 32]. Nonspecific binding of an abundant protein to ribosomal RNA (see above) might be an explanation.

Association of PAB II with interchromatin granules and perichromatin fibrils observed by electron microscopy is also in good agreement with the immunofluorescence data. The nucleolar and the much weaker cytoplasmic labeling were not detectable by immunofluorescence. This difference may largely reflect different signal thresholds detectable by the two methods.

Perichromatin fibrils contain hnRNP core proteins and snRNPs [33], U1 and U2 snRNAs [34], as well as the splicing factor SC-35 [35]. Since the perichromatin fibrils have previously been shown to represent nascent pre-mRNA [36, 37], it is highly probable that splicing takes place on these structures. As 3'-end processing is thought to be initiated on nascent transcripts [38], the association of PAB II with perichromatin fibrils is consistent with its proposed role in mRNA polyadenylation. The interchromatin granule clusters contain only low levels of rapidly labeled RNA [for review, see 39] and hnRNP proteins [33]. However, they do contain poly(A) (see above) and large amounts of different splicing factors [23, 33–35, 40]. Although it is generally assumed that the interchromatin granule clusters do not represent splicing organelles, their true function in nuclear RNA metabolism remains unknown [see 30 for a recent discussion].

The nuclear localization of PAB II demonstrated in this paper, its colocalization with factors known to be involved in pre-mRNA processing, and the association with perichromatin fibrils are in agreement with a role of the protein in polyadenylation. In contrast, immunofluorescence studies with PAB I in yeast and mammalian cells show that the major localization of this protein is cytoplasmic [13, 41]. This is consistent with the genetic data showing an involvement in two cytoplasmic reactions, translation and poly(A) shortening [2]. The localization of the two poly(A) binding proteins in two different cellular compartments confirms their roles in different aspects of poly(A) tail metabolism.

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