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IMMUNOHISTOCHEMICAL DETECTION OF RIBOSOMAL TRANSCRIPTION FACTOR UBF: DIAGNOSTIC VALUE IN MALIGNANT SPECIMENS

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SUMMARY

The nucleolar organizer regions (NORs) of human chromosomes can be identified in interphase and mitotic cells by localization of some intrinsic components such as the associated enzyme RNA polymerase I. A new sensitive staining method for NORs is described using a specific antibody to the ribosomal transcription factor UBF. By indirect immunofluorescence and enzyme-labelling methods, NORs stained in benign and malignant cells from a variety of tissues with monospecific anti-UBF serum showed significant morphological differences which correlated well with histopathological evaluation. The number of NORs per cell in malignant preparations increased significantly. Furthermore, the staining of a NOR protein component such as UBF appears to be as sensitive as the silver-staining technique (AgNOR) and might be a better alternative for detecting ribosomal activity in malignant tissues. © **1998 John Wiley & Sons, Ltd.**

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KEY WORDS—**nucleolar organizer regions; upstream binding factor; UBF; AgNORs**

INTRODUCTION

Nucleolar organizer regions (NORs) are loops of chromatin which contain the rRNA genes (rDNA). The argyrophilic staining technique developed by Plotton *et al.*[1] known as the AgNOR method, detects proteins associated with these regions as black dots whose number and surface area are used as a parameter to estimate proliferative activity. This technique has been accepted in histopathology as an aid in the diagnosis and prognosis of malignancy.[2] Several studies have suggested that the staining assay correlates with cell proliferation rather than with biological malignancy.[2,3] However, it remains a matter of controversy whether the changes observed in NOR area reflect only activated metabolism and rRNA synthesis, or a specific role of some NOR-associated protein in cell duplication.

The contribution of each NOR protein to nucleolar activity as measured by the AgNOR technique is largely unknown and thus specific evaluation of individual NOR components seems mandatory. This approach might not only lead to a better understanding of the functions of different NOR proteins, but could also turn out to be of diagnostic value. In addition, an immunohistochemical technique might advantageously replace or complement the silver-staining technique, making it more reproducible and easier to interpret and sparing many laboratories the sophisticated computer-aided image analysing system which is required to evaluate the AgNOR technique objectively.

The RNA polymerase I transcription factor UBF remains closely associated with rDNA in mitotic cells and is therefore a NOR protein,[4] although its silver affinity and contribution to AgNOR staining have not been established. We have previously cloned the UBF gene from a hamster expression library (CHO cells) and produced rabbit polyclonal antibodies against this factor.[5] As expected for a highly conserved gene product, these antibodies are universal markers for NOR structures from animals to plant cells. In this report, we assayed an anti-UBF serum in a wide array of benign and malignant lesions seen in the daily practice of a pathology laboratory, to test the validity of UBF evaluation as an alternative to the silver staining of NORs in the diagnosis of malignancy.

MATERIALS AND METHODS

Pathological samples

These were taken as touch imprints or frozen sections. Paraffin blocks were not used, as formalin fixation seems to mask UBF localization. Biopsies from the lymphoma specimens studied were sent to experts from the Spanish Lymphoma Club in Madrid for confirmation of the reported diagnosis. Where touch imprints were used, sections from the same area were embedded in paraffin wax and stained with haematoxylin and eosin in order to confirm that they were representative of the neoplastic cell population.

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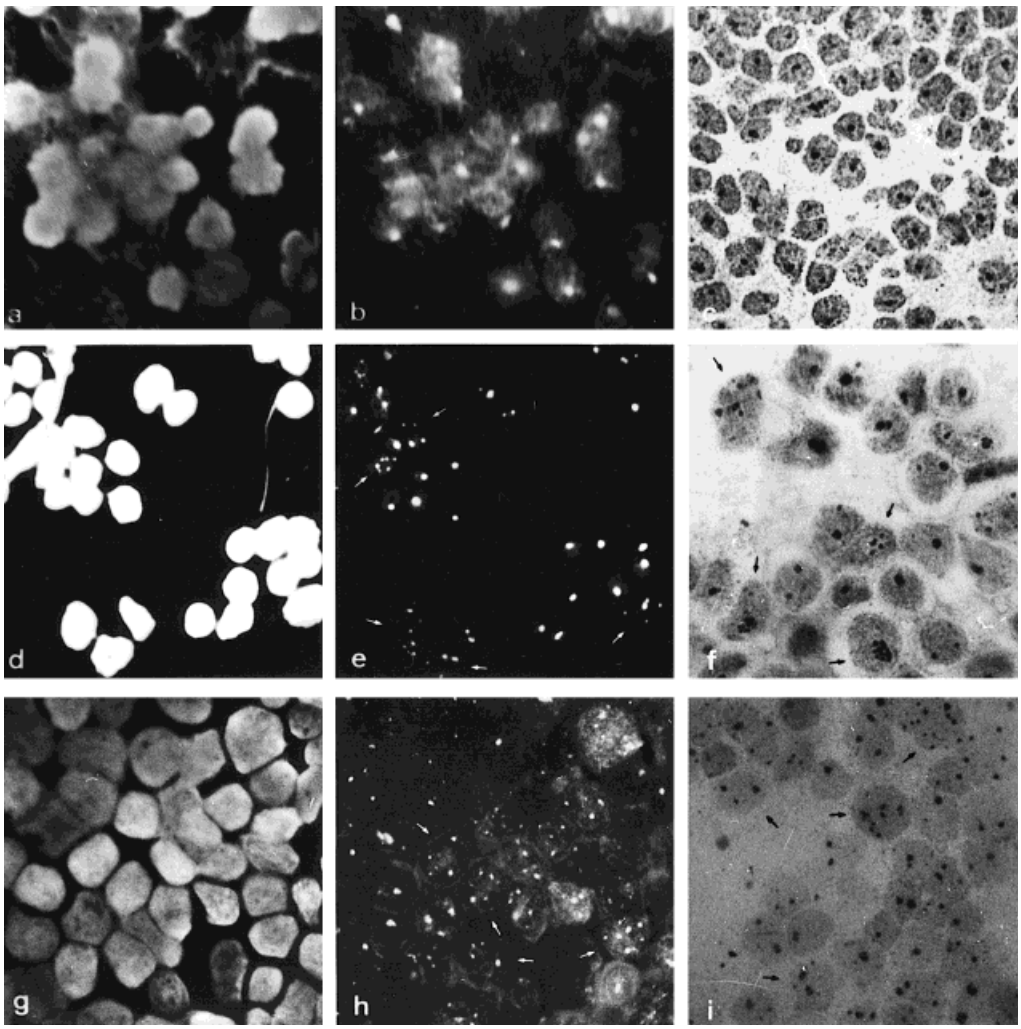

Fig. 1—Immunofluorescence staining of the ribosomal transcription factor UBF and AgNOR labelling of three different lymphomas. A small lymphocytic lymphoma is shown in a, b, and c; a centroblastic–centrocytic lymphoma is shown in d, e, and f; and a centroblastic–centrocytic lymphoma in progression to diffuse centroblastic lymphoma is shown in g, h, and i. The same cells are shown by immunolocalization of UBF (b, e, h) and Hoechst staining of DNA (a, d, g). AgNOR staining is shown in c, f, and i. On these three specimens, there is a good correlation in the number of dots per cell with the AgNOR technique and the UBF fluorescence method

Silver staining

AgNOR staining was carried out on cytological preparations by the method of Plotton *et al.*[1] with the following modifications:[6] preparations were fixed in 80 per cent ethanol for 5–24 h. Afterwards, they were briefly washed in distilled water and laid upside down in a grooved crystal plate over a heated plate at 37°C. The staining solution consisted of a 1:1 mixture of 1 per cent formic acid and 50 per cent silver nitrate. After 10 min, the slides were thoroughly washed in distilled water, dehydrated, and mounted using mounting medium acidified with 1 per cent formic acid. Counterstaining was done either with methyl green or by adding to the first 95 per cent ethanol a few drops of eosin solution.

Anti-UBF staining

For immunostaining, an anti-recombinant UBF serum was used. The production of this and other anti-UBF sera will be described elsewhere. Briefly, truncated hamster UBF protein (220–764 amino acid residues) was expressed in the pET-3a system under the T7 RNA polymerase promoter and used for the production of specific rabbit anti-UBF antibody. This serum recognizes the typical 94–97 kD polypeptide doublet corresponding to UBF1 and UBF2 in Western blots of human cell extracts.

Cytological samples of frozen sections in polylysine or silane-coated slides were fixed in acetone for 1–3 h. Slides were incubated for 45 min at 37°C with polyclonal rabbit anti-UBF serum diluted 1:300 in phosphate-buffered saline (PBS) in a humidified chamber. After washing in PBS, a second fluorescein-labelled anti-rabbit antibody was applied for 45 min at 37°C. The preparations were washed in PBS and mounted in PBS–glycerol, 1:9 (vol/vol), containing 1 μg/ml Hoechst 33342. The dye attaches to DNA, allowing easy recognition of the nucleus. Fluorescent spots were clearly localized

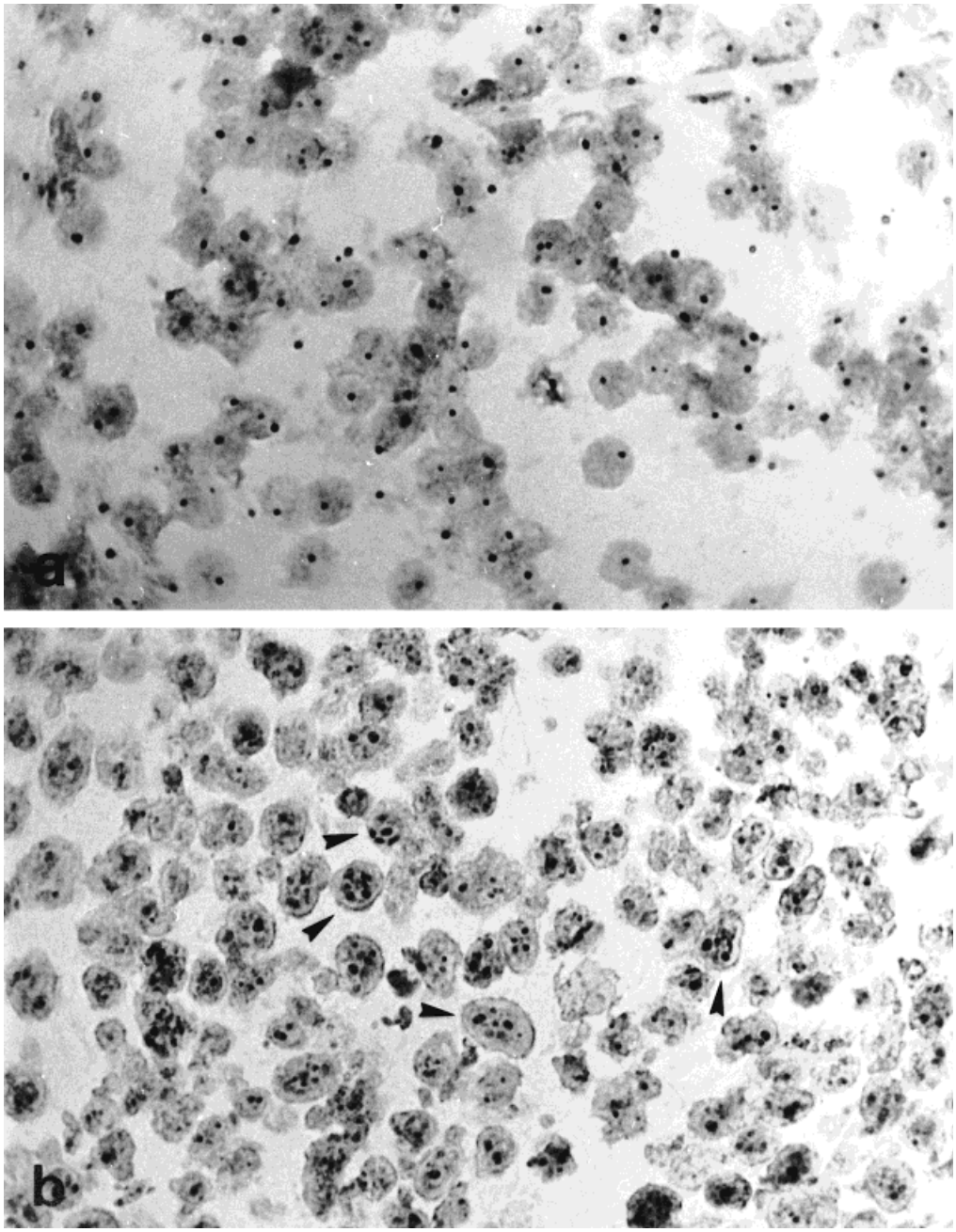

Fig. 2—Immunohistochemical detection of UBF by peroxidase labelling. Small lymphocytic lymphoma (a) and large B-cell lymphoma (b) stained with anti-UBF serum and peroxidase-labelled second antibody developed with AEC (aminoethyl carbazole). Whereas in a a single dot is found in each cell, note the multiple dots per cell corresponding to UBF activity in b, indicated by arrows

inside the nucleus and comparison of positive dots and nuclear area was facilitated. In some cases, a second antibody labelled with peroxidase (Vectastatin kit) was used and subsequently developed with diamino benzidine (DAB), counterstained with methyl green, dehydrated, and mounted. In other cases, antibodies were developed with aminoethyl carbazole (AEC; Biomeda Corporation). The proliferation index was estimated by Ki-67 staining. Observations were made using a Zeiss Axiophot fluorescence microscope equipped for epi-illumination.

RESULTS

UBF staining by the immunoperoxidase or immunofluorescence methods yielded good quality preparations on the different types of cells tested. We have been preferentially using fluorescence staining, because it can be combined with Hoechst 33342 labelling of nuclei. AgNOR and UBF staining carried out in parallel showed a similar pattern and degree of expression, correlating with each other as well as with the proliferation index and/or the malignant grade. This can be

seen in Fig. 1, which shows three lymphomas (a small lymphocytic, a centroblastic–centrocytic, and a centroblastic–centrocytic in progression to a diffuse centroblastic lymphoma) whose proliferative indices were 4, 15–20, and 20 per cent respectively, as assessed by Ki-67 staining.

A gradual increase in positive dots with higher proliferative index and/or malignant histological grade can be appreciated even by simple inspection. Incidentally, diagnosis was also facilitated in case 2 in Fig. 1 by this technique, as it showed two cell types with different morphologies by Hoechst staining, differing in the number of positive dots. These two cell types were identified as centroblasts and centrocytes. A similar clear-cut difference in the number of dots between high- and low-grade lymphomas was seen when a peroxidase-labelled second antibody was used (Fig. 2).

A good correlation between AgNOR and UBF staining was seen in many other lesions studied (e.g., breast carcinoma, data not shown). In benign thyroid lesions (nodular hyperplasia), most cells contained 2 dots and some of them 4–5, as revealed by the silver technique; this again was mirrored by UBF staining (Fig. 3). However, we were surprised to find a divergence between AgNOR and UBF dot number in a case of thyroid papillary carcinoma. By the silver technique, most cells displayed one or two dots per nucleus (Fig. 4c). This low AgNOR number has been reported for thyroid neoplasms by other authors, who concluded that the AgNOR technique was not useful for distinguishing benign from malignant lesions of the thyroid.[7] In contrast, preparations stained with anti-UBF showed frequent cells displaying a sprinkling of small positive dots (Fig. 4). This was demonstrated by immunofluorescence (Fig. 4b) and immunoperoxidase (Fig. 4d) techniques, although the latter seems to underestimate the number of dots, as focusing up and down was necessary to obtain an accurate measurement. The proliferation index of this carcinoma was estimated to be low (<1 per cent). Further studies on thyroid carcinomas will be necessary to clarify the advantage of UBF immunostaining over AgNOR labelling in this type of malignancy.

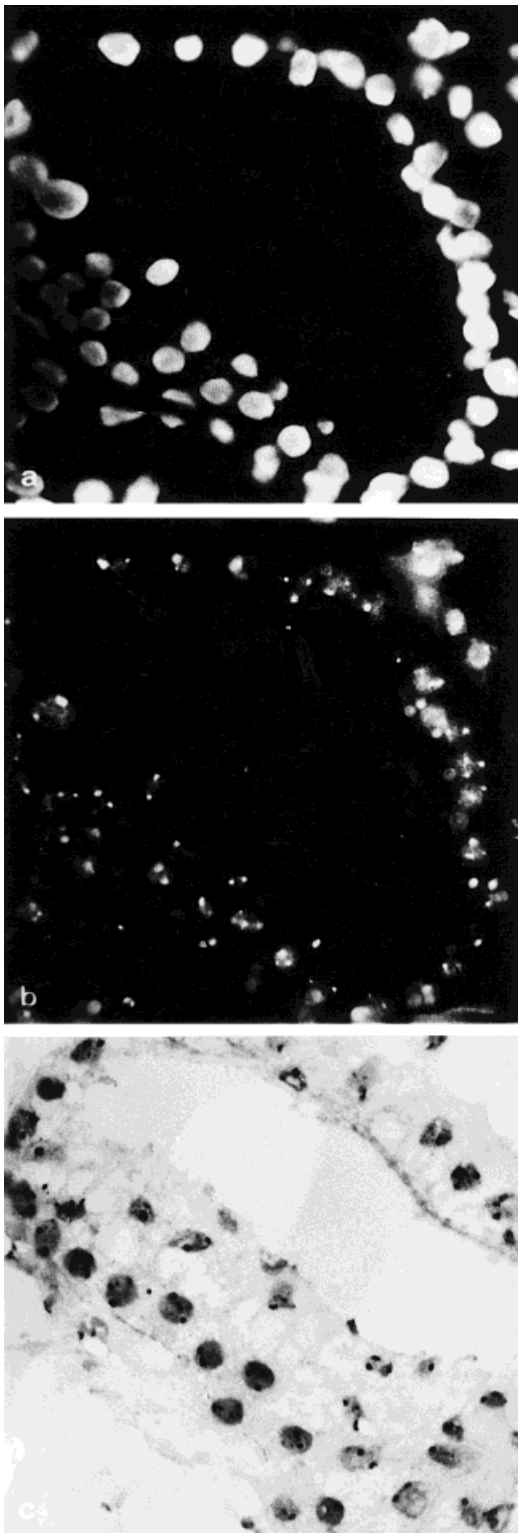

Fig. 3—Immunofluorescence staining of ribosomal transcription factor UBF (b) and AgNOR labelling (c) in thyroid nodular hyperplasia. On this tissue, the number of dots per cell (usually 2) detected by both techniques is similar. (a) Hoechst staining for DNA of the same cells as in b.

DISCUSSION

Immunohistochemical analysis using specific staining of a NOR component, the ribosomal transcription factor UBF, shows a significant difference in the distribution of this protein between benign and malignant lesions (Figs 1 and 2). This finding suggests a clear difference in nucleolar activity, which increases in the malignant specimens. A similar distribution was obtained using the standard AgNOR staining technique and a close correlation between AgNOR and anti-UBF staining has been obtained in most of the lesions tested to date. Although this is not surprising, it cannot be altogether anticipated, since UBF is not a major target protein for AgNOR staining of interphase cells.[4] In fact, after gel electrophoresis and blotting analysis, we could not visualize any reactivity of a recombinant UBF protein by the AgNOR technique, in spite of using a large excess of the protein as detected by both Ponceau Red staining and specific immune reaction with anti-

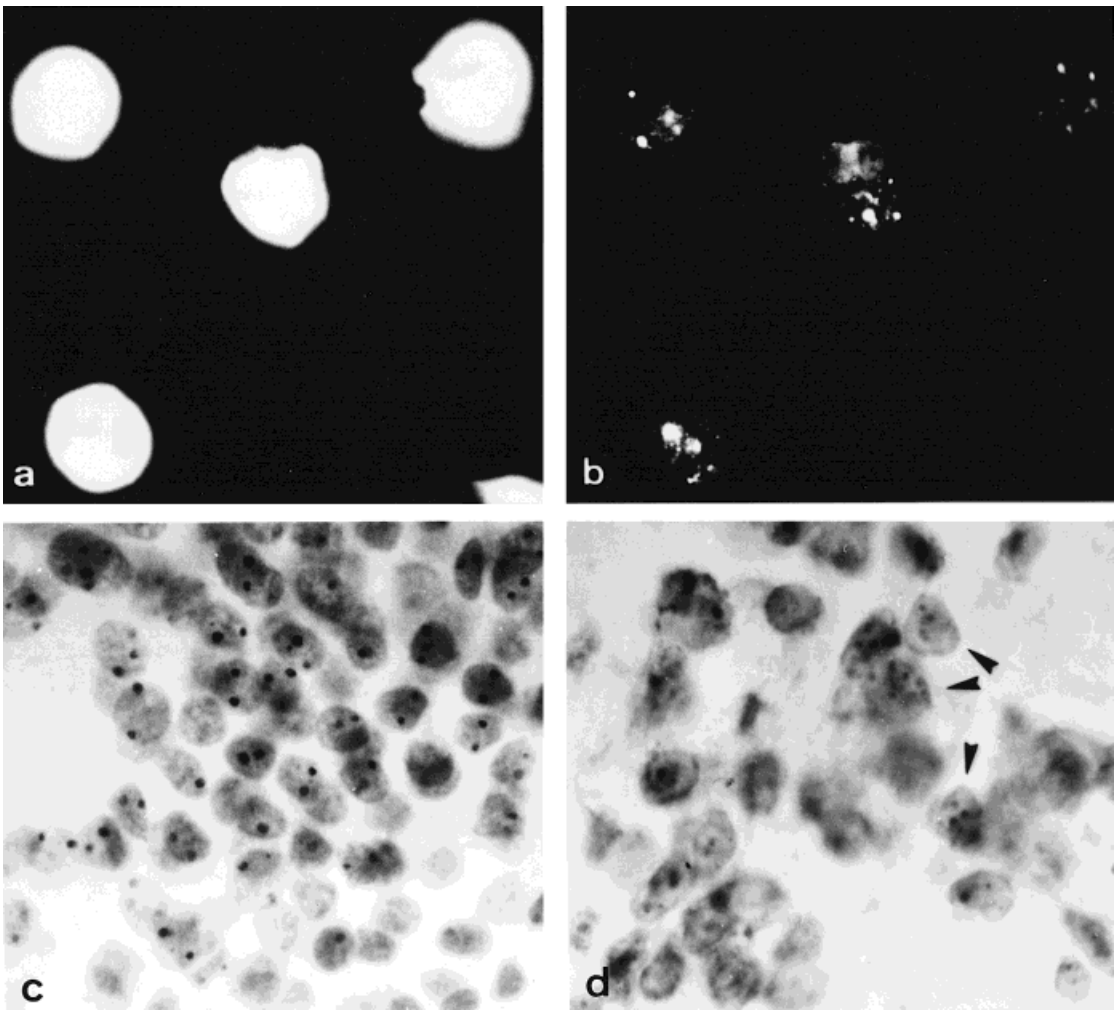

Fig. 4—Immunofluorescence (b) and peroxidase staining (d) of thyroid papillary carcinoma cells with anti-UBF serum. AgNOR labelling for this type of specimen is shown in c. Note that the UBF multi-dot pattern found in this tissue by fluorescence and peroxidase immunolabelling is not revealed by the AgNOR method. (a) Hoechst staining for DNA of the cells shown in b.

UBF (data not shown). This result clearly suggests that UBF is not an AgNOR stainable protein.

Many studies have demonstrated the usefulness of interphase AgNOR evaluation in tumour pathology for diagnostic and prognostic purposes. However, the applicability of this technique is limited to a few lesions and it suffers from some drawbacks regarding both its interpretation and its significance. Simple enumeration of black dots (the counting method) was found to have low reproducibility, mainly due to frequent overlap between AgNORs and to observer subjectivity. This difficulty was overcome by resorting to computer-aided analysing systems which take account of the number and surface area. Nevertheless, areas are highly dependent on the fixative employed, the temperature, and the time of staining, so that comparison of data between different laboratories is sometimes difficult, although a new refinement has recently been proposed for the standardization of the technique.[8]

The main difficulty, however, lies in our ignorance about the underlying significance of the AgNOR method, as it stains several proteins in proportions not known and some of which have not yet been characterized. Data gathered from descriptive histopathological studies, as well as more basic research, suggest that AgNOR amount reflects mainly proliferative activity,[9] although some claims to the contrary have also been made.[10] Hernandez-Verdún and co-workers have shown that increased AgNOR amount correlates well with increasing amount of two major nucleolar proteins, nucleolin and B23.[9] It has been suggested that nucleolin interacts with histone H1, implying that it could participate in chromosome decondensation,[11] thus being mainly related to cell duplication. Instead, UBF as specific cofactor of RNA polymerase I must be directly involved in ribosome biogenesis. The two functions of cell proliferation and ribosome biogenesis are usually coupled, but they may dissociate in some conditions.

The divergence in the number of silver and UBF dots in a case of thyroid papillary carcinoma is of interest. Although this result needs confirmation from the study

of more cases, it underlines the advantages of UBF staining over the traditional AgNOR method. It might also provide a model for the study of the intricate relationship between cell proliferation and ribosome biogenesis. An earlier study by Zatsepina *et al*.,[12] using a human autoimmune serum against NORs, demonstrated an increase in the number of positive dots in exponentially growing cultures compared with stationary cultures of the pig cell line PtK. They noticed that a reduction in the number of fluorescent dots was accompanied by an increase in their size. O'Mahony *et al*.[13] did not find a diminution in the total amount of UBF after neoplastic CHO cells were growth-arrested by serum starvation. Instead, they observed decreased phosphorylation and migration of UBF from the nucleus to the cytoplasm. Both results taken together suggest that it is the spatial redistribution of UBF, and not its total amount, which changes in relation to proliferation. From the diagnostic viewpoint, this could result in a simplification of the AgNOR technique, making granule counting a reliable parameter of proliferation without the need for the simultaneous determination of surface area. The present report is consistent with this view, showing a scattering of UBF dots in relation to malignant potential and proliferative activity. However, our methods do not allow us to conclude that it is only the spatial redistribution of UBF, and not its total amount, that changes in relation to these cellular features, and some pictures suggest that the size of UBF dots does increase in relation to malignancy.

While UBF and AgNORs have been superimposable in most of the cases studied in this report, their values were clearly divergent in a case of thyroid papillary carcinoma. The fact that the proliferative index of this tumour measured by Ki-67 was very low suggests that UBF could be related to metabolic rather than proliferative activity and that it may in some conditions be a better indicator of malignancy than AgNOR number. Further comparisons of these two techniques may yield very useful information on the functions of NOR proteins in normal and neoplastic cells.

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