

## Short Communication

# Nucleolar Localization of Upstream Binding Factor in HeLa Cells Depends on rRNA Synthesis Activities

(HeLa cells / nucleolus / transcription factor UBF / nascent rRNA / immunogold labelling / electron microscope)

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**Abstract.** During Pol I-mediated rRNA synthesis, UBF plays a key role in transcription. Numerous localization studies provided, however, rather divergent results with regard to its presence in either of the two structural nucleolar subcompartments, FC and DFC. These observations suggest different roles of UBF within nucleoli. In order to expand our knowledge in this respect, we have performed the ultrastructural localization of UBF and DNA in HeLa cell nucleoli and complemented it with mapping DNA/RNA hybrids, which testify for the presence of newly synthesized rRNA. With immunogold UBF mapping, the results fell, depending on the type of nucleoli, basically into two categories. In nucleoli exhibiting one, or at most a few, large FC, UBF molecules were mapped mainly into FC. Such nucleoli are known to possess lower activity in rRNA synthesis. In the second category of reticulated nucleoli with many tiny FCs, which are characterized by high rRNA gene transcription, the gold particles were enriched mainly in DFC/FC borders and DFC. The established differential localization of UBF in nucleoli of HeLa cells thus has a functional meaning. It reflects both the level of rRNA synthesis activities and the architectural role of UBF in nucleoli of these cells.

## Introduction

Besides other functions (Raška et al., 2006), the nucleolus serves one major function: ribosomal RNA is transcribed, processed and assembled into pre-ribosomal subunits in this nuclear organelle. Ribosomal genes are organized in clusters of tandem repeats termed nucleolar organizer regions (NORs) at chromosomes bearing NORs. Thin-sectioned mammalian nucleoli exhibit a simple ultrastructure as basically just three nucleolar structural components are seen. The electron lucent fibrillar centres (FCs) are mostly surrounded by the dense fibrillar component (DFC). These two components are embedded in the granular component (GC) that has a grainy appearance (Raška et al., 2006). Although nucleolar functions in rRNA synthesis and ribosome formation were demonstrated more than 40 years ago, identification of the transcription sites of rRNA genes in the nucleolus remained controversial for a long time. In recent years, researchers have come to an agreement that the rRNA synthesis sites are found in the boundary region between FCs and DFC, and the products of transcription firstly emerge in DFC, and then gradually move to the GC (Raška et al., 2006).

The efficient transcription of rDNA is mediated by RNA polymerase I (Pol I) and its associated transcription factors such as upstream binding factor (UBF) and selectivity factor 1, which form a pre-initiation complex on the rDNA promoter (Raška et al., 2006). Recently, important studies have appeared showing that only a part of UBF molecules are “directly” involved in the active transcription, while many UBF molecules have architectural role, binding over the whole ribosomal gene, and generating a loosened structure of ribosomal chromatin (O’Sullivan et al., 2002, Prieto and McStay, 2005). Accordingly, binding of the UBF molecules all over to the ribosomal gene generates an “open” chromatin structure providing the necessary (but not sufficient) condition for their transcription (Raška et al., 2004).

Importantly in this context, data from previous reports concerning the localization of UBF diverged. UBF molecules were located to both DFC and FC by some

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Abbreviations: DFC – dense fibrillar component(s), FCs – fibrillar centros, GC – granular component, NORs – nucleolar organizer regions, Pol I – RNA polymerase I, rRNA – ribosomal RNA, UBF – upstream binding factor.

authors, whereas other groups situated them either solely in FC or in DFC (Rodrigo et al., 1992; Roussel et al., 1993; Raška et al., 1995; Cmarko et al., 2000; Mais and Scheer, 2001). A possibility exists that these divergent localization results reflect different functions of UBF, either in the synthesis of rRNA or the architectural function. In order to expand the knowledge in this respect we have, using human HeLa cells, implemented the ultrastructural cytochemistry staining technique together with ultrastructural immunocytochemistry using two kinds of antibodies: (1) nucleolar DNA was visualized by the NAMA-Ur method for the DNA-specific staining (Testillano et al., 1994); (2) since rRNA genes are the only active genes present within nucleoli (Raška, 2003), the newly born rRNA was mapped in nucleoli by means of anti-DNA/RNA hybrid antibody (Rudkin and Stollar, 1977; Testillano et al., 1994); (3) UBF was localized with the help of specific anti-UBF antibodies (Rodrigo et al., 1992; Tao et al., 2001).

## Material and Methods

### *Cell culture*

HeLa cells were grown in DMEM medium (Biocompare, San Francisco, CA) supplemented with 10% foetal bovine serum (Biochrom AG, Berlin, Germany) at 37 °C under 95% air/5% CO<sub>2</sub> and antibiotics.

### *Processing of cells for electron microscopy*

Lowicryl K<sub>4</sub>M was purchased from Chemische Werke Lowi GMBH & Co. (Waldkraiburg, Germany). Samples of HeLa cells were fixed in 2% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h. After washing in the buffer three times, 30 min each, they were dehydrated in an ethanol series and infiltrated by 100% ethanol : K<sub>4</sub>M (1 : 1) mixture for 12 h at 0 °C, 100% ethanol : K<sub>4</sub>M (1 : 2) mixture for 12 h at -10 °C, and 100% K<sub>4</sub>M for 60 h at -30 °C. They were then placed in fresh Lowicryl K<sub>4</sub>M and the resin was polymerized at -30 °C under UV irradiation over 24 h, and then irradiated again for 2–3 days at room temperature. Embedded HeLa cells were sectioned in a Leica ultracut R ultramicrotome (Leica Microsystems, Bensheim, Germany). Ultrathin sections processed for affinity cytochemistry were stained with 2% uranyl acetate and observed in JEOL JEM 1010 electron microscope (JEOL USA, Inc., Peabody, MA).

### *DNA-specific staining*

The ultrastructural NAMA-Ur procedure for DNA-specific staining was carried out as described by Testillano et al. (1994) and Tao et al. (2003).

### *Immunogold labelling*

Immunogold labelling was carried out as described (Tao et al., 2003). The anti-UBF antibody was kindly provided by Prof. Manuel M. Valdivia (Department of Biochemistry, Faculty of Science, University of Cadiz)

and/or purchased from upstate Biotechnology (Rodrigo et al., 1992; Tao et al., 2001). Protein A bound to 10-nm colloidal gold particles was purchased from Sigma (St. Louis, MO). In the text, we used the term labelled cells for these mapping experiments.

In control experiments, the use of antibodies to UBF and RNA/DNA hybrids was omitted. In the text, we used the term unlabelled cells for the control mapping experiments.

For each mapping, a density of gold particles was assessed from 10 electron micrographs by analyses and calculations with the IBAS image processing system (Kontron, Munich, Germany).

## Results and Discussion

After DNA-specific staining, the components containing DNA in nuclei of HeLa cells exhibited high electron density, while the cytoplasm and interchromatin regions showed much lower density or were even completely bleached (Fig. 1, A and B). In nucleoli, there was still a weak DFC staining of DFC, and thus FCs could still be identified (Testillano et al., 1994; Tao et al., 2003). Nucleolar DNA was shown to extend continuously in a reticulated pattern within DFC and the DFC/FC borders (Fig. 1, A and B), but some DNA fibres were also seen in FCs (Fig. 1, B). Such nucleolar distribution of DNA was in agreement with numerous studies dealing with normal as well as transformed mammalian cells (e.g. Raška et al., 1983a, b, 1995).

During transcription of rRNA genes, a double-stranded structure of an RNA/DNA hybrid is temporarily formed and the immunocytochemical mapping of the RNA/DNA hybrids is, besides non-isotopic mapping of modified bases incorporated into nascent rRNA chains (Raška et al., 1995; Raška, 2003), a convenient approach to identification of the nucleolar sites of rRNA synthesis (Stoller, 1975; Testillano et al., 1994; Tao et al., 2003). Using anti-DNA/RNA hybrid antibody to label the transcription sites of rRNA genes, the results showed that the border region between FCs and DFC as well as the whole DFC were mainly labelled (Fig. 2A,B). No or little label was seen in FCs and GC. The quantification analysis showed that gold particles were enriched in DFC and the DFC/FC borders of labelled HeLa cells as compared to the controls (Fig. 2C).

In HeLa cell nucleoli, the results of the UBF mapping could be divided, depending on the type of nucleoli, into two classes (Fig. 3). First, FCs were heavily labelled in the nucleoli exhibiting one, or at most a few, large FC (Fig. 3A, D); the second class involved reticulated nucleoli, or nucleoli with nucleolonemas (Busch and Smetana, 1970), with many tiny FCs. In these nucleoli, the highest incidence of gold particles was observed in the border region between DFC and FC as well as in DFC (Fig. 3B, E). No or just a few particles were observed over FCs. In the first type of nucleoli, the densities of gold particles in the FC were about eight-fold higher in labelled cells than those in controls, whereas the analy-

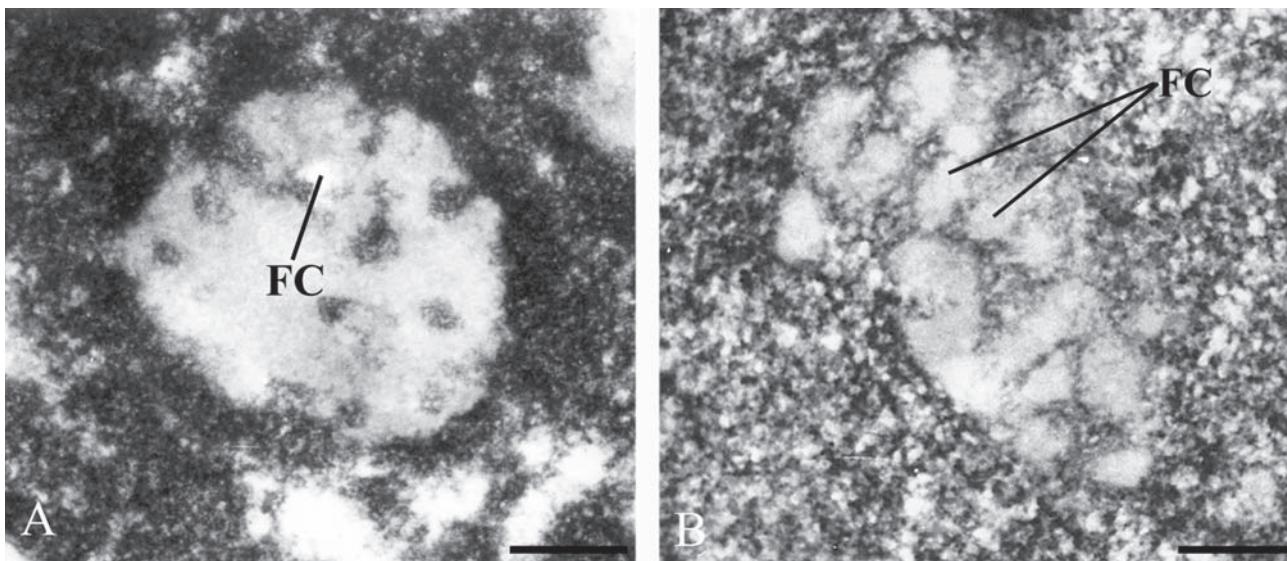


Fig. 1. Distribution of DNA in the nucleoli of HeLa cells. NAMA-Ur DNA-specific staining showing that DNA was mainly distributed in the DFC and the border region (A and B). However, DNA fibres could be seen extended within some FCs (B). Bar = 0.5  $\mu$ m.

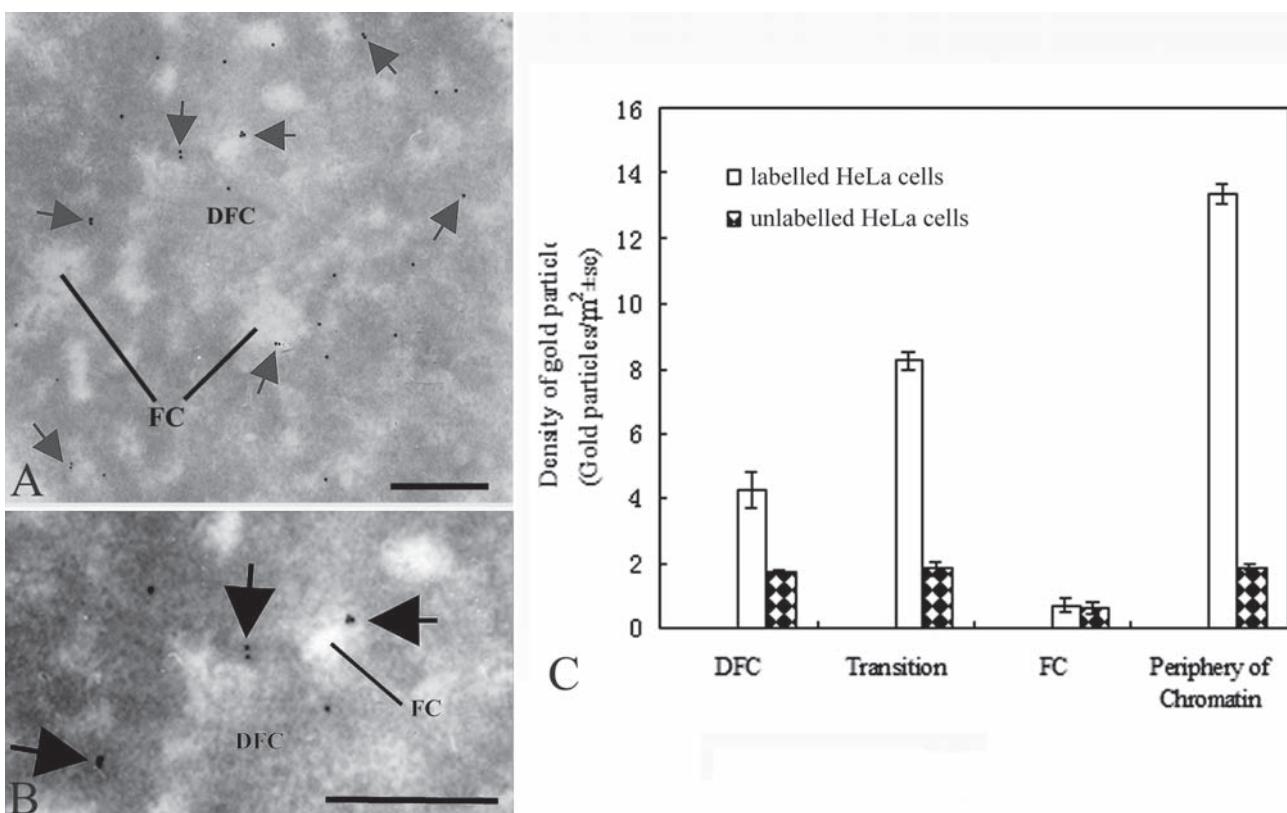
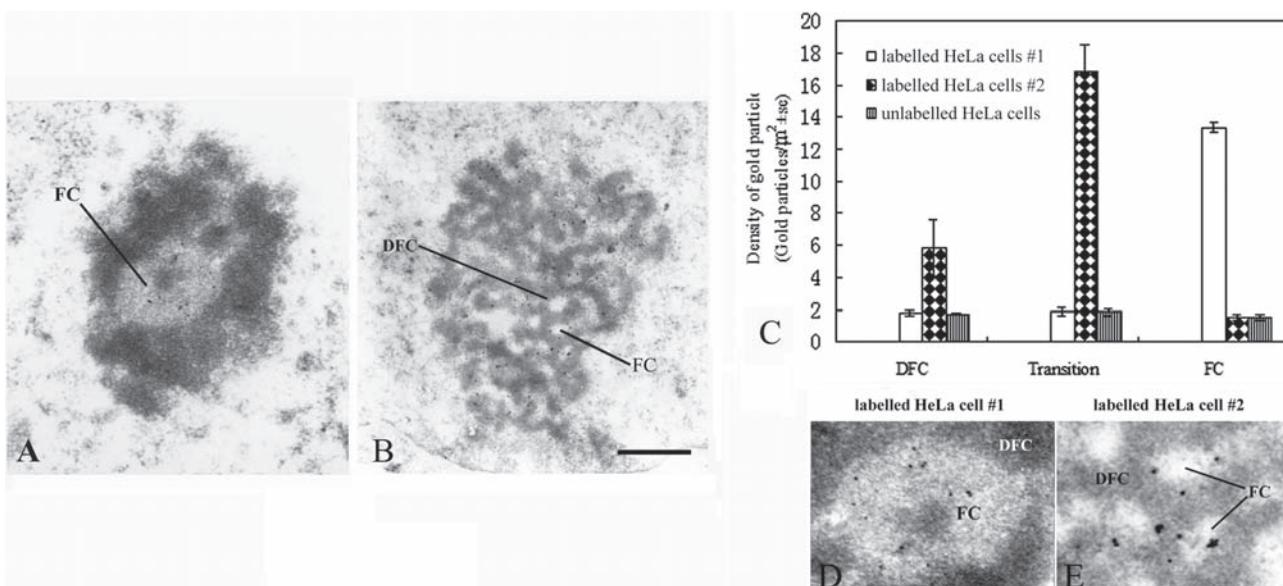


Fig. 2. Immunogold labelling of anti-RNA/DNA hybrid antibody. In HeLa cell nucleoli, gold particles are enriched in DFC and DFC/FC borders (A, B, arrowheads). The signal is almost missing in FCs. C, Quantification of gold particle densities in sectioned HeLa cells labelled and not labelled with anti-RNA/DNA antibody. Bar = 0.5  $\mu$ m.

sis of the UBF label present in DFC and the border region between FC and DFC showed that the difference of the density labels did not show differences between controls and labelled cells (Fig. 3C). In the second category

of nucleoli, the densities of gold particles in DFC and the DFC/FC borders were always higher (about two-fold and four-fold, respectively) in labelled cells than those in controls, while gold particle densities in FC



**Fig. 3.** Immunogold labelling with anti-UBF antibody. UBF was located either in FC in the nucleoli with one or a few large FC (A and D; D is a detail of A) or in DFC and DFC/FC borders in the reticulated nucleolus with many tiny FCs (B and E, E is a detail of B). C, Quantification analysis of gold particle densities labelled or not labelled with anti-UBF antibody in HeLa cell nucleoli. Labelled nucleolus #1 (A and D) indicates the category of nucleoli in which UBF labelling signals were associated with FC; labelled HeLa cell #2 (B and E) denotes the second category of nucleoli in which signals were mainly located in DFC and the border region between FC and DFC. Control (= unlabelled) nucleoli mean cells incubated with buffer instead of primary antibodies. Transition means the border region between DFC and FC. Bar = 0.5  $\mu$ m.

were almost unchanged between unlabelled and labelled cells (Fig. 3C).

The previous localization results with UBF can be divided into three groups. First, UBF was usually observed over FC (Rodrigo et al., 1992). The second group of results showed that UBF was detected almost exclusively in DFC, not in the interior of FC (Cmarko et al., 2000; Tao et al., 2001). Finally, UBF exhibited a high incidence both in DFC and FC (Rendon et al., 1992; Rousset et al., 1993). The reason for these apparently divergent results may be due to the regulation of rRNA gene expression and to the fact that UBF is not only located at the promoter, but also binds extensively across the rDNA repeats *in vivo*, with UBF targeting both active and inactive rRNA genes (O'Sullivan et al., 2002; Raška et al., 2004; Prieto and McStay, 2005). We are of the opinion that our UBF mapping results may reconcile the apparently divergent results obtained in the mentioned studies.

The regulation of ribosomal gene transcription can be divided into two basic categories (Raška et al., 2004). Either the relevant NORs are completely silenced, i.e. their chromatin structure is “closed”, or the relevant NORs exhibit an “open” structure, i.e. the UBF molecules are bound to such ribosomal genes. This second category can still be subdivided into two subcategories that involve differential modulation of the transcription level reflecting the status of the RNA Pol I machinery. While our mapping results of nascent rRNA identified DFC and border DFC/FC region as sites of transcrip-

tion, the UBF molecules were mapped in nucleoli exhibiting large FCs, but in reticulated nucleoli to DFC and the border of DFC/FC. Importantly in this respect, classical studies by Smetana and later on by Smetana and collaborators (Smetana et al., 1967, 1968, Busch and Smetana, 1970; Raška et al., 1983a, b; Ochs and Smetana, 1989; Raška et al., 1995; Koberna et al., 2002) established that the reticulated nucleoli, or the nucleoli with nucleolonemas, which exhibit many tiny FCs, are highly active in the transcription of ribosomal genes, while the nucleoli exhibiting one or a few large FC are characterized by lowered rRNA biosynthetic activities. Accordingly, the presence of the UBF molecules in FCs in the latter types of nucleoli reflects the presence of ribosomal genes in this nucleolar sub-compartment that has an “opened” chromatin structure, but they are not transcribed or their level of transcription is highly down-regulated. For example, dormant human lymphocytes from peripheral blood usually contain just one FC in a single nucleolus (Smetana et al., 1967, 1968; Raška et al., 1983a). Once the cells are activated, human lymphocytes exhibit many tiny FCs in one or a few large nucleoli present in the nucleus (Smetana et al., 1967; Busch and Smetana, 1970; Raška et al., 1983b; Ochs and Smetana, 1989).

In summary, our results reconcile the apparently divergent UBF mapping established previously in the literature. The differential localization of UBF in different types of nucleoli in HeLa cells established in this study thus has a functional meaning. It reflects the involve-

ment of two mechanisms, one acting directly at the level of rRNA synthesis activities and the other at the architectural level of UBF in the structure of ribosomal chromatin in these cells.

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