Locating transcribed and non-transcribed rDNA spacer sequences within the nucleolus by *in situ* hybridization and immunoelectron microscopy

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ABSTRACT

Immunoelectron microscopy and *in situ* hybridization have been used to investigate the precise location of transcribed and non-transcribed rDNA spacer sequences. Whereas a 5'-extemal transcribed spacer sequence is predominantly visualized in the fibrillar centers of nucleoli, a non-transcribed spacer sequence is preferentially detected in the interstices, in close contact with the fibrillar centers and which interrupt the surrounding dense fibrillar component. Occasionally these two spacers are also observed in clumps of dense nucleolus-associated chromatin. These observations provide insights into the organization of ribosomal repeats within the nucleolus.

INTRODUCTION

The rRNA genes of higher eukaryotes are present in multiple copies and are organized as tandem repeats (1, 2). Each repeating unit comprises a transcribed region flanked by a stretch of DNA known as the non-transcribed spacer. The transcription unit contains, in addition to the genes coding for 18S, 5.8S and 28S rRNAs, an external transcribed spacer at the 5' end and two internal transcribed spacers separating the coding sequences.

In interphase cells, rRNA genes are expressed in the nucleolus. Extensive electron microscopic studies have provided in-depth knowledge of this nuclear compartment's ultrastructure. These studies have led to recognize the following elements as general components of the mammalian interphase nucleolus (3, 4): the fibrillar centers, the dense fibrillar component, the granular component, the nucleolar interstices, and nucleolus-associated chromatin. On the other hand, the precise relationships between these structural compartments and their molecular functions have been but partially elucidated.

We have recently investigated the location of rRNA genes within Ehrlich tumor cells by *in situ* hybridization at the electron microscope level (5). Using a 1.95 kb probe containing 1.45 kb of 18S rDNA, we detected hybrids principally at the periphery of the fibrillar centers. In this report, we have extended our analysis to other regions of rDNA repeats. We have investigated the precise location of two rDNA spacer sequences: a transcribed external spacer and a non-transcribed spacer. The latter represents about two-thirds of the (approximately) 44 kb mouse rDNA repeat (6).

MATERIALS AND METHODS

Strains

Ehrlich ascites tumor cells from the peritoneal cavity of C 57 Bl mice were cultured as monolayers in Petri dishes according to Lepoint and Bassleer (7). The Escherichia coli strain C 600 PyrFΔ(Pro-ArgF-Lac) Argl PyrF thi rΔ- mΔ was used for transformation (8).

Preparation of cells for electron microscopy

The monolayer cultures were scraped off the dishes and centrifuged at low speed to form a pellet. Small fragments of the pellet were fixed in 0.2% glutaraldehyde or in 4% formaldehyde in 0.1 M Sörensens's buffer (pH 7.4) at 4°C for 15 min or 60 min, respectively. After fixation, the cells were washed in Sörensens's buffer, dehydrated through graded ethanol solutions and then processed for embedding in Lowicryl K4M using the technique of Roth et al. (9). Ultrathin sections were collected in platinum rings and stored on distilled water until use.

Enzymes

Restriction endonucleases were purchased from Boehringer Mannheim/FRG and Bethesda Research Laboratories Inc. (BRL; Gibco Europe SA, Gent, Belgium) and were used as suggested by the manufacturer. T4 ligase was from Boehringer and E. coli DNA polymerase I was from BRL.

DNA

The mouse ribosomal fragment Mr974(10) was a kindly gift, as a 11.35 kb EcoRI insert in pUC9 from I. Grummt (University of Würzburg, Würzburg, FRG). DNA manipulations were as described by Maniatis et al. (11).

In vitro DNA labeling

Biotinylated plasmid DNA was prepared by nick-translation (12) with Bio-11-dUTP. Purification of the different labeled probes was achieved by gel filtration on Sephadex G-50, tracking the
DNA by 0.5% blue dextran, and a subsequent ethanol precipitation.

**In situ hybridization**

Sections of cells were either heated in water at 100°C for 5 min or incubated for 30 min at 37°C in 0.1 mg/ml protease of Bacillus polymyxa (Boehringer Mannheim) in 0.1 M Sorensen's buffer (pH 7.4) followed for 120 min in 0.1 N NaOH at room temperature and immediately cooled to 4°C before being hybridized as previously described (5).

These denaturation conditions have previously been shown to provide an intense labeling after the application of an immunocytochemical approach involving a monoclonal anti-single-stranded DNA (5).

After hybridization, biotinylated hybrids were detected according to Thiry and Thiry-Blaise (5).

Finally, the sections were stained with uranyl acetate and lead citrate before examination in JEOL CX 100 II electron microscope at 60 KV.

**RESULTS**

A few years ago, Grummt et al. (10) cloned a 11.35 kb EcoRI fragment Mr974 containing parts of the 18S region and adjacent spacer sequences of mouse rDNA. Figure 1 illustrates the organization of transcribed and non-transcribed regions on this cloned ribosomal fragment and shows the Sall cleavage site positions determined by these authors (13). Fragment C contains 1.45 kb of the 18S sequence and has previously been used as an hybridization probe (5).

Here, we have biotinylated two fragments: large fragment A, representing 3.8 kb of non-transcribed spacer, and small fragment E, which contains 0.55 kb of external transcribed spacer. For this purpose, the EcoRI–Sall fragment A was subcloned in pBR325 (=pBRMrE) and the Sall fragment E was inserted into pBR325 (=pBRMrE).

Both labeled probes were hybridized with denatured Lowicryl ultrathin cell sections and the biotinylated hybrids were then visualized by means of an indirect immunogold labeling procedure using an anti-biotin antibody in consort with secondary antibody coupled to colloidal gold particles 5 nm in diameter. Under these conditions, observed labeling of many Ehrlich tumor cells is restricted to the nucleoli, whatever the ribosomal repeat probe or the fixation used. The labeling is represented by small clusters of gold particles. This clustering label results from the fact that each hybrid contains several biotinylated nucleotides which may be independently detected by our immunogold procedure. In addition, each biotin molecule itself may be revealed by several gold particles due to amplification effect of indirect immunoglobulin-gold methods. However, the analysis of about a hundred nucleoli reveals that the location of label within the nucleolus differs for the two ribosomal repeat spacer sequence probes. With pBRMrE (Fig. 2), gold is found distributed in small clusters located preferentially in the fibrillar centers, frequently at the periphery near the dense fibrillar component and interstices. With pBRMrA (Figs. 3 and 4), on the other hand, label is preferentially distributed over the small clumps of intranucleolar chromatin which occupy the interstices interrupting the layer of dense fibrils. It is rarely detected in the fibrillar centers. Neither probe ever gives rise to labeling over the dense fibrillar component or over the granular component. A few gold particles can occasionally be seen over the perinucleolar condensed chromatin.

The reaction’s specificity was tested in several ways. First of all, no labeling occurs when the spacer-sequence probe is replaced by pBR322. Secondly, labeling is completely abolished if the denaturation step or the probe is omitted. Finally, no labeling occurs when only the colloidal-gold-coupled secondary antibody is used.

**DISCUSSION**

As already suggested by previous biochemical data (14) and recent in situ hybridization experiments (5), these results show that Ehrlich-tumor-cell ribosomal repeating units are predominantly located inside the nucleolus. They specifically reveal that an external transcribed spacer sequence is located preferentially in the fibrillar centers whereas a non-transcribed spacer sequence is to be found primarily in the interstices at the boundaries of the fibrillar centers.

Although autoradiographic studies seemed to point to the dense fibrillar component as the site of pre-rRNA synthesis (4, 15), recent data strongly suggest that rDNA transcription occurs, rather, in the fibrillar centers. Among these data is the fact that DNA has never been clearly visualized in the dense fibrillar component of mammalian-cell nucleoli (16, 17, 18, 19). In contrast, many studies based on a variety of methods have demonstrated its presence in the fibrillar centers (4, 16), especially at their periphery (17, 18). Furthermore, electron microscopy has shown that the enzymes RNA polymerase I and DNA topoisomerase I, involved in transcribing rRNA genes, are selectively concentrated in the peripheral regions of the fibrillar centers (20, 21). Our results, reported here and previously (5), indicate that two different transcribed rDNA sequences are located preferentially in the fibrillar centers, especially near their periphery. The fibrillar centers are thus the sole nucleolar structures where transcribed regions of ribosomal repeats and the enzymes involved in rDNA transcription are located together.

This report further shows that a non-transcribed ribosomal repeat spacer sequence is predominantly detected outside the fibrillar center, in the interstices which interrupt the layer of dense fibrils. The presence of DNA in these small nucleolar cavities bordering on the fibrillar centers has been evidenced by various ultrastructural techniques (17, 18, 22, 23). It is also interesting to note that, contrary to the DNA contained in the fibrillar centers, this DNA is generally quite conspicuous and exhibits an electron density similar to that of perinucleolar chromatin. Moreover, an in situ Feulgen-like osmium-ammine reaction reveals this intranucleolar DNA as chromatin clumps or fibers with a nucleosomal configuration, whereas the fibrillar-center DNA looks more like extended non-nucleosomal filaments (24).
However, one has to ask whether the absence of gold particles in the fibrillar centers observed with a non-transcribed probe could not result from detection problems rather than from a true absence of non-transcribed rDNA in these nucleolar regions. As a first point, a lower density of DNA in the fibrillar center could limit its detection, but this restriction would also apply to the two transcribed regions and it is not the case. Secondly, the probe size could affect the labeling efficiency. Nevertheless, whatever

Figure. 2. Electron microscopic immunolocalization of ribosomal external transcribed spacer sequence on sections of Ehrlich tumor cell nucleoli after in situ hybridization. Each of the two fibrillar centers (FC) exhibits a cluster of gold particles (arrowheads). F: dense fibrillar component; G: granular component; I: nucleolar interstices. 0.2% glutaraldehyde/Lowicryl K4M/ protease-NaOH. Bar = 0.1 μm.
Figures 3 and 4. Electron microscopic immunolocalization of ribosomal non-transcribed spacer sequence. General view (Fig. 3) or details (Fig. 4) showing a cluster of gold particles (arrowheads) over interstice (I) contiguous to the fibrillar components. C: condensed chromatin; FC: fibrillar center; F: dense fibrillar component; G: granular component. 0.2% glutaraldehyde/Lowicryl K4M/ protease-NaOH. Bar = 0.1 μm.
In electron microscopic spread preparations of transcribing ribosomal repeats, transcription-unit chromatin invariably appears as a thin, smooth axis and is clearly distinguishable from spacer chromatin which is often characterized by variable numbers of particles, irregular in shape and distribution, and generally somewhat smaller than those of nucleosomes (26, 27). In some species (28, 29, 30), particularly in mammalian cells (31), spacer chromatin reportedly looks identical to the bulk of transcriptionally inactive chromatin, whose regular, beads-on-a-string appearance indicates a nucleosomal organization (26, 27). Furthermore, DNase I-hypersensitive sites have recently been visualized in situ in the fibrillar centers as well as in the interstices surrounding them (32). In previous biochemical experiments, such sites were identified in the actively transcribed ribosomal genes of various species, especially in their promoter sequences (33), which have been mapped in the non-transcribed spacer (34, 35).

Taken together, these observations strongly suggest that the non-transcribed spacer sequences visualized in the interstices are indeed of two kinds: they belong to both transcribing and inactive ribosomal repeats.

In conclusion, a new level of compartmentalization of ribosomal repeats appears to emerge. First, there is the morphological distinction between inactive repeats contained in the nucleolus-associated dense chromatin and transcriptionally active repeats to be found inside the nucleolus. The active repeats are further distributed into distinct nucleolar compartments: the transcribed part in the fibrillar centers and the non-transcribed part in the interstices in contact with the fibrillar centers.

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