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Fluorescent labelling of nascent RNA reveals nuclear transcription domains throughout plant cell nuclei

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Summary. Transcription and RNA processing are main functions of the nucleus. These processes are found localized in specific nuclear domains. We have investigated the presence of nuclear transcription domains in plants by incorporating 5-bromouridine 5'-triphosphate in nascent RNA in isolated nuclei of *Brassica napus*. Bromo-uridine labelled RNA was visualized by a FITC-labelled biotin-avidin system in combination with confocal laser scanning microscopy. Labelled domains were found throughout the nucleus, in some cases including the nucleolus. This shows that the distribution of transcription sites in plant nuclei is similar to that of mammalian nuclei and that the same labelling procedures can be used.

Keywords: *Brassica napus*; 5-Bromouridine 5'-triphosphate incorporation; Confocal scanning laser microscopy; Immunolabelling; Isolated nuclei; Transcription sites.

Introduction

In the interphase nucleus RNA synthesis, RNA processing and RNA transport are important processes. Three RNA polymerases are involved in the transcription process, termed RNA polymerase I (RP I), RNA polymerase II (RP II) and RNA polymerase III (RP III). RP I synthesizes the 45S ribosomal pre-RNA in the nucleolus. RP II and RP III are active in the nucleoplasm. RP II synthesizes the heterogeneous nuclear RNA (hnRNA) and most of the small nuclear RNAs (snRNA). RP III synthesizes several small RNAs, like 5S ribosomal RNA and transfer RNA (Sentenac 1985).

In recent years it has become clear that the nucleus is a highly organized organelle and that several steps in

the metabolism of RNA are localized in specific nuclear domains (Stuurman et al. 1992, Testillano et al. 1993; reviewed in Spector 1993). Also, tracks and foci of specific RNAs have been reported (Lawrence et al. 1989, Huang and Spector 1991, Raap et al. 1991, Dirks et al. 1995). The most conspicuous nuclear compartment is the nucleolus. Other nuclear domains can be visualized only after, e.g., immunolabelling (see Testillano et al. 1993, Risueño and Testillano 1994, Beven et al. 1995).

One step towards understanding the functional organization of the interphase nucleus is the in situ visualization of the sites of active chromatin, i.e., sites at which nascent RNA is found. In mammalian cells, these sites of transcription were labelled with fluorescent probes after incorporation of the UTP-analogue 5-bromouridine 5'-triphosphate (BrUTP) into nascent RNA in run-on experiments. This was done with permeabilized and microinjected human bladder carcinoma cells and human skin fibroblasts (Wansink et al. 1993), with permeabilized HeLa cells (Jackson et al. 1993) and with nuclear spreads of monkey kidney cells (Garcia-Blanco et al. 1995). After permeabilization of the cells, these in vitro systems continue the transcription processes that already had been initiated in vivo.

In plants, less is known about the structural organization of RNA processing. Recently, Sjö Dahl et al. (1993) have incorporated ³²P-UTP into nascent RNA of isolated nuclei of *Brassica napus* seeds in order to identify the synthesis of transcripts belonging to three cruciferin gene families. This, however, does not

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allow a precise localization of the sites of transcription because of the long path-length of the β -radiation. Therefore, we decided to use the BrUTP incorporation to visualize nascent RNA in plant cells.

This study is part of a larger project on nuclear activity during plant embryogenesis, in which embryogenic microspore cultures of *B. napus* are used (see, e.g., B. Hause et al. 1993, G. Hause et al. 1995). In earlier experiments we were able to detect DNA synthesis in cells from microspore cultures using bromodeoxyuridine (BrdU) (Binarova et al. 1993), but we did not succeed to detect incorporated BrU in nascent RNA in the nucleoplasm, using permeabilized cells from both microspore cultures and root tips of *B. napus*. In all mammalian cells studied so far for BrUTP incorporation, permeabilization of the membrane or microinjection of BrUTP proved to be necessary, because nucleotide triphosphates cannot cross the cell membrane. For plant cells, the cell wall forms an extra barrier. To investigate whether the BrUTP incorporation method can be used in plant systems, we decided to use isolated nuclei.

Material and methods

Isolation of nuclei

Plants of *Brassica napus* cv. Topas were grown in the greenhouse at 18–23 °C. Young leaves were harvested and nuclei were isolated at 4 °C according to the protocol of Gustavsson et al. (1991) with some minor changes. The leaves were homogenized in 30 ml of MG-buffer (50% glycerol (Merck, Darmstadt, Federal Republic of Germany), 25 mM morpholinoethane sulfonic acid (MES, pH 6.0), 250 mM sucrose, 5.0 mM MgCl₂, 1 mM 1,4-dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM EDTA) in a mortar. The homogenate was filtered through an 80 HD filter and centrifuged in 15 ml M-buffer (as MG buffer, but without glycerol and with 750 mM sucrose) for 30 min in a HB4 swing-out rotor at 6000 g. The pellet was resuspended in 5.0 ml M-buffer containing 0.4% Triton X-100 (BDH, Poole, England), layered on a 40%/80% Percoll gradient and centrifuged for 30 min at 6000 g. The nuclei were harvested from the gradient and washed twice in HG-buffer (as MG-buffer, but with 10 mM HEPES instead of 25 mM MES). During the first wash, 0.2% Triton X-100 was added. The nuclei were stored at –20 °C in HG-buffer.

BrUTP incorporation

The protocol for BrUTP incorporation was according to Wansink et al. (1993), adapted for plant nuclei with the transcription buffer according to Sjö Dahl et al. (1993). A drop of HG-buffer containing isolated nuclei was put on an aminopropyl-triethoxysilane-coated slide (Sigma Chemical Co., St. Louis, MO, U.S.A.). The nuclei were incubated for 3 min in glycerol buffer (25% glycerol, 20 mM Tris (hydroxymethyl)-aminomethane (Tris)/HCl, pH 7.4, 5 mM MgCl₂, 0.5 mM EGTA) containing 1 mM PMSF followed by a 3 min incu-

bation in glycerol buffer containing 0.5% Triton X-100, 1 mM PMSF and 20 U/ml RNasin (Promega, Madison, WI, U.S.A.). Run-on transcription was performed in transcription buffer (20 mM N-[2-hydroxyethyl]piperazine-N'-[2 ethanesulfonic acid] HEPES, pH 8.0, 12 mM MgCl₂, 200 mM (NH₄)₂SO₄, 50 mM KCl, 100 µg/ml tRNA, 100 U RNasin/100 µl, 0.5 mM ATP, CTP, BrUTP (Sigma Chemical Co.) and GTP (Boehringer, Mannheim, Federal Republic of Germany) each, for 45 min at 37 °C. Subsequently, the nuclei were washed 3 min in TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM MgCl₂) containing 0.05% Triton X-100, 1 mM PMSF and 6.6 U/ml RNasin, followed by a 3 min wash in TBS containing 1 mM PMSF and 6.6 U/ml RNasin. In control experiments α -amanitin (10 µg/ml; Sigma) or actinomycin-D (0.2 µg/ml; Sigma) was included until the wash steps. Also, BrUTP was replaced by UTP (Boehringer) to test the specificity of the first antibody.

Fixation and immunocytochemistry

Nuclei were fixed in 2% freshly prepared formaldehyde in PBS (140 mM NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄ · 2H₂O, 1.5 mM KH₂PO₄; pH 7.4) for 15 min at room temperature followed by 2 × 5 min PBS wash steps. After this, the nuclei were treated with 0.5% Triton X-100 in PBS and rinsed in PBS. The slides were blocked as follows: 10 min 100 mM glycine in PBS, 20 min 2% acetylated BSA (Aurion, Wageningen, The Netherlands) in PBS and 5 min PBG (0.5% BSA, 0.1% gelatin from cold water fish skin in PBS). The nuclei were incubated overnight at 4 °C with a mouse monoclonal antibody against BrdU (1 : 500 in PBG; Sigma), washed four times 5 min in PBG, incubated with a biotin conjugated goat anti-mouse IgG (H + L) (1 : 3000 in PBG; Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.), washed four times 5 min in PBG, incubated for 45 min at 37 °C with ExtrAvidin-FITC (1 : 1500 in PBG; Sigma) and washed twice 5 min in PBS. Nuclei were incubated for 10 min in 0.1 µg/ml propidium iodide in PBS to stain the DNA. Finally the slides were mounted in Citifluor in glycerol (Citifluor UKC, Chem. Lab., Canterbury, U.K.).

In a control experiment the slides were treated after fixation with 1 mg/ml RNase A (Boehringer) in STE buffer (10 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) for 2 h at 37 °C, washed in STE and blocked as described above.

Confocal scanning laser microscopy (CSLM)

Images of double labelled nuclei were recorded with an MRC 600 Bio-Rad using an argon-krypton ion laser attached to a Nikon Diaphot inverted microscope equipped with a ×100/1.3 N.A. oil immersion lens. The images were collected with a pinhole setting at 6 units (about 3 mm), a K1/K2 filter combination and a Kalman filter (about 64 scans per image), both simultaneously in one scan or separately in two scans. In the last case the FITC scan was made first.

Processing of images

Images were processed with the Comos software package (Bio-Rad, Veenendaal, The Netherlands). The contrast was modified by contrast stretch and the 3 × 3 smooth filter. The background was measured in the images and subtracted (mean – 2 × S.D.) from the original signal.

To keep the signals of the scans of the controls in the same value as the positive labelled nuclei, they were scaled 16 times before the background was subtracted. This is the average factor with which the BrUTP incorporated nuclei were stretched. Prints were made on a Kodak XLS 8600 printer.

Fig. 1. Distribution of transcription domains in nuclei of *Brassica napus*. After isolation of the nuclei, BrU was incorporated into nascent RNA in a run-on experiment. Incorporated BrU was detected by indirect immunofluorescence. One optical section is shown. **a** Transcription domains are found throughout the nucleoplasm and **b** occasionally also the nucleolus was positive. **a'** and **b'** DNA staining with propidium iodide of the same nucleus as in **a** and **b**, respectively. *nu* Nucleolus. Bar: 1 μ m

Results and discussion

To study the spatial distribution of transcription domains in the interphase nuclei of *Brassica napus*, an immunocytochemical technique was used to visualize newly synthesized RNA after incorporation of BrUTP. To detect incorporated BrU, an antibody that specifically recognizes BrU and BrdU was used (Jackson et al. 1993, Wansink et al. 1993). The sites

of BrU incorporation were visualized by using a secondary antibody that was conjugated with FITC.

In preliminary experiments only a faint positive labeling was found in the nucleolus of a few microspores in culture after incorporation of BrU but there was never a positive signal in the nucleoplasm. The labeling with antibodies was done on 2 μ m polyethylene glycol couples (not shown). This gave the indication that there were penetration problems, either from the

Fig. 2. Controls. **a** BrUTP was substituted by UTP to examine the specificity of the anti BrU-antibody. Almost no label was found. **b** 10 $\mu\text{g/ml}$ α -amanitin was present during run-on transcription to inhibit RP II and RP III. Only transcription by RP I in the nucleolus was sometimes observed. **c** 0.2 $\mu\text{g/ml}$ actinomycin-D blocks all RP activity, so no label was found. **d** Also, no label was found when after BrU incorporation nuclei were treated with 1 mg/ml RNase A for 2 h. *nu* Nucleolus. Bar: 1 μm

BrUTP or from the antibodies or that BrUTP or BrU-labelled RNA was broken down by enzymes. Therefore, BrUTP incorporation experiments were done on isolated nuclei from leaves of *B. napus*.

After incorporation and immunolabelling of BrU in nascent RNA of these isolated nuclei, a typical punctate labelling pattern was observed throughout the nucleus (Fig. 1 a). In some nuclei the nucleolus was also labelled (Fig. 1 b). Labelling in the nucleolus gave always a brighter signal than the domains in the nucleoplasm. These brighter labelled domains in the nucleolus might be caused by the fact that ribosomal genes belong to the most actively transcribed genes in the nucleus and because they are organized in tandemly repeated units. The fact that most nucleoli were not labelled might be due to penetration problems of the antibodies into the nucleoli.

The results are in agreement with observations by Wansink et al. (1993) on human skin fibroblasts and Jackson et al. (1993) on HeLa cells. These authors have also shown that only a limited amount of BrU was incorporated in nascent RNA before the transcription stopped. Most of the RNA, in that case, was still connected with RNA polymerase or the underlying nuclear matrix structure. Wansink et al. (1993) further showed that the punctated pattern distribution remained unchanged after a longer period of BrUTP

incorporation. There was only an increase in labelling intensity. Therefore, nuclear domains that are rich in BrU labelled nascent RNA are in majority sites of transcription. However, the possibility that some Br-labelled RNA is diffused or transported from the sites of transcription, producing a faint label, cannot be excluded.

A striking difference in the intensity of labelling of the various spots in a nucleus was often found. This difference in labelling intensity may have several reasons. The spatial resolution of this light microscopical study is not sufficiently sensitive to distinguish between single gene products when several active genes are situated close to each other or when a gene is transcribed by several RNA polymerases, as was shown in electron micrographs by Hozák et al. (1994). It is possible that one domain reflects the activity of a single gene or of a cluster of genes, which can result in a brighter spot. Secondly, the transcription of some genes may almost be finished in vivo when the nuclei were isolated, so only a small amount of Br-labelled RNA is produced in vitro, resulting in a faint label. Furthermore, there will be a difference in BrUTP incorporation between AT or GC-rich genes. AT-rich genes produce BrU-rich RNA, which might give a brighter signal. On the other hand, it is unknown whether there is a difference in

incorporation efficiency between genes. It is known that the efficiency of incorporation of BrUTP is about 40% lower than RNA synthesis in the presence of UTP in different cell systems (Nakayama and Saneyoshi 1984, Wansink et al. 1993, Hozák et al. 1994). This can mean that BrUTP decreases the incorporation efficiency of BrU in RNA from GC-rich genes. Figure 2 a–d shows control experiments. When the nuclei were treated with UTP instead of BrUTP no label was found (Fig. 2 a). This indicates that the antibodies are specific for BrU-labelled RNA. Low concentrations of α -amanitin inhibit RP II. At higher concentrations RP III is also inhibited (Roeder 1976). The α -amanitin treated nuclei showed little or no label in the nucleus, except in some cases, where label was found in the nucleolus (Fig. 2 b). This is due to the fact that RP I is not inhibited by α -amanitin and therefore transcription of ribosomal genes can take place. All RNA polymerases are blocked by actinomycin-D, which binds to DNA and in this way inhibits all RNA synthesis. In nuclei treated with 0.2 μ g actinomycin-D/ml only background labelling was observed (Fig. 2 c). Also, when cells were treated with RNase A after BrU incorporation, only background label was found (Fig. 2 d). These controls show that the labelling seen in the BrU positive nuclei (Fig. 1) is due to the incorporation of BrU into RNA. We conclude that it is possible to visualize sites of RNA synthesis after incorporation of BrUTP in nascent RNA in isolated nuclei of *B. napus*. The results are in good agreement with those obtained in animal and human cells (Wansink et al. 1993, Jackson et al. 1993, Garcia-Blanco et al. 1995) and are promising for further studies on various plant systems.

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