Relationships between transcription, silver staining, and chromatin organization of nucleolar organizers in *Secale cereale*

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Summary. The nucleolar organizer regions (NORs) are composed of hundreds of rRNA genes, typically spanning several megabases. Cytologically, NORs include regions that are highly condensed and regions that are decondensed, the latter corresponding to regions at which associated proteins stain intensively with silver (Ag-NORs) and where active rRNA gene transcription is thought to occur. To test the relationship between rRNA gene activity, NOR silver staining, and rDNA (genes coding for rRNA) chromatin condensation, we used the DNA methyltransferase inhibitor 5-azacytidine to evaluate the correlation between the epigenetic regulation of rRNA genes and NOR silver staining in the plant *Secale cereale*. Following 5-azacytidine treatment, we observed an increase in rRNA gene transcription as well as a reduction in the number of cells showing a significant difference in the size of the silver-stained domains in the two NORs. These transcriptional changes occurred concomitantly with an increase in nuclear and nucleolar size and were associated with the reallocation of most of the rDNA from perinucleolar heterochromatin into the nucleolus. Collectively, these results suggest that rRNA gene transcription, silver staining, and NOR decondensation are interrelated in *S. cereale*.

Keywords: Nucleolar organizer region; Transcription; Silver staining; 5-Azacytidine; *Secale cereale*.

Introduction

Nucleolar organizer regions (NORs), the chromosomal loci where the ribosomal RNA genes are tandemly arrayed, form distinctive structures. Commonly employed ultrastructural cytological techniques make use of the ability to selectively stain these regions and have been used in attempts to correlate the structure of stained NORs with ribosomal gene transcription. Silver staining of chromosomes (Goodpasture and Bloom 1975) distinctly labels RNA polymerase I transcription machinery, including nucleolin, the RNA polymerase I large subunit RPA195, and UBF (Roussel et al. 1996; Dundr et al. 1997, 2000; Dundr and Olson 1998). The NOR not only stains prominently with silver but also forms a secondary constriction along the chromosome at metaphase. These constrictions are thought to arise in regions that are actively transcribed. Despite much study, however, this has still not been confirmed. Support for this idea comes from numerous lines of experimentation. For example, inhibition of protein synthesis in *Allium cepa*, which would necessarily block rRNA gene transcription, leads to a reduction in NOR silver staining (Moreno et al. 1990). In human cell lines, it has been established that the presence of ectopic binding sites for the Pol I transcription factor UBF at sites outside the NOR is sufficient to recruit a subset of the Pol I transcriptional machinery to these sites and to induce the formation of silver-stained secondary constrictions at metaphase, even though no Pol I transcription occurs at these sites (Mais 2005). In an effort to integrate molecular analyses of rRNA transcript abundance with cytological observations of chromatin condensation and silver staining, we analyzed root tip meristematic cells of *Secale cereale* treated with the hypomethylating agent 5-azacytidine (5-AC), an inhibitor of cytosine methyltransferase activity which causes the derepression of rRNA genes silenced by nucleolar dominance (Vieira et al. 1990, Chen et al. 1998, Lawrence et al. 2004). We show that 5-AC treatment causes an increase in steady-state rRNA transcript levels

that is accompanied by an enlargement of the nucleolus. At metaphase, prominent silver-stained secondary constrictions of similar size were observed at the two NORs in *S. cereale* following 5-AC-treatment, whereas in untreated cells, one large and one small silver-stained secondary constriction were typically observed. Furthermore, 5-AC treatment causes decondensation of interphase NOR chromatin, as well as reallocation of rDNA (genes coding for rRNA) from the condensed perinucleolar heterochromatin into the nucleolus. Collectively, these studies provide direct evidence that increased silver staining, NOR decondensation, and increased nucleolar volume at interphase, which can be observed by cytogenetic approaches, reflect changes in rRNA gene transcription as determined by molecular assays that measure transcript abundance.

Material and methods

Plant material

Secale cereale L. (cultivar Imperial) diploid seeds were germinated for three days at 24 °C in either distilled water or a 10 μg/ml solution of 5-AC (prepared fresh every day) (Castilho et al. 1999). For chromosomal spreads, all root tips were fixed in 3:1 (v/v) absolute ethanol-glacial acetic acid for 24 h at room temperature and transferred to a fresh solution of FAA (50% ethanol, 37% formaldehyde, glacial acetic acid, 18:1:1, v/v) for 3 days at −20 °C. Cell spreads were obtained after enzymatic digestion with cellulase and pectinase, and squashes were made in 45% acetic acid (Caperta et al. 2002).

Tissue sectioning and nucleus analysis

Medial sections were made from the meristematic zone of primary roots. Root tips were excised and treated in distilled water or in a 10 μg/ml solution of 5-AC (prepared fresh every day) (Castilho et al. 1999), fixed in 3:1 (v/v) absolute ethanol-glacial acetic, acid, and stored at −20 °C. They were sectioned (approximately 30 μm thick) with a vibratome (Series 1000; TAAB Laboratories Equipment Ltd., Aldermaslon, U.K.), placed in cleaned multiwell slides (ICN, Biomedicals Inc.), and dried, as described by Abranches et al. (1998). After 4′,6-diamidino-2-phenylindole staining of the root tip sections, nuclei and nucleoli from the meristematic zone of primary roots were observed by epifluorescence microscopy (Leitz Biomed). Images were collected using an AxioCam digital camera (Zeiss) controlled by AxioVision 3.0. Measurements of nuclear area were performed using the AxioVision measurement module 3.0.0.0 by Axion 3.0.6.38 (Carl Zeiss Vision GmbH).

Silver staining

Silver staining of the chromosome preparations was performed as described in Caperta et al. (2002). The preparations were observed under a light microscope (Leitz-Dialux-22) and photographed with a Zeiss Axio-Cam digital camera. The images were composed by the Photoshop 6.0 software (Adobe Systems, San Jose, Calif., U.S.A.).

Fluorescent in situ hybridization

The fluorescent in situ hybridization (FISH) procedure for *S. cereale* was carried out as described by Schwarzacher and Heslop-Harrison (2000) using the pTa71 probe containing the wheat 45S rDNA sequence and spacer (Gerlach and Bedbrook 1979). Probes were labeled with biotin or digoxigenin and were detected after hybridization with streptavidin-Cy3 or fluoresceinated anti-digoxigenin. DNA was counterstained with 4′,6-diamidino-2-phenylindole. The cells were analyzed with a Zeiss Axioskop2 microscope equipped with white light (for silver staining) and epifluorescence (for in situ hybridization). Nuclei and chromosomes were photographed with a Zeiss AxioCam digital camera. The images were composed by the Photoshop 6.0 software (Adobe Systems).

S1 nuclease protection experiments

RNA was isolated as described by Chen et al. (1998). S1 nuclease protection experiments were performed according to McStay and Reeder (1986). The *S. cereale* external transcribed spacer sequence was amplified using the forward and reverse primers TGTGCCGGATTATGACT GAA and GAGCGAGGTTGCCTTGATAG, respectively, and then cloned into the pCR 4.0 vector (Invitrogen). The vector was linearized with *Xma*I and dephosphorylated with shrimp alkaline phosphatase (New England Biolabs). Five micrograms of the linearized vector was radioactively end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (New England Biolabs) and then digested with *Pml*I, releasing a 676 bp fragment. The end-labeled DNA was subjected to electrophoresis on a 5% polyacrylamide gel and the 676 bp fragment was excised and eluted in 1 ml of Tris-EDTA at 37 °C overnight. Ten percent of the Tris-EDTA-eluted labeled DNA was hybridized with 30 μg of *S. cereale* total RNA and digested with 50 U of S1 nuclease (Invitrogen) at 37 °C for 45 min. The S1-digested probe was then run on an 8 M urea 10% polyacrylamide sequencing gel and exposed to a phosphoimager cassette for 48 h. The resulting gel was visualized and quantified by a Bio-Rad Personal Molecular Imager FX.

Results and discussion

Effects of hypomethylating agent 5-AC on nuclear and nucleolar size and ribosomal chromatin decondensation

Exploiting the well-established cytogenetics of the plant *S. cereale*, we sought to test whether a direct relationship exists among rRNA gene transcription, silver staining, and NOR decondensation. Advantages of this model system include its having chromosomes with a high level of NOR cytological resolution and two NORs which show unequal degrees of condensation and differences in silver staining at metaphase (Caperta et al. 2002). Using the DNA methyltransferase inhibitor 5-AC, which has been found to derepress silent rRNA genes in triticale, *Brassica* sp., and *Arabidopsis thaliana* (Vieira et al. 1990, Chen et al. 1998, Lawrence et al. 2004), we were able to modulate rRNA transcription and to test whether changes in rRNA transcription are correlated with changes in NOR condensation and NOR silver staining.

Previous experiments with other species have suggested that ribosomal gene silencing is a direct consequence of cytosine methylation (Neves et al. 1997, Lawrence et al. 2004) and that treatment with the DNA methyltransferase inhibitor 5-AC causes a loss of ribosomal gene silencing (Neves et al. 1997, Chen et al. 1998, Lawrence et al.

2004). In order to determine whether an inhibition of DNA methylation derepresses inactive rRNA genes in *S. cereale*, we grew seedlings in the presence or absence of 5-AC. We then isolated DNA from 3-day-old seedlings and measured ribosomal gene transcription in an S1 nuclease protection assay with a probe that detects rRNA transcripts with 5′ ends corresponding to the transcription initiation site of the rRNA gene promoter. In this assay, the end-labeled DNA probe is supplied in vast excess over rRNA transcripts, such that resulting S1 nuclease protection signals reflect the abundance of accurately initiated rRNA transcripts. In two independent pools of control and treated seedlings, 5-AC treatment triggered a significant increase in newly initiated transcripts at rRNA gene promoters (Fig. 1C), as expected on the basis of previously published results. The increase in transcription could be due to an increased fraction of *S. cereale* rRNA genes that are transcriptionally active following a loss of DNA methylation or could be due to an increased rate of tran-

scription initiation from the same set of rRNA genes. To address these alternative possibilities, we investigated whether the 5-AC-induced changes in rRNA gene transcription are correlated with cytological changes in NOR morphology. Firstly, we used FISH to analyze NOR localization and organization within the cell. Secondly, we used silver staining of the nuclei to directly analyze the effects of 5-AC on the rDNA loci.

In agreement with earlier studies (Leitch and Heslop-Harrison 1992, Delgado et al. 1995, Caperta et al. 2002), the NORs detected by FISH form two prominent foci along the periphery of the nucleolus (n = 306) (Fig. 1A). Previous ultrastructural studies of plant nucleoli have shown that inactive rRNA genes are located along the nucleolar periphery (Leitch and Heslop-Harrison 1992, Shaw et al. 1993, Delgado et al. 1995), suggesting that the prominent FISH signals represent the inactive and highly compacted rRNA genes. In contrast, the active rRNA are localized within the nucleoli and are highly
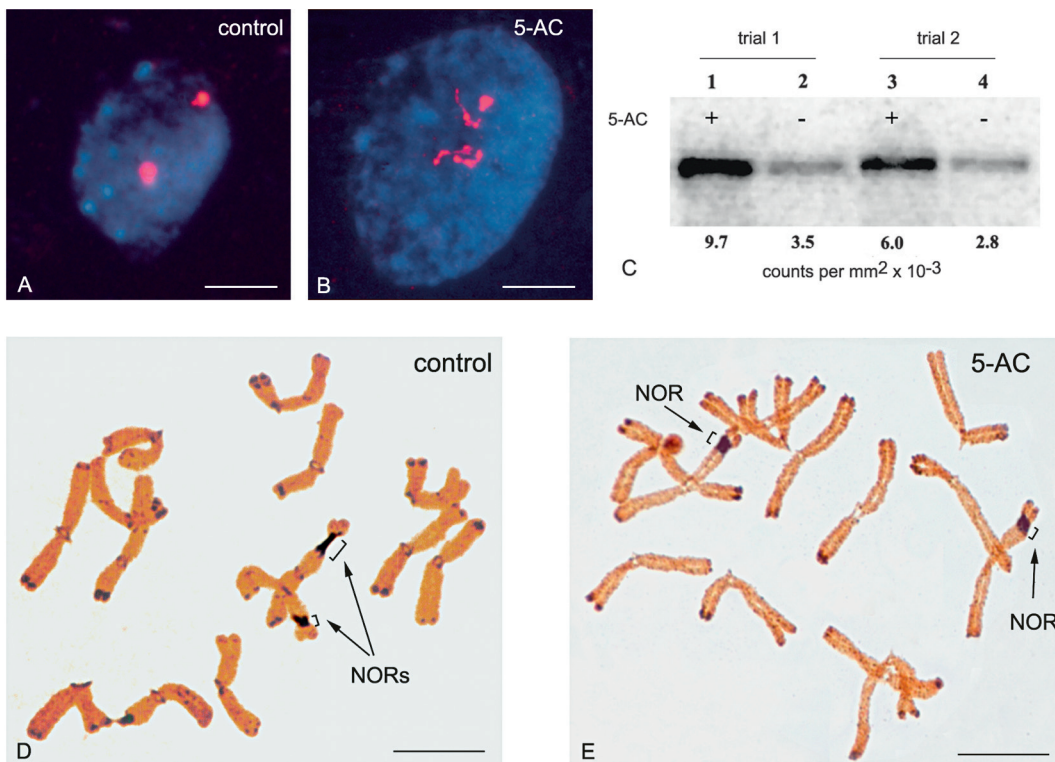

Fig. 1 A–E. *Secale cereale* rRNA gene chromatin modifications following 5-AC treatment in interphase root tip meristematic cells. **A** and **B** Nuclei were subjected to FISH using a rRNA gene probe (pTa71, red) and counterstained with 4′,6-diamidino-2-phenylindole (blue). **A** In control seedlings, most interphase nuclei show two large condensed rDNA knobs at a perinucleolar location with thin filaments emerging from them. **B** Following 5-AC treatment, NOR chromatin is decondensed and the perinucleolar knobs are absent. Bar: 5 μm. **C** S1 nuclease protection was used to quantitatively detect rRNA transcripts accurately initiated from the rRNA gene promoters of untreated and 5-AC-treated *S. cereale* seedlings. *1* and *3* RNA from 5-AC-treated seedlings, *2* and *4* RNA from untreated seedlings. The total radioactive counts per minute were quantified for each lane. **D** and **E** Silver-stained *S. cereale* root tip meristematic metaphase cells following 5-AC treatment. **D** Control nuclei show two Ag-NORs with significantly unequal sizes (brackets), suggesting differential expression of the two rDNA loci in the preceding interphase. **E** 5-AC-treated nuclei show two Ag-NORs with equal sizes (brackets), suggesting equivalent expression of the two rDNA loci in the preceding interphase. Bars: 5 μm

decondensed so that the chromatin strands are not visible by conventional light microscopy techniques. Conversely, 43% (n = 1013) of the cells from 5-AC-treated plants exhibited a change in the NOR conformation relative to untreated control seedlings. Treatment with 5-AC caused a shift from compact NOR foci to a decondensed configuration in which discontinuous FISH-positive signals formed a pattern resembling beads on a string. The condensed "beads" likely represent inactive, compact rRNA genes that are interspersed with nonstained actively transcribed regions of rRNA genes (Fig. 1B). These data suggest that NOR condensation is regulated by DNA methylation and that clusters of inactive and active rRNA genes can be interspersed amongst one another at *S. cereale* NORs.

Next, we sought to test whether the 5-AC-induced changes in rRNA transcription and condensation correlate with changes in NOR silver staining. Silver-positive metaphase NORs are composed of two regions: a region that stains poorly with silver, which is thought to represent a compact domain composed of rRNA genes that were transcriptionally silent during the preceding interphase, and a region that stains prominently, which is less condensed and is thought to correspond to the region composed of transcriptionally active genes (Caperta et al. 2002). A prediction of this hypothesis is that any increase in rRNA gene transcription should lead to a change in the extent of the silver-stained domains within the NORs. Therefore, we compared the length of the two silver-stained constrictions in control and 5-AC-treated plants. In control plants, 94% of the cells (n = 37) had metaphase chromosomes on which the two NORs had secondary constrictions of significantly unequal size (Fig. 1D), suggesting that the two NORs are transcribed to different degrees. In contrast, only 28% (n = 78) of cells from plants treated with 5-AC had silver-stained secondary constrictions with different sizes, suggesting that after DNA methylation had been inhibited, and thus rRNA transcription increased, the two NORs were transcribed to similar extents, causing the lengths of the secondary constrictions to become more equal (Fig. 1E). Moreover, 71% (n = 121) of the interphase nuclei in control (untreated) plants contained a pair of nucleoli that differed significantly in size, also suggesting a difference in rRNA transcription between the NORs. As a result of treatment with 5-AC, only 44% of the nuclei contained nucleoli of unequal size (n = 105). Collectively, these data indicate that increases in rRNA transcription parallel a change in the extent of these domains, providing direct evidence for a correlation between rRNA gene transcription levels and patterns of NOR silver staining.

Effects of transcription on nuclear and nucleolar size

In human cancer cell lines, a direct relationship between nucleolar volume and RNA polymerase I transcription has been suggested (Derenzini 2000). Therefore, we asked whether treatment of *S. cereale* roots with 5-AC induced changes in nucleolar volume that correlate with the increases in rRNA transcription and NOR silver staining that we observed. We found that medial cross sections of interphase nuclei from roots of three different pools of control plants had an average diameter of 77.0 μm (n = 105). In contrast, roots from *S. cereale* seedlings treated with 5-AC showed a significant increase in nuclear size. On average, the nuclei from three independent pools of 5-AC-treated plants had an average nuclear diameter of 99.5 μm (n = 121). To evaluate the nucleolar contribution to the increase in nuclear size, the ratio between the nuclear area and the nucleolar area was determined for each cell by first measuring the area of the nucleus and then dividing this by the area of the nucleolus. The results revealed a decrease in the nucleus-to-nucleolus ratio from 7:1 to 5:1, indicating that increases in rRNA gene transcription result in an overall increase in the volume of the nucleolus relative to the nucleus.

Our work establishes for the first time a relationship between transcription, silver staining, and decondensation of NORs in *S. cereale*. We show that 5-AC treatment causes an increase in steady-state rRNA transcript levels that is accompanied by an enlargement of the nucleolus. At metaphase, differences in the size of silver-stained secondary constrictions between the two NORs in *S. cereale* were also reduced following 5-AC treatment. Furthermore, decondensation of interphase NOR chromatin and reallocation of rDNA from perinucleolar heterochromatin into the nucleolus occurred. Collectively, these studies provide direct evidence that increased NOR silver-staining domains at metaphase and rDNA chromatin decondensation at interphase, evaluated through cytogenetic approaches, reflect changes in rRNA gene transcription.

Acknowledgments

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