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Z-DNA, a new *in situ* marker for transcription

A. Černá,[1] A. Cuadrado,[2] N. Jouve, S.M. Díaz de la Espina, C. De la Torre

Centro de Investigaciones Biológicas, CSIC, Madrid, Spain; [1]Institute of Experimental Botany, ASCR, Prague, Czech Republic and [2]Departamento de Biología Celular y Genética, Universidad de Alcalá de Henares, Madrid, Spain

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Z-DNA forms transiently behind the active RNA polymerases, because of the mechanical torsional stress produced during transcription. In this paper, we explore the possibility that the distribution of Z-DNA stretches signals the sites related to nuclear transcription. To localize transcription, the *in situ* assay for active RNA polymerases, that allows the elongation of the already initiated transcripts but no initiation of new ones (run-on experiments), was carried out in isolated nuclei of *Allium cepa* L. root meristems. Both nucleolar and non-nucleolar sites appeared labelled. Nucleoli were most active in transcription than the multiple non-nucleolar foci altogether. *In situ* immunodetection of Z-DNA provided images that were comparable to those obtained after the run-on assay, with one exception: while Z-DNA and transcription sites were scattered throughout the whole nucleus, Z-DNA also accumulated in the nuclear periphery, where no transcription foci were detected in the run-on assays. The peripheral Z-conformation signals might correspond to dsRNA segments present in the pre-mRNA in the process of their export to cytoplasm. The Z-containing structures nearly disappeared when non-nucleolar RNA polymerase II-dependent transcription had been previously abolished by the adenosine analogue DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole). This inhibition selectively decreased the amount of all nucleoplasmic Z structures. On the other hand, the inhibition of the nucleolar RNA polymerase I by cordycepin (3'-deoxyadenosine) prevented the presence of Z-DNA in nucleoli. We propose to use the *in situ* immunodetection of Z-DNA as a marker of the transcription level in both nucleolus and non-peripheral nucleoplasmic regions of nuclei. Co-detection of Z-DNA and of intermediate filament (IF) proteins, the major components of the nuclear matrix, was also carried out. The IFA antibody recognises a conserved epitope essential for dimerization of the multiple IF proteins. They co-localized with most nucleolar Z-DNA, but not with the nucleoplasmic ones. In the nuclear periphery, the Z-positive signals were adjacent to the IF proteins constituting the lamina, though both signals did not often co-localize.

Key words: *Allium cepa*, Z-DNA immunodetection, *in situ* run-on transcription, FISH of rDNA, nucleoli, intermediate filaments.

Correspondence: Consuelo de la Torre, CIB-CSIC
Ramiro de Maeztu 9. E-28040-Madrid, Spain.
E-mail: delatorrec@cib.csic.es

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Z-DNA is a configuration alternative to the normal B-one. It forms behind an active RNA polymerase because of the topological stress produced during transcription (Wittig et al. 1989). It can also be produced in the local regions of short complementary base-pairing of the otherwise single-stranded DNA (Herbert and Rich, 2001). In the Z-DNA, the helix axis zig-zags back and forth, hence its name, while the base pairs are located nearly perpendicular to it. Z-DNA represents a more elongated left-handed helix than the right handed B-configuration, with the phosphate groups closer than in the B-form. Z-DNA is a moving and transient target. Alternating purine-pyrimidine sequences favours and stabilises such a conformation, GpC repeats being more efficient than the CpG ones (Rich and Zhang, 2003). The left-handed DNA structure is stabilizedd by DNA negative supercoiling (Peck et al., 1982), and by cations such as Ca^{2+}, spermine and spermidine, and cytosine methylation at C5, but not at C8 (Kawai et al., 1996), as well as by a high salt environment (Behe and Felsenfeld, 1981).

More than 10 years ago it was shown that Z-DNA was preferentially formed near the 5´ ends of genes, i.e. near the site of transcription initiation (Schroth et al., 1992). Moreover, the potential Z-DNA sequences are distributed throughout the whole genome in a specific pattern that can be followed both in interphase nuclei and in metaphase chromosomes. In the insect polytene chromosomes, they are more abundant in the heterochromatic regions and in telomeres, i.e. in the untranscribed DNA regulatory sequences (Arndt-Jovin et al., 1985), even when these sequences are mostly devoid from any active role in promoting transcription.

Two main proteins bind to Z-DNA. The first of them, WP900 is an enantiomer of daunorubicin that actually favours the conversion of B- into Z-DNA. The second protein is the adenosine deaminase (ADAR) enzyme that edits RNA. ADAR

anchors to Z-DNA to slide along newly synthesized RNA. ADAR produces small changes in the spontaneously formed double-stranded regions of pre-mRNA, deaminating adenosine to give rise to inosine, while uracil is replaced by cytosine. When inosine is read as guanine in the ribosomes, modified proteins can appear after the editing process (Herbert and Rich, 1991). RNA editing can also create or destroy protein splice sites (Herbert, 1996). Z-DNA is involved in the cellular defence against pox viruses attach (Rich and Zhang, 2003).

In this paper we explore the possibility that the distribution of Z-DNA stretches in the nucleus may correspond to transcription-related sites. For this, we have carried out immunodetection of Z-DNA in the nuclei of the plant proliferating cells of *Allium cepa* root meristems. The assays were performed both in control conditions as well as after preferential inhibition of non-nucleolar transcription by DRB, a treatment that secondarily unravels the rDNA in the nucleolar organizer region (Le Panse et al. 1999). Immunodetection was also carried out under preferential inhibition of the RNA polymerase I-dependent nucleolar transcription, by using cordycepin (3′-deoxyadenosine). This drug also collapses without blocking the (non-nucleolar) transcription going on in the Balbiani rings of insect polytene chromosomes (Díez et al., 1973).

The set of assays here used proves that the *in situ* evaluation of Z-DNA is a reliable marker for ongoing transcription sites, where multiple active RNA polymerases are clustered (Martin and Pombo, 2003). It discerns the transcription sites both in nucleolus and nucleoplasm. The data suggest that the antibody also detect Z-RNA in local regions of double-stranded RNA in pre-mRNA that becomes adjacent to the internal face of nuclear envelope while waiting for export to cytoplasm.

Materials and Methods

Isolation of nuclei

Root meristem cells from *Allium cepa* L. bulbs were grown in filtered tap water at room temperature. Isolation of nuclei was performed as previously described (Mínguez and Moreno, 1993).

Drug treatment

Two day-old roots growing from bulbs were treated either with DRB (50 mM, 6h) or with cordycepin (10^{-4}M, 4h) and then extensively washed in running tap water before being used for the experiments.

Primary antibodies

As primary antibodies we have used the monoclonal anti-BrdUrd antibody (from Sigma) and the sheep polyclonal antibody to Z-DNA (AbCam, Cambridge, UK).

Secondary antibodies

The following secondary antibodies were used: goat anti-rabbit polyclonal to sheep IgG FITC-conjugated antibody (Abcam, dilution 1:200) (wavelength: 488/518 nm) was used. In the case of the pTa71 probe from whole 45S pre-ribosomal RNA, it was directly labelled with TRITC falloidin (wavelength: 514/610 nm) or goat anti-mouse secondary antibodies from Molecular Probes: Alexa 488 (wavelength: 495/519 nm) and Alexa 546 (wavelength: 556/573 nm).

Run-on in situ assay for active RNA polymerases

Experiments of Br-UTP incorporation were carried out in isolated onion root meristematic cell nuclei. We followed the protocol published by Wansink et al. (1993), modificated by De Cárcer and Medina (1999). Freshly isolated nuclei were washed in glycerol buffer (25% glycerol, 20 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EGTA, 25 units RNasin (Plus RNase inhibitor, Promega) for 5 min at 4°C. Then they were permeabilized in the same buffer, which was supplemented with 1% Nonidet-P40, for 5 min at 4°C and washed again in washing buffer for 3 min at 4°C. In these steps, centrifugation was at 1000g for 10 min at 4°C. Incubation was performed in the transcription solution (25% glycerol, 100 mM KCl, 50 mM Tris/HCl, pH 7.5, 5 mM MgCl2, 0.5 mM EGTA), supplemented with 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.5 mM Br-UTP, 25 units/mL RNasin, 100 µg/mL tRNA and 0.1 mM PMSF, for 30 min at 37°C. The reaction was stopped by plunging the tube into ice and adding cold (0°C) paraformaldehyde up to a 4% final concentration. Nuclei were fixed under these conditions for 30 min. Finally, they were repeatedly washed in washing buffer. Sterile materials were used throughout the procedure. For light microscope observation, nuclei were dropped onto round, polylysine-coated coverslips. Br-UTP was revealed by immunofluorescence with the undiluted monoclonal antibody anti-BrUdR (Sigma).

Fluorescence in situ hybridization

The isolated nuclei were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS: 1.4 mM H2KP04, 8 mM Na_2HPO_4, 2.7 mM KCl, 3.7 mM NaCl, pH 7.4) for 30 min, then extensively washed in 0.05% Tween in PBS, followed by 10 min permeabilization with 1% Triton-X in PBS (all at 4°C). After 10 min washing with 0.05% Tween in PBS, the nuclei were incubated with the hybridization solution (15 μL of 100% formamide, 6 μL of 50% dextran sulphate, 3 μL 20×SSC,1 μL 10% SDS in water, 1 μL salmon DNA (10 ng/μL), the wheat pTa71 probe (a 9kb fragment from *Triticum aestivum* containing the 18S-5.8S.25S rDNA and intergenic spacers (Gerlach and Bedbrook, 1979), that was directly labelled with TRITC falloidin (wavelength: 514/610 nm), in distilled H_2O up to a final volume of 30 μL/eppendorf. It has been previously denaturated for 10 min and then incubated on ice for 5 min. Then the nuclei were placed in the programmable thermal controller with a selected programme (75°C 5 min, 55°C 2 min; 50°C 0.30 min; 45v 1 min; 42°C 2 min; 40°C 5 min; 38°C 5 min). Once the programme was finished, the eppendorfs were placed at 37°C in a humid chamber and let them hybridize for 2h. After hybridization the non-specifically bound and the weakly hybridized probe was removed by extensively washing in 0.05% Tween in PBS. The nuclei were then incubated with a 1:10 dilution of the primary sheep polyclonal antibody to Z-DNA (Abcam), left overnight at room temperature. This incubation was followed by 1h incubation at the room temperature with the rabbit polyclonal to sheep IgG FITC-conjugated secondary antibody (Abcam, dilution 1:200). In all these steps centrifugation at 1000g was provided. After washing in 0.05% Tween in PBS, the nuclei were spread on coverslips and directly stained with 1 mg/mL 4′,6-diamino-2-phenylindole (DAPI) and mounted, using Vectashield/Vector as the anti-fading mounting medium.

Immunofluorescence

Immunofluorescence was performed either on coverslips containing a layer of isolated nuclei (for run-on and Z-DNA immunolocalization experiments) or in eppendorfs containing the nuclear fractions, that after incubation with the secondary antibody were dropped on coverslips (for FISH and Z-DNA immunolocalizaton experiments). For the Z-DNA and IFA detection the nuclei were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. Then extensively washed in 0.05% Tween in PBS, followed by 10 min permeabilization with 1% Triton-X in PBS (all at 4°C). After 10 min washing with 0.05% Tween in PBS the nuclei were incubated 10 min with 20 mM glycin in PBS solution, then washed and blocked for 30 min in 2% BSA in PBS. After blocking the nuclei were incubated with the primary sheep polyclonal antibody to Z-DNA (Abcam, dilution 1:10) overnight, at room temperature. The incubation with the primary antibody was followed by 1h incubation at the room temperature with the anti-rabbit polyclonal to sheep IgG FITC- conjugated antibody (Abcam, 1:200 dilution). After each step nuclei were recovered by centrifugation at 1000g. After washing in 0.05% Tween in PBS the nuclei were dropped on coverslips and directly stained with 1mg/mL DAPI and mounted using Vectashield.

Slides containing nuclei after the run-on reaction were blocked directly with 2% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Samples were then incubated with the first antibody diluted in blocking solution, for 1 h at 37°C. In the co-localization experiments, they were washed and incubated again with the other first antibody, under the same conditions. After extensive washing in PBS, slides were incubated with the secondary antibody or antibodies, also for 1 h, at 37°C. After a final washing, they were mounted on slides, using Vectashield as anti-fading mounting medium. In controls, the first antibody was omitted. After labelling, preparations were stained with 1 mg/mL DAPI. Confocal microscopy was performed with a Laser Scanning Spectral Confocal Microscope (Leica TCS-SP2-AOBS-UV) supplied with an argon-krypton laser and also with UV (for DAPI). A 63X/1.4-0.1 mm immersion objective was used. Digital images were processed with Adobe Photoshop 4.01.

Results

In situ assay for active RNA polymerases (run-on experiments)

BrUTP-incorporation after the in situ assay for active RNA polymerases detects transcription at discrete sites in the nucleolus and nucleoplasm of plant cells (Morcillo et al., 1976; González-Melendi et al., 2000). Isolated nuclei were used to perform the *in situ* assay for active RNA polymerases that allows the completion of the elongat-

ing RNA chains without allowing the initiation of new transcripts. The assay requires the addition of the four nucleotides. Br-UTP instead of UTP was one of the nucleotides added to the assay, so that its incorporation into the growing RNA chains could be directly detected by immunofluorescence.

The use of this run-on assay provided an image of the topological distribution of the transcription foci in the nuclei (Figure 1A). While the nucleoli are dark roundish regions in the blue DAPI-stained nucleus, the green foci in the nucleus represent the place where the elongating chains of RNA are being completed. The largest positive Br-UTP signals correspond to nucleoli, while the non-nucleolar transcription foci are smaller and appear dispersed throughout the whole nucleus. The total amount of nucleoplasmic transcription was usually smaller than that of nucleolar transcription. Most of the transcription foci in nucleoplasm appeared as dots, differing in size. There was no preferential location of transcription foci apposed to nuclear envelope.

Distribution of Z-DNA to discrete transcription sites

Z-DNA is a highly immunogenic structure and there are poly- and monoclonal antibodies to detect it. Moreover, these antibodies are not species-specific, so that they can be used in different materials. As far as we know this is the first time one of these antibodies is used to detect Z-DNA in plant cells.

Under control conditions, immunodetection of Z-DNA showed a distribution pattern (Figure 1B) slightly different from the topological transcription pattern obtained after the run-on experiments (Figure 1A). On one side, Z-DNA occupied a larger portion of both nucleolar masses than the signals produced by the residual transcription in the already initiated nascent RNAs. Secondly, there was an important peripheral layer of signals positive to the Z-configuration with no clear correlate in the in situ assay. The accumulation of signals in the nuclear periphery did not took place in conditions where the non-nucleolar transcription has been prevented by the use of a preferential inhibitor of the non-nucleolar transcription dependent on RNA polymerase II (Figure 1C). These signals might correspond to prem-RNA sequences in the process of editing during its export from nucleus, as double-stranded RNA regions pack in the Z-configuration and editing of mRNA apparently continues in the cytoplasm (Herbert and Rich, 2001). The Z-DNA spots dispersed throughout these nuclei display a pattern fully compatible with the nucleoplasmic transcription sites.

In order to detect whether changes in the pattern of transcription may modify the topology of Z-DNA, we impaired the transcription of polymerase I and II with drugs. The transcription of the RNA polymerase II was preferentially inhibited by the use of the adenosine analogue DRB. Its use diminished the nucleoplasmic foci that contained Z-DNA (Figure 1 C). Under this treatment, the continuous layer of Z-DNA apposed to the internal part of the nuclear envelope in the control cells (Figure 1D) had also disappeared, as commented above. Few foci of Z-DNA were still observed in the nucleoplasm of these cells. On the other hand, in the DRB-treated cells, Z-DNA dispersed throughout the whole nucleoli with multiple small positive dots (Figure 1 E). This dispersion is best seen in the left nucleolus of Figure 1C. There appears to be a small Z-DNA spot for each of the ribosomal genes transcribing in a nucleolus, as their spacing occur every 3 nm, the size of each of the whole transcriptional unit known as Christmas tree. Sometimes, their coalescence provide strong continuous signals instead, as also seen in portions of the right nucleolus in Figure 1C. The DRB secondary effect on unravelling and dispersion of nucleolar rDNA sequences was described in mammalian cells (Le Panse et al., 1999).

Converserly, cordycepin, another adenosine analogue that preferentially inhibits the transcription by RNA polymerase I, prevented indeed the detection of Z-DNA in the nucleoli (Figure 1D). Simultaneously, the amount of Z-DNA increased and displayed a dispersed pattern in these nuclei, with accumulation of Z-signals in the nuclear periphery, as again occurred under control unimpeded transcription (Figure 1B).

Finally, the co-detection by FISH of the rDNA codifying for the 45S ribosomal RNA precursor (pTa71 probe directly labelled with rhodamine.dUTP by nick translation to reduced any background signal) and of Z-DNA immunodetection requires additional efforts to try to find a compatible protocol. Thus, when FISH was carried out overnight on the slides, the detection of rDNA was perfect but the detection of Z-DNA was very poor. Converserly, when FISH was reduced to only 2 hours in the nuclear suspension, the rDNA signals were greatly reduced in comparison to control, while the immunodetection of Z-DNA reached a level similar to controls (*data not shown*).

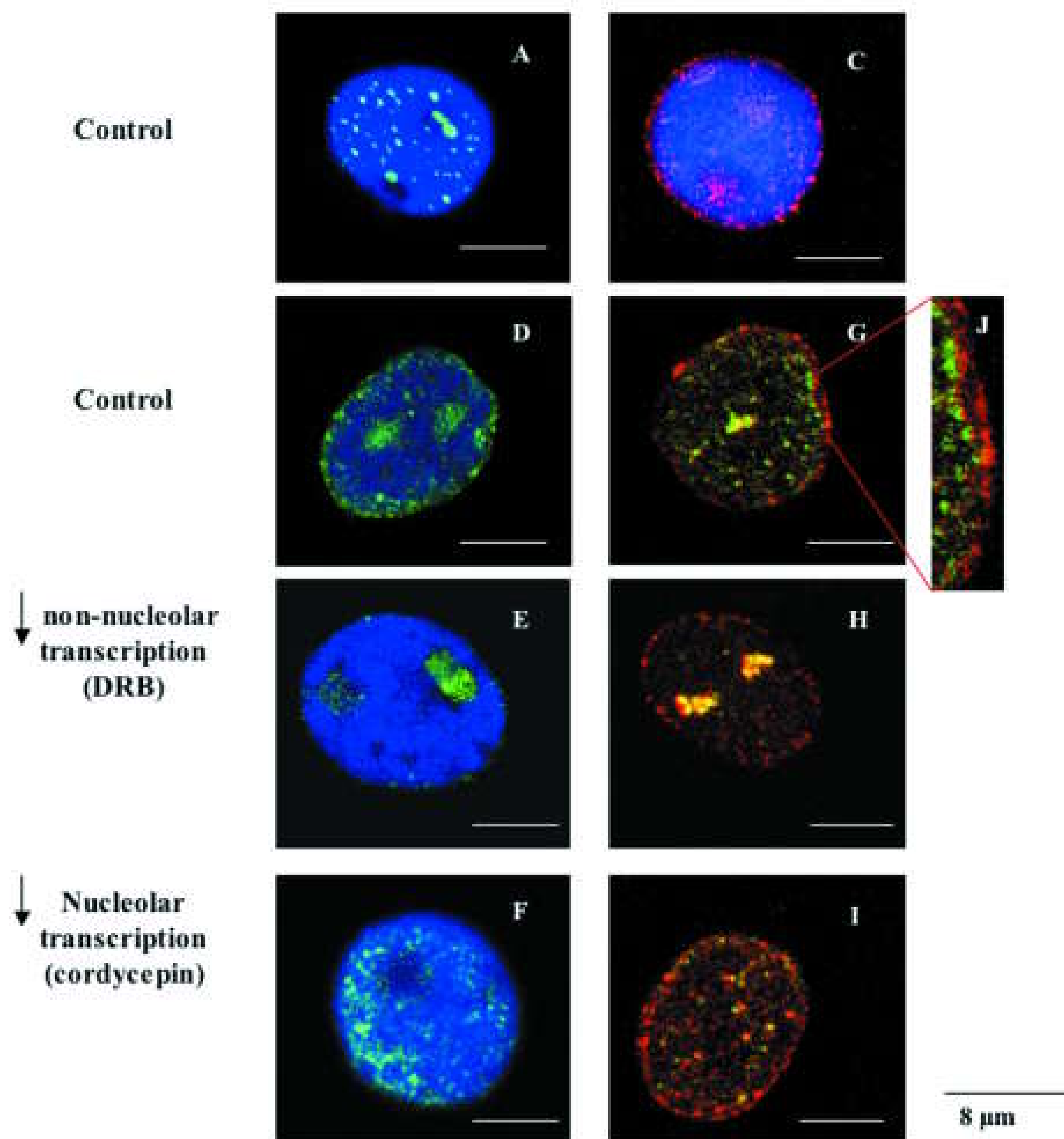

Figure 1. Nuclei of ***Allium cepa*** root meristematic cells, under different RNA transcription conditions. A and B: Control conditions. Detection of active RNA polymerases by the in situ assay using Br-UTP. Blue, DAPI staining of nuclear DNA; light green, immunodetection of Br-UTP. The two nucleoli appear relatively poor in DNA in comparison with other nuclear regions, because the large amount of proteins they have. Both have active transcription sites. B: Immunodetection of Z-DNA. Green, Z-DNA; Blue, DAPI. Z-DNA is mostly concentrated in nucleoli, but also distributed throughout nucleoplasm. There is an enrichment in the Z-signals in the peripheral part of nuclei in comparison to the distribution of the transcription foci in A. C: Cell treated with DRB, the preferential inhibitor of non-nucleolar transcription. Z-DNA has diminished in comparison to D, while unravelling of one of the two nucleoli is clearly seen, while the multiple dot signals present in the rightmost nucleolus seems to have a fixed distribution pattern. (Colours as in B). D: Cell treated with cordycepin, the preferential inhibitor of nucleolar transcription. Z-DNA is missing from nucleoli (darker region in this nucleus), while it is still dispersed in the nucleoplasm. (Colours as in B). E. Control nucleus. Immunodetection of intermediate filament (IF) proteins. Red, IFA; blue, DAPI. The IF-proteins localize to a nuclear network pervaiding the nucleus, with a denser distribution in nucleoli and in the nuclear periphery, corresponding to the lamina and nucleolar matrices, respectively. F: Control nucleus. Co-detection of Z-DNA and IF proteins. Both nucleolar and peripheral distribution of Z-DNA are evident. Z-DNA co-localizes with the IF proteins in the nucleoli. However, co-localization is not frequent in the nucleoplasmic signal. The inset at the right of this particular figure shows the topological distribution of IF-proteins and Z-signals distribution in an enlarged region of the nuclear envelope. The Z-signals concentrate underneath the lamina that contains the IF antigens, though they do not co-localize. G: Prominent Z-DNA in the nucleoli of the cells treated with DRB to selectively depress the RNA polymerase II transcription taking place in the nucleoplasm. H: Lack of any Z-DNA signals in the nucleoli of cells treated with cordycepin, the drug that preferentially inhibits the nucleolar RNA polymerase I-dependent transcription. There is again distribution of Z-signals close but not co-localizing with the IF proteins of the nuclear lamina.

Z-DNA sites and the nuclear network of intermediate filaments

The use of the IFA antibody, against a conserved region essential for dimerization and present in most nuclear IF proteins, showed that the IF protein made up by a loose intranuclear network in control nuclei (Figure 1E). These proteins (contrasted in red) were enriched in the nucleoli, and also in the nuclear periphery. In this particular nucleus, there is retraction of the nuclear DNA content (blue, DAPI signal). This is a common observation after the processing steps it has been submitted. In order to analyze whether the distribution of Z-DNA may be conditioned by the IF presence, co-detection of IF and Z-DNA was carried out both in control conditions (Figure 1F) and under inhibition of nucleoplasmic (Figure 1G) and nucleolar transcription (Figure 1H). The data show adjacent positioning but not co-localization of IF and Z-signals, both in nucleoli and also underneath the internal membrane of the nuclear envelope. This is better seen in the inset located at the right of Figure 1F. In it, it is possible to visualize how the IF proteins are closer to nuclear envelope than their adjacent Z-signals. In the nucleoli, however, there appears to be co-localization of most IF proteins with Z-DNA (Figure 1H), though this may result from the fine and overlapping dispersion of both nucleolar components.

Discussion

Though it is known for a long time that plants posses Z-DNA binding proteins (Lafer et al., 1985), to our knowledge, this work is the first that shows cross-reactivity of a polyclonal antibody against Z-DNA with plant nuclei. The present results show that indirect immunodetection of the moving and transient Z-DNA is a reliable marker for active transcription. This assay presents some advantages over alternative ones. Thus, Z-DNA immunodetection is faster and cheaper than the in situ assay for active RNA polymerases (run-on), which has being used for comparison in this work. Some patients with systemic lupus erithematosus produce antibodies against Z-DNA (Lafer et al., 1983). Z-DNA is highly antigenic so that there are available commercial monoclonal and polyclonal anti-Z-DNA antibodies (Lafer et al. 1981) that cross-react with the left hand conformation of DNA irrespective from the species. It is known that the domain of the RNA editing enzyme that anchors to Z-DNA to edit nascent RNA recognizes the B-DNA to Z-DNA transition (Kim et al., 2000).

Z-DNA forms as a consequence of the operation of the RNA polymerase I in the nucleolus and also of the RNA polymerase II during the synthesis of pre-messenger RNAs in the non-nucleolar region of the plant nuclei, as a consequence of the DNA negative supercoiling produced behind the progressing polymerases (Liu and Wang, 1987). The already formed Z-DNA becomes the target of DNA topoisomerases that relax such a structure in around 15 min after transcription is turned off (Wolft et al., 1995, 1996). This is supported by data showing the recognition of Z-DNA by topoisomerase II in *Drosophila* (Arndt-Jovin et al., 1993).

It would be possible to increase the amount of Z-DNA by stabilizing it through the addition of cations as spermine, spermidine and Ca^{2+} (Behe and Felsenfeld, 1981) and high salt. Thus, stabilized Z-DNA could be detected in the nuclear matrices isolated after high salt extraction where proteins of the intermediate filament family have also being detected (Moreno Díaz de la Espina, 1995). Their co-detection with specific sequences as those constituting the MAR sequences (Matrix Attachment Regions) in *Arabidopsis* (Van Drunen et al., 1997) may be possible.

The topological transcription pattern for both RNA polymerase I and II in Allium is similar in plant and animal cells (Wansick et al., 1993). By changing the *in vivo* conditions of the cell it would be possible to favour the discernment of transcription that depends on a single of the different RNA polymerases. Thus, making the ionic requirements more strict for one single polymerase will ensure the recording of one of the three transcription systems that coexist in the eukaryotic cell.

An intriguing fact deserved our attention. There is an abundant Z-DNA signal inside the nuclear envelope in control conditions. It does not correlate with the presence of Br-UTP incorporation, and it fades away after inhibition of the non-nucleolar transcription. It could be thought that this signal result from the flow of pre-mRNAs towards cytoplasm in the nuclei that are actively transcribing in the proliferating cells. The Z-conformation may also be formed in the double-stranded regions of RNA (Davis et al., 1986). This hypothesis deserves to be carefully explored.

Finally, the easy detection of Z-DNA will favour

the still missing study to define some putative functional role of this DNA, apart from being the mechanical consequence of transcription. We are aware of the many questions on Z-DNA that still remain open, but the potential of the *in situ* immunodetection of Z-DNA as an easy marker of transcription is already clear.

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