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Spatial expression of DNA topoisomerase I genes during cell proliferation in *Daucus carota*A. Balestrazzi¹, G. Bernacchia², L. Pitto³, G. Luccarini³ and D. Carbonera¹¹Department of Genetics and Microbiology "A. Buzzati-Traverso", University of Pavia, Via Abbiategrasso 207, 27100 Pavia, Italy; ²Department of Biology, University of Ferrara, Via Borsari 46, 44100 Ferrara, Italy and ³Istituto di Mutagenesi e Differenziamento, C. N. R., via Svezia 2/105, 6100 Pisa, Italy.

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Key words: DNA topoisomerase I, *in situ* RNA hybridization, cell proliferation, somatic embryo, carrot**SUMMARY**

The spatial expression of carrot (*Daucus carota* L.) *top1* genes encoding the two isoforms of the enzyme DNA topoisomerase I (EC 5.99.1.2) was investigated. *In situ* hybridization analysis performed with a probe recognizing both *top1* transcripts provided evidence that in explanted hypocotyls induced to proliferate *in vitro* by the addition of the growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D), the mRNA accumulation parallels the proliferation of provascular cells of the stelar cylinder. During somatic embryogenesis, the histological distribution of *top1* transcripts was strongly evident at the stage of torpedo-shaped embryos, but gene expression was not only restricted to meristematic regions. When the spatial localization was extended to carrot vegetative apices and the investigation was carried out with specific probes for *top1* α and *top1* β , both transcripts preferentially accumulated in tissues having mitotic activity.

INTRODUCTION

In plants, the understanding of molecular events that regulate cell division is relevant for studies

concerning development. Many of the genes that in animals have been described to regulate cell transition to S- or to M-phase have been found also in plants and it is likely that the cell cycle machinery is characterized by the presence of similar components in all eukaryotic cells (Hirt and Heberle-Bors, 1994). Nevertheless, due to the different life strategies characterizing animals and plants, it is unlikely to imagine that the cell cycle machine might be regulated by identical environmental and endogenous signals (Steeves and Sussex, 1989).

DNA topoisomerase I (EC 5.99.1.2) is an ubiquitous enzyme involved in basic processes such as DNA replication, recombination and transcription (Wang, 1996). Due to its ability to transiently break and rejoin phosphodiester bonds, topoisomerase I controls the topological state of DNA molecules. In eukaryotes, the nuclear type I protein forms covalent but transient 3'-phosphotyrosine intermediates with DNA molecules, then relieving the torsional restraints of DNA.

Among the possible roles played in cell metabolism, the involvement of topoisomerase I in the regulation of gene transcription appears to be of great interest. It has been suggested that the

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enzyme could be involved in the activation of chromatin by inducing topological modifications. Moreover, a recent study has demonstrated the involvement of the type I enzyme in the activation and repression of the basal transcription as component of the TFIID complex (Merino *et al.*, 1993).

The expression of *top1* gene, which is tightly regulated at the transcriptional level, is both constitutive and induced by stimuli among which those inducing cell proliferation, as demonstrated in several mammalian systems: in cultured fibroblasts upon stimulation with serum (Romig and Richter, 1990a) or with a phorbol ester (Hwong *et al.*, 1989) and following adenovirus infection (Romig and Richter, 1990b), in lymphocytes induced to proliferate with phytohemagglutinin (Hwong *et al.*, 1993), in liver cells after partial hepatectomy (Kelly *et al.*, 1983).

Recently, additional activities have been demonstrated for mammalian topoisomerase I: a specific kinase activity, phosphorylating RNA splicing factors (Rossi *et al.*, 1996), and a ribonuclease activity (Sekiguchi *et al.*, 1997). These newly discovered roles suggest the presence of additional functions besides the well known involvement in DNA replication.

The nuclear localization of topoisomerase I is associated with the presence of several NLS (nuclear localization signals) consensus sequences located in the NH₂-terminal domain of the protein (Alsner *et al.*, 1992). In proliferating cells the enzyme has been demonstrated to have essentially a nucleolar distribution, even if the localization pattern seems to depend also on cell type (Meyer *et al.*, 1997). Recently, it has been demonstrated that leucine-starved CEM cells are characterized by a perinuclear redistribution of topoisomerase I (Baker *et al.*, 1995). This localization is also observed in response to specific topoisomerase I inhibitors; however, the mechanism leading to this event is still unknown (Buckwalter *et al.*, 1996). Differently from animal systems, information about tissue distribution and subcellular localization of this enzyme are completely missing in the plant kingdom.

In a previous paper, we provided evidence for the presence of two distinct DNA topoisomerases I (designated α and β) in carrot nuclei (Balestrazzi *et al.*, 2000). Moreover, we showed that both *top1 β* and *top1 α* genes are expressed in cycling

cultured cells; however, the *top1 β* transcript represents the most abundant species in proliferating cells, where the ratio between the β and α mRNA levels was calculated to be 2.5.

As a start point for future investigations, we report the analyses of the spatial distribution of both *top1* transcripts in two systems offering the opportunity of correlating the induction of cell division to the time of mRNA accumulation. Finally, using two distinct probes recognizing specifically *top1 α* or *top1 β* transcripts, we compare their spatial distribution in the vegetative apices from carrot plants.

MATERIALS AND METHODS

Plant materials

Embryogenic cell lines of *Daucus carota* (cv. Lunga di Amsterdam) were initiated as described by deVries *et al.* (1988). Carrot cell suspensions were subcultured by diluting cells with B5 fresh medium (1:10) every 10 d (Cella *et al.* 1987). Somatic embryogenesis was started by diluting and transferring embryogenic cultured cells to B5 medium devoid of 2,4-D, essentially as described by Giuliano *et al.* (1983). Carrot seeds were sterilized essentially as described by Balestrazzi *et al.* (1991). Seeds were induced to germinate in sterilized soil and plantlets maintained in a growth chamber at 25°C with a 16/8 day/night cycle.

In situ ³⁵S-labelled RNA hybridization analysis

In situ hybridization analyses with paraffin-embedded tissue sections from carrot hypocotyls and embryos were essentially carried out as described by Smith *et al.* (1987). The ³⁵S-labelled sense and antisense RNA probes for *top1* RNA were derived from the 1.5 kb *EcoRI-SpeI* fragment from pTop28 clone (Balestrazzi *et al.*, 1996).

In situ digoxigenin-labelled RNA hybridization analysis

In situ hybridizations were performed on paraffin-embedded longitudinal sections of apical meristems of 50-days old carrot plants in accordance to the method described by Coen *et al.* (1990). Digoxigenin-labelled RNA probes corresponded to restriction fragments containing the 5' region specific for *top1 α* and *top1 β* cDNAs

(Balestrazzi *et al.*, 2000) were added to the hybridization solution at 600 ng/ml. Slides were then treated as described by Coen *et al.* (1990) in accordance with manufacturer's instructions (Boehringer Mannheim) for detection and colour development. Bright field photographs were taken on an Axiophot Zeiss microscope with 400ASA Kodak Gold films.

Histone H1 kinase assay

Histone H1 kinase assay was performed as described by Sauter *et al.* (1995) with the following modifications: proteins were extracted by freezing and grinding carrot somatic embryos in liquid N₂. The crude extract was centrifuged at 12000 rpm for 30 min at 4°C and the supernatant utilized in kinase assays. Each sample (0.2 µg of protein) was incubated in the reaction mixture in the presence of 1.25 µCi [γ -³²P] ATP (3000 Ci mmol⁻¹), with or without histone H1. Sample was then analysed on 10% SDS-polyacrylamide gels followed by autoradiography. Protein concentration was determined according to Bradford (1976).

RESULTS

Spatial and temporal expression of *top1* genes in auxin-stimulated carrot hypocotyls

When hypocotyls are explanted from carrot seedlings and cultivated *in vitro* in the presence of auxin (2,4-D) in the medium, only provascular cells are stimulated to actively proliferate in response to the phytohormone exposure, whereas cells of the cortical parenchyma enlarge but do not contribute to the dividing population. Moreover, the contribution of wounding to this event can be excluded, since the same process was also induced in the entire carrot plantlets (Guzzo *et al.*, 1994).

In order to study the relationship between *top1* gene expression and the induction of cell proliferation, we decided to utilize this experimental approach analysing the temporal and spatial accumulation of both *top1* transcripts in explanted hypocotyls by *in situ* RNA hybridization.

Preliminary data obtained by northern blot analysis had shown that the accumulation of *top1* transcripts reached the maximum level (8-fold the amount present at zero time) after 20 d from exposure (Balestrazzi *et al.*, 1996). In this study, ³⁵S-

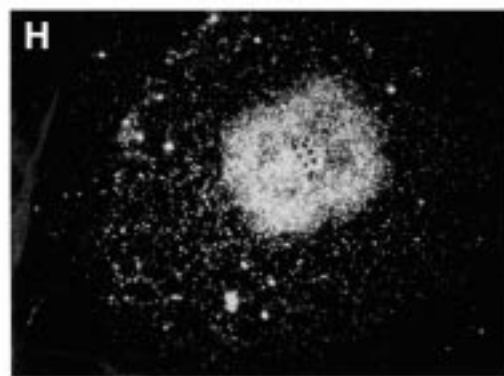
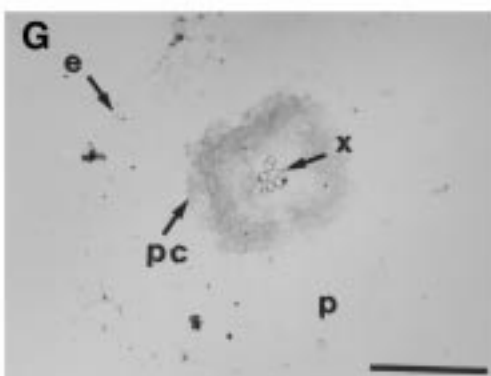
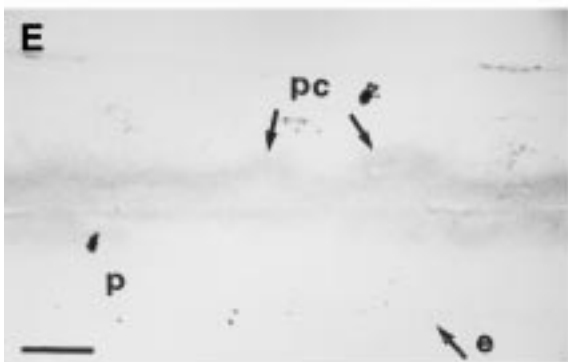
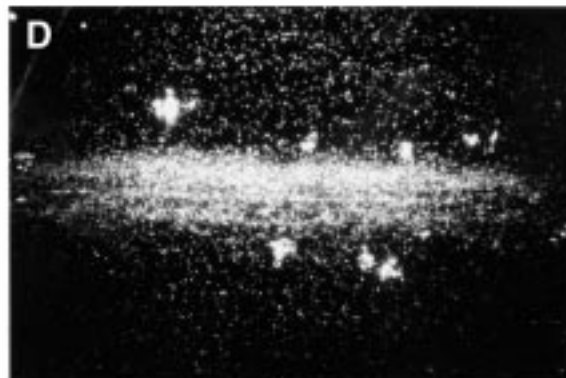
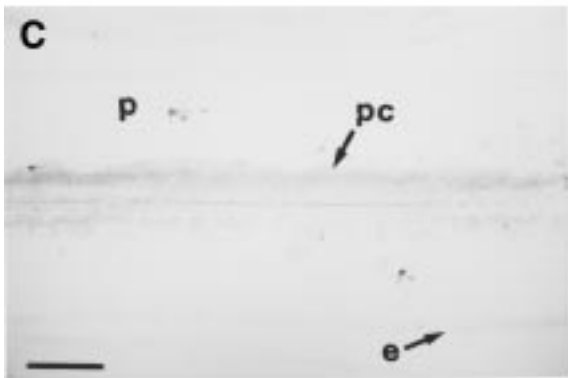
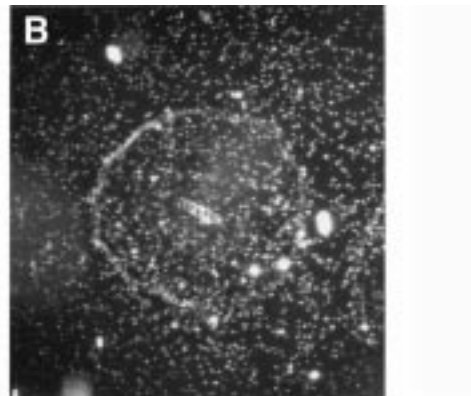
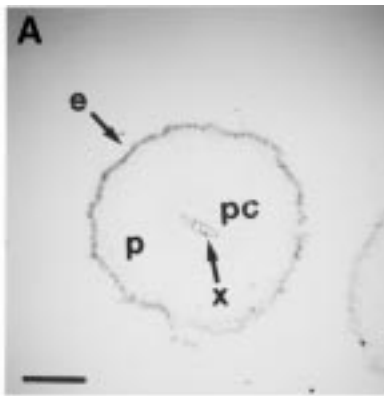
labeled sense and antisense riboprobes able to detect both *top1* α and β mRNAs were *in situ* hybridized to hypocotyl sections after 1, 2, 4 and 7 d from the induction event. No signal above background was observed at zero time in the epidermis, cortical parenchyma, procambial cells and vascular elements (Fig. 1, B) and very little accumulation of the *top1* transcripts appeared to have occurred after 1 d (not shown). However, after 2 d, hybridization signals were clearly detectable in close proximity of vascular elements where procambial tissue is localized (D). Transcript accumulation was far more evident after 4 (not shown) and 7 d from induction (F and H). As evidenced by staining with Giemsa, the presence of proliferating cell masses was observed near the vascular tissue (E and G) and the accumulation of *top1* transcripts was clearly localized only in those regions (F and H). For each time, control sections, which were hybridized with the sense riboprobe, did not give any signal above the background (not shown).

These results show that the transcript accumulation is spatially restricted to provascular tissue, namely the only tissue of an explanted hypocotyl which proliferates in response to the presence of the synthetic auxin 2,4-D.

Expression of the *top1* genes during somatic embryogenesis

Somatic embryogenesis represents a useful system for the dissection of molecular events that occur when the induction of cell proliferation is associated to a developmental program. The process is characterized by localized and rapid cell divisions and therefore we decided to gain some insight into the regulation of *top1* gene expression during carrot embryogenesis.

Somatic embryos were collected at 1, 8 and 14 d after transfer of proembryogenic masses (PEMs), isolated from carrot suspension cultures, to fresh medium in the absence of auxin. It is worth noting that somatic embryos originate *in vitro* from these cell clusters (Halperin, 1966). To gain information on the proliferative state of the embryo populations, we decided to analyse the level of p34^{cdc2/CDC28}-like histone kinase activity in embryos collected at 1, 8 and 14 d after induction. This protein kinase, which is required for entry into mitosis, represents an essential component of the regulatory machine of cell division in eukaryotic



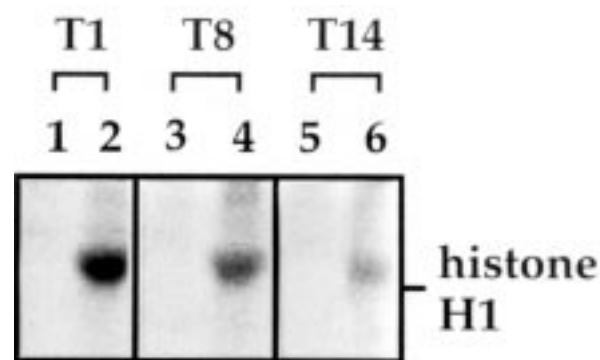


Fig. 2 - Histone H1 kinase activity during carrot somatic embryogenesis. Somatic embryos were collected 1 (T₁), 8 (T₈) and 14 d (T₁₄) after induction and crude extracts from each sample were prepared. An aliquot (0.2 µg of protein) from each extract was incubated in the reaction mixture containing 1.25 µCi [γ -³²P] ATP, in the presence (lanes 2, 4, 6) or in the absence (lanes 1, 3, 5) of histone H1 (20 µg). The presence of ³²P-labeled histone H1 was analysed by SDS-PAGE followed by autoradiography.

organisms (Gorst *et al.*, 1991) As shown in Fig. 2, *in vitro* phosphorylation of histone H1, performed to assay the p34 protein kinase activity, peaked at the T₁ stage at the beginning of somatic embryogenesis (lane 2) and slightly decreased in T₈ and T₁₄ (lanes 4 and 6, respectively) populations. When exogenous histone H1 was omitted, no labeled protein was detected, thus demonstrating the specificity of the assay (lanes 1, 3, 5).

Information on the spatial distribution of the *top1* transcripts were achieved by *in situ* RNA hybridization performed on somatic embryos using the same riboprobes detecting both α and β transcripts previously described. *Top1* transcripts appeared to be distributed quite uniformly in early embryos (data not shown). In later embryo stages such as in late torpedos (Fig. 3, A), *top1* mRNAs were clearly located in root and shoot meristems, in cotyledons and in the provascular region, namely in tissues characterized by cell proliferation, but the localization was not strictly restricted to meris-

tematic regions (Fig. 3, B). All tissue sections were also analysed with the sense *top1* riboprobe, but no signal above the background was in all cases detected (not shown).

Distribution of *top1* α and β transcripts in vegetative apices from carrot plantlets

Differently from previous experiments, the spatial localization of both *top1* α and β transcripts was evaluated using gene-specific probes and *in situ* RNA hybridization was performed using a nonradioisotopic labeling system (digoxigenin-labeled probes), since this technique seems to offer the opportunity of detecting more precisely the expression pattern in single cells (Schmidt *et al.*, 1996). Longitudinal sections of vegetative apices from 50-day-old carrot plants were used as starting material. In these experiments, sections were hybridized with antisense and sense RNA probes. Both *top1* α (Fig. 4, A) and β (Fig. 4, B) mRNAs were evenly distributed within regions such as meristems and leaf primordia where active cell proliferation occurs. However, no significant differences between *top1* α and *top1* β hybridization patterns were observed in these tissues. As expected, hybridizations with sense RNAs gave no specific labelling (data not shown). The uniform distribution of both *top1* α and *top1* β exactly reflected the spatial distribution observed when the same experiment was performed with the *ubi-CEP* (ubiquitin-carboxyl extension protein) probe (data not shown), a gene whose expression level was shown to be enhanced in rapidly dividing cells (Balestrazzi *et al.*, 1998).

DISCUSSION

In mammalian cells the regulation of DNA topoisomerase I occurs at the transcriptional and post-translational level throughout the cell cycle. Nevertheless opposite opinions on the relationship

Fig. 1 - Spatial and temporal expression of *top1* genes in carrot hypocotyl-cells induced to proliferate in 2,4-D-containing medium. Tissue sections were *in situ* hybridized with ³⁵S-labeled antisense RNA probe derived from pTop28 and recognizing both *top1* α and β transcripts. Sections were then stained with 5% Giemsa. Bright- (A) and dark-field (B) photographs of a transverse section of hypocotyl at zero time. Bright- (C, E) and dark-field (D, F) photographs of longitudinal sections of hypocotyls after 2 d (C, D) and after 7 d (E, F) from the induction of proliferation. Bright- (G) and dark-field (H) photographs of a transverse section of hypocotyl after 7 d from the induction.

e, epidermis, p, cortical parenchyma, pc, provascular cells and x, xylematic elements. Proliferating cell masses of provascular cells (pc) are showed by arrows. Bar, 100 µm.

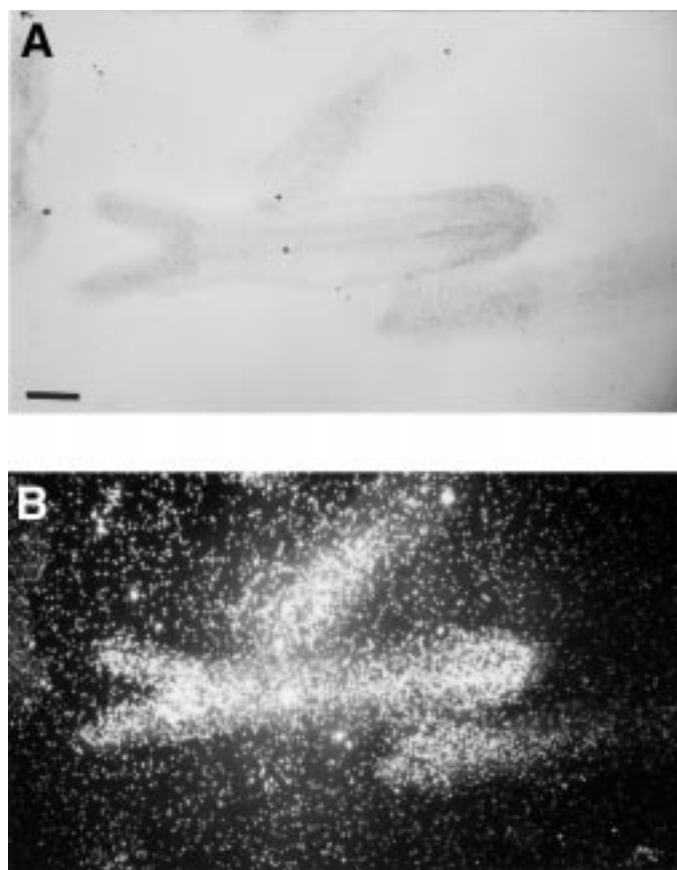


Fig. 3 - *In situ* localization of *top1* mRNAs in 14 d-old somatic embryos. Tissues were hybridized as described in Fig. 1. Bright- (A) and dark-field (B) photographs of longitudinal sections of torpedo somatic embryos. Bar, 200 μ m.

between topoisomerase I expression and cell proliferation have been expressed, over the past few years several data on the presence of *top1* transcript fluctuations during cell cycle have been reported (Kelly *et al.*, 1983; Hwong *et al.*, 1989; Romig and Richter, 1990a and 1990b). In particular, recent experiments suggested that the human *top1* gene is expressed just before the G₁/S transition in T lymphocytes that are induced to enter cell cycle by PHA stimulation and that both mRNA and protein accumulations parallel DNA synthesis. In addition, human topoisomerase I is subjected to a degradation process during proliferation with the aim, as hypothesized by authors, to maintain the enzyme at low level in the cell (Hwong *et al.*, 1993).

In this study, we have examined the response of *top1* genes to events inducing proliferation in plant

systems which, differently from cell suspension cultures, allow to obtain information on *top1* transcript localization at the tissue level. Results of the *in situ* RNA analysis of the transcript distribution during hypocotyl-cell proliferation provide evidence that only procambium, induced to proliferate by the presence of 2,4-D in the medium, reveals an enhanced level of *top1* transcripts. An additional information supplied by these experiments concerns the kinetics of *top1* accumulation in response to 2,4-D. We found that hybridization signals were only detectable after 24 h, then excluding a direct effect of the synthetic phytohormone on *top1* gene induction, since the transcriptional activation of several auxin-induced genes has been described as a rapid event which frequently requires only minutes (Theologis, 1986).

Embryogenic cell cultures may be utilized to produce large amounts of somatic embryos by selecting, from the heterogeneous population, cell clusters named proembryogenic masses (PEMs) and transferring them to 2,4-D-free medium at a low density (Sung and Okimoto, 1983). Embryo development is generally described as a process characterized by a strong boost to cell division in early stages.

It has been reported that a reprogramming of gene expression occurs at the transition from globular to more mature embryo forms (Zimmerman, 1993). At that stage both division and differentiation events give rise to the tissues and organs of the future adult plant. Since topoisomerase I is directly involved in the regulation of transcription (Merino *et al.*, 1993) and, in addition, is located in regions of active transcription (Fleischmann *et al.*, 1984; Gilmour and Elgin, 1987), we can speculate that the spatial distribution of the *top1* mRNAs during somatic embryogenesis may be also related to a reprogramming of the expression of genes whose products are necessary in the organization of new tissues and then particularly at the heart-torpedo stages.

It is interesting to note that the carrot gene *Met2-21*, encoding the enzyme DNA (cytosine-5)-methyl-

transferase and having a maximal expression in proliferating cells, shows a transcript distribution mainly concentrated in apical and root meristems at the torpedo-stage (Bernacchia *et al.*, 1998).

Finally, we investigated the spatial distribution of each *top1* transcripts by *in situ* hybridization of carrot vegetative apices, in order to verify the possibility that one of these genes could show a cell cycle-dependent expression pattern. Cell cycle-related genes are characterized by "spotty" expression patterns in apical meristems because their transcripts accumulate during specific phase(s) of the cell cycle and meristematic cells are not synchronized (Fobert *et al.*, 1994). Both *top1* mRNAs appear to be uniformly distributed within tissues having a high rate of division, such as the meristem and the leaf primordium, confirming that *top1* gene expression and cell proliferation are strongly correlated. However, there is evidence neither for a periodicity of *top1* expression throughout the cell cycle nor for a spatial difference in their mode of expression.

ACKNOWLEDGEMENTS

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Fig. 4 - *In situ* localization of *top1 α* (A) and *top1 β* (B) transcripts in carrot shoot apices. Longitudinal sections of shoot apices from 50-d old carrot plants were hybridized with digoxigenin-labeled RNA probes generated by *in vitro* transcription of *top1 α* and *top1 β* cDNAs, as outlined in Materials and Methods. **A.** Section hybridized with the *top1 α* antisense RNA probe. **B.** Section hybridized with the *top1 β* antisense RNA probe. The bar equals 300 μ m.

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