Original Articles

Analysis of Transcription of Plant 7SL RNA Gene Variants in HeLa *in Vitro* Transcription System

(RNA polymerase III / 7SL RNA gene / *in vitro* transcription)

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Abstract. We have performed an analysis of transcription of hop 7SL RNA genes in heterologous human extract from HeLa cells. Several variants of the *Hl7SL-1* gene with a truncated or mutated 5' non-transcribed part revealed a crucial importance of TATA box for transcription. The USE element was found important but dispensable for transcription. Transcription of mutants in the A-like box and experiments with hop 7SL RNA pseudogenes E44 and G32 revealed the importance of internal elements. The A-like box and possibly CG doublet at position +15/+16 described by Bredow et al. (1990a) are according to our results indispensable for *in vitro* transcription of plant 7SL RNA genes in human extract.

7SL RNA genes, which code for an RNA component of the signal recognition particle (SRP) (for review see Lutcke, 1995), are transcribed by RNA polymerase III. Genes controlled by this polymerase feature several different types of promoters (for recent review see Geiduschek and Kassavetis, 2001). The first type is represented by 5S RNA genes. Its promoter, composed of box A, intermediate element and box C, is included in the transcribed sequence. Box A contains the domain common for polymerase III promoters (Ciliberto et al., 1983). The intermediate element and box C are specific for the 5S RNA promoter and determine binding of 5S RNA specific transcription factor TFIIIA (Pieler et al., 1987). TFIIIC then interacts with TFIIIA and box A. Further binding of TFIIIB initiate transcription of 5S RNA genes. tRNA genes belong to the second group. They also include most important promoter elements – boxes A and B – in the transcribed sequence (Galli et al., 1981). These two boxes are recognized directly by TFIIIC without requirement for an additional factor. TFIIIC directs TFIIIB together with TBP (TATA-box binding protein) to the upstream parts of the gene and initiates transcription of TATA-less genes (White and Jackson, 1992; Joazeiro et al., 1996). Although it was thought that polymerase III promoters of the first and second group usually do not require elements upstream of the transcription start, there are cases where at least external TATA elements are required. 5S RNA and tRNA genes in fission yeast are not expressed without TATA elements (Hamada et al., 2001). TATA elements are important for re-initiation of tRNA transcription in plants (Yukawa et al., 2000), TATA-like element promotes transcription of tRNA genes in insect cells (Trivedi et al., 1999; Ouyang et al., 2000). According to Hamada et al. (2001), promoters that require the TATA element probably form an evolutionarily older promoter type. U6 RNA (Kunkel and Pederson, 1988), 7SK RNA (Murphy et al., 1987), plant 7SL RNA (Heard et al., 1995) and H1 RNA (Myslinski et al., 2001) genes are examples of the third polymerase III promoter type. It is similar to promoters of genes transcribed by RNA polymerase II comprising PSE and TATA elements and no internal elements. Finally, 7SL RNA genes in certain organisms like humans (Ullu and Weiner, 1985; Bredow et al., 1990a, b), trypanosome (Ben-Shlomo et al., 1997) and budding yeast (Dieci et al., 2002) form the fourth type promoter. These promoters include elements upstream of the transcription start (PSE, TATA) and also conserved elements inside the transcribed sequence resembling tRNA elements. Internal A and B blocks are necessary for SCR1 7SL RNA gene expression in budding yeast (Dieci et al., 2002).

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Abbreviations: PSE – proximal sequence element, SRP – signal recognition particle, TBP – TATA-box binding protein, USE – upstream sequence element.

The regulation of plant 7SL RNA genes has been postulated to occur from external elements (Heard et al., 1995). However, as we have shown previously, the hop 7SL RNA gene *Hl7SL-1* was also successfully and specifically transcribed *in vitro* in human nuclear extract from HeLa cells (Matoušek et al., 1999). An internal promoter element is strictly required for transcription of the human 7SL RNA gene (Bredow et al., 1990a). It is possible that although not required for transcription in plants, internal promoter elements were conserved in certain plant 7SL RNA genes throughout evolution due to 7SL RNA structure requirements. These elements could facilitate transcription of plant 7SL RNA genes in human extract. The *Hl7SL-1* gene contains USE and TATA elements, one more distant upstream conserved region (UCR) specific for hop and internal conserved blocks α and β. The α conserved motif includes the sequence motif AACCCAAGTGG matching the consensus sequence RRYN-NARYGG of the A-like box for RNA polymerase III (Poritz et al., 1988; Matoušek et al., 1999). The goal of this paper is to show which of these elements are important for *in vitro* transcription of hop 7SL RNA genes in human nuclear extract.

Material and Methods

Mutants in the A-like box of α block were prepared by introduction of either *Nco*I or *Sal*I restriction sites into the ends of fragments generated by PCR using primer pairs UCR (CATGTATAAACTTTCTGC) × *Nco*mut5' (CCCATGGGTTACAGGCCCACGTTGC) + *Nco*mut3' (AACCCATGGGGGGGCATGTGGGAAT) × T1 (GCCTAACAAAAAAGAAATGGT) and UCR × *Sal*mut5' (TGGTCGACAGGCCCACGTTGCTA) + *Sal*mut3' (GAGTCGACCAAGTGGGGGCATGTGG) × T1, respectively, and *Hl7SL-1* genomic clone (Matoušek et al., 1999; EMBL accession number AJ 236706) as a template. Fragments were treated with appropriate restriction endonuclease, ligated together and second PCR was performed using primers UCR × T1. The final PCR product was cloned into the pCR-Script SK(+) vector.

E44 and G32 natural pseudogenes were isolated from hop cultivars Eroica and Galena, respectively, by the method used for the *Hl7SL-1* clone described in Matoušek et al. (1999).

PCR fragments for transcription were prepared using one of the following 5' primers: UCR, USE-W (AACATAAGTCCCACATGGAAAACGAA), USE-M (AACATAAGGCCCACAGCGAAAACGAA), TATA-W (TAGTATATGAATTGTTCTCA), TATA-M (TAGGAGATGAATTGTTCTCA) and Hl7SL-1-Start (GCCGGGCTTAGCAACGTGGG) in combination with the T1 primer as 3' primer and the *Hl7SL-1* genomic clone, *Nco*I or *Sal*I A-like box mutant of the *Hl7SL-1* clone and E44 or G32 pseudogene clones, respectively, as templates. PCR fragments were separated from the template by electrophoresis in agarose gels and extracted using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) before transcription. The *in vitro* transcription was performed in nuclear extract from HeLa S3 cells (Dignam et al., 1983). Transcription assays were carried out in 10 µl reaction mixture with 0.6 mM each ATP, CTP, UTP, 0.03 mM GTP including 8 MBq/ml [α-^{32}P] GTP (8 Ci/mmol), 10 mM creatine phosphate, 2 mM $MgCl_2$, 25 mM KCl, 6 µl of HeLa S3 extract and 15 ng of purified PCR fragments 90 min at 30°C. Transcription products were run on 6% PAA sequencing gels. Autoradiograms were scanned and quantified using STORM PhosphoImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Results

To analyse which elements are important for specific *in vitro* transcription of hop 7SL RNA genes in HeLa extract, several PCR fragments with 5' truncation and/or with mutations in promoter elements of the wild-type *Hl7SL-1* gene were prepared (Figs. 1A, 2). Moreover, to analyse the importance of internal elements, two natural pseudogenes designated E44 and G32, lacking conserved motifs α, were included in the experiments. It has been proved in our previous experiments (Matoušek et al., 1999) that the addition of 2 µg α-amanitin per ml does not inhibit transcription of the 7SL RNA genes, therefore this polymerase II transcription inhibitor was not further included in the reaction mixtures.

Mutations in USE decreased transcription to 73.8% of the wild-type clone. The variant with deleted USE starting from TATA box resulted in the transcription level of 70.5%. Mutations introduced in TATA box resulted in a decrease of transcription to 50.2% (Fig. 1). Templates without TATA box starting from the transcribed sequence were transcribed at a much weaker level. The transcription reached 3.6% of the full-length clone or 5.1% of that of template starting from wild-type TATA box (Fig. 1). This indicates the importance of the TATA element for transcription of plant 7SL RNA genes in HeLa extract. Moreover, there appeared several shorter transcription products from a template lacking TATA box (Fig. 1B), probably due to initiation of transcription downstream of the regular transcription start.

Mutations in the A-like box within the α block changed the sequence 5'-AACCCAAGTGG-3' to 5'-AACCCATGGGG-3' when *Nco*I or to 5'-CGACCAAGTGG-3' when *Sal*I restriction sites were introduced, respectively (Fig. 3). Both changes decrease transcription considerably. Templates with *Nco*I provided 20.3% of the wild-type transcription level. The level of 42.4% was reached in the case of *Sal*I

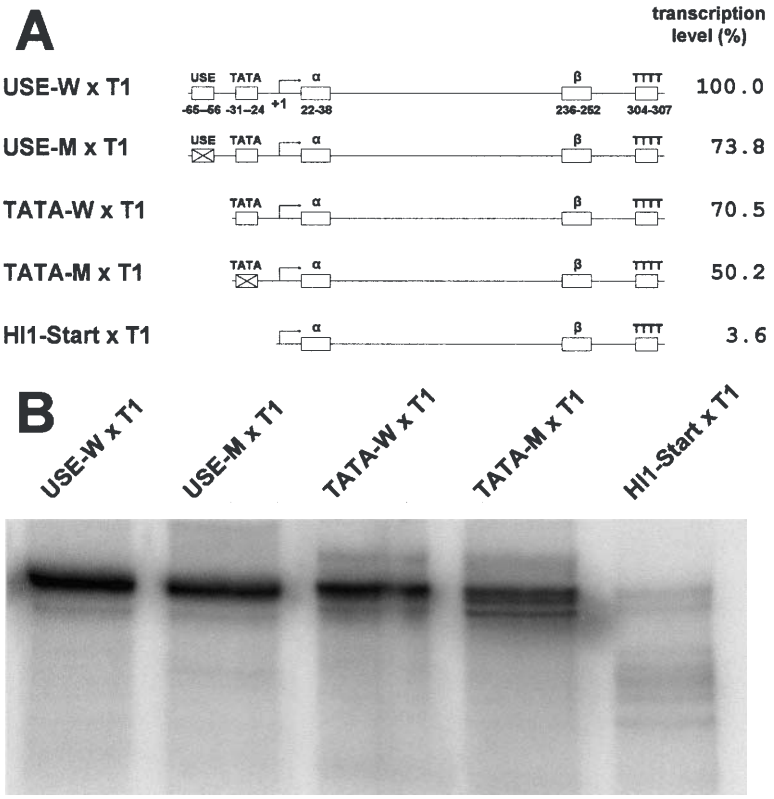

Fig. 1. A – schematic drawing of templates and transcription levels with a modified 5' non-transcribed region of the *Hl7SL-1* gene. USE-W × T1, USE-M × T1 – templates starting from the wild-type USE element and mutated USE element, respectively. TATA-W × T1, TATA-M × T1 – templates starting from the wild-type and mutated TATA element, respectively. Hl7SL-1-Start × T1 – template starting from the transcribed sequence of *Hl7SL-1*. +1 marks the transcription start, α – conserved sequence block with A-like box homology, β – another conserved sequence block, TTTT – termination signal. The relative transcription level (%) of a particular template in HeLa extract is shown on the right side. B – example of autoradiogram of transcription products

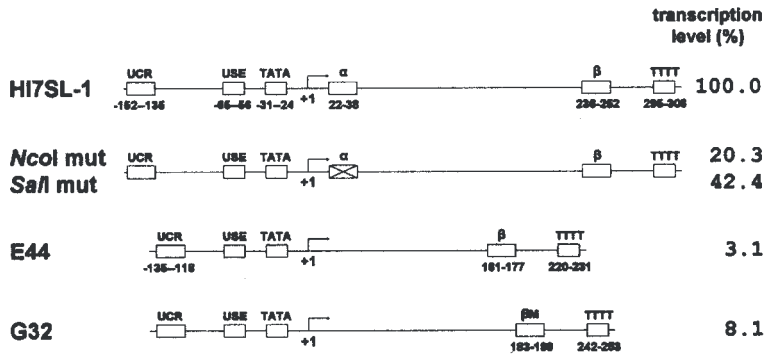

Fig. 2. Schematic drawing of templates and transcription levels with a mutated α block of the *Hl7SL-1* gene and of pseudogenes lacking the entire α block. *Nco*I mut and *Sal*I mut – mutants of *Hl7SL-1* in the α block with introduced restriction sites *Nco*I and *Sal*I, respectively. E44, G32 – hop 7SL RNA pseudogenes. The relative transcription level of a particular template in HeLa extract is shown in the right column. UCR – upstream conserved region specific for hop (Matoušek et al., 1999). Other symbols are the same as in Fig. 1.

mutants (Fig. 2), suggesting the importance of the A-like box for transcription in human extract.

Pseudogenes E44 and G32 were selected from hop cultivars Eroica and Galena, respectively. Their transcripts are detectable by RT-PCR in hop (data not shown). Both pseudogenes lack the α block and part of the sequence downstream of this block. The deletions are 77 and 55 bp long for E44 and G32, respectively (Fig. 3). Although both pseudogenes are transcribed quite efficiently in plant extract from tobacco at the approximate level 60% of that of the wild-type *Hl7SL-1*, they were nearly not transcribed in HeLa extract. Their transcription achieved only 3.1 and 8.1% for E44 and G32 pseudogenes, respectively, in comparison to *Hl7SL-1* (Fig. 2). This suggests that conserved motifs missing in E44 and G32 are important for transcription in human extract.

Discussion

Our experiments revealed that the USE element does play a role in the *in vitro* transcription of hop 7SL RNA genes in HeLa extract. However, the TATA element and some internal elements are essential for transcription in HeLa extract. This is consistent with the fact that the mutated TATA box is functional at a lower level and a gene without TATA signal is nearly not transcribed at all. Moreover, the TATA element probably plays an important role in the recognition of the transcription start in our system, as we observed shorter transcription products when a TATA-less template was used. The TATA element or analogous sequence is probably important for binding of TBP and direction of TFIIIB in human extract to the hop 7SL RNA promoter.

Mutations in the internal A-like box decreased transcription substantially. The *Nco*I mutation of the A-like box is located further downstream of the *Sal*I mutation (Fig. 3) and affects transcription considerably more strongly. We can conclude that the A-like box, especially its 3' part, is important for initiation of transcription, presumably for proper binding of TFIIIC in HeLa extract.

Hop 7SL RNA pseudogenes Eroica 44 and Galena 32 have homologous parts to A, B, C, D1 and D2 conserved blocks

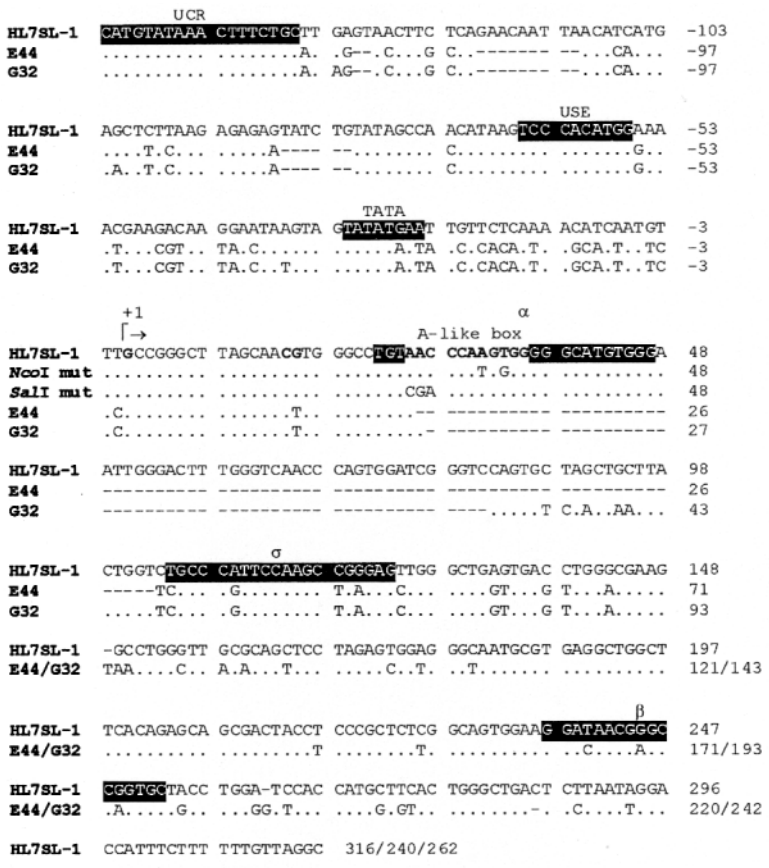

Fig. 3. Alignment of the *Hl7SL-1* gene with A-like box mutant clones *Nco*I mut and *Sal*I mut and with E44 and G32 pseudogenes. Only lines that differ from *Hl7SL-1* or from E44 in the case of G32 are shown. Dots mark the same nucleotides as in *Hl7SL-1*, dashes mark deletions.

described by Riedel et al. (1995) in tomato 7SL RNA genes. These pseudogenes were quite well transcribed *in vitro* in plant extract (unpublished). Moreover, products of these pseudogenes were detected by RT-PCR in hop. The doublet CG at position +15/+16 (Bredow et al., 1990a) and the external element (Bredow et al., 1990b) are the only elements essential for transcription of the human 7SL RNA gene in human extract. This CG doublet is present in the *Hl7SL-1* gene; however, it is mutated in hop 7SL pseudogenes to doublet CT (Fig. 3). Hop pseudogenes lack the α block including the A-like box, which apparently is not required for transcription in plants. However, as these pseudogenes were nearly untranscribed in HeLa extract, we can assume that this part, probably together with the CG doublet, plays an essential role for transcription in HeLa extract.

The elements most important for transcription of hop 7SL RNA genes in HeLa extract are the TATA element and some internal promoter elements, most probably the doublet CG and the consensus of the A-like box. This is not the case of plant extract, where the same internal promoter does not play such a significant role *in vitro*. However, internal promoter elements could play some role in fine regulation of plant 7SL RNA expression. There exist many variants of 7SL RNA genes in each plant species analysed so far (Heard et al., 1995; Riedel et al., 1995, Matoušek et al., 1999) and it can be assumed that the transcription of these gene variants is under intimate cellular control. It was shown by Heard et al. (1995) that internal elements, although not required for high-level transcription, have some effect on transcription. Analogous function of putative internal elements of hop 7SL RNA genes could be one of the reasons why these elements have been preserved in evolution. Recently, it has been shown that in the most primitive eukaryotic model organism *Saccharomyces cerevisiae* both, A and B internal block homologues are crucial elements for yeast 7SL RNA transcription (Dieci et al., 2002). It is possible that originally 7SL RNA genes were controlled by internal elements that during evolution were gradually loosing they role as major promoter elements. Some of them were conserved due to requirements for a functional 7SL RNA structure or for binding of SRP proteins. The others could be preserved in 7SL RNA genes because of their regulative effect on the main promoter elements, now located upstream of the transcription start.

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References

Ben-Shlomo, H., Levitan, A., Beja, O., Michaeli, S. (1997) The trypanosomatid *Leptomonas collosoma* 7SL RNA gene. Analysis of elements controlling its expression. *Nucleic Acids Res.* **25,** 4977-4984.

Bredow, S., Kleinert, H., Benecke, B. J. (1990a) Sequence and factor requirements for faithful *in vitro* transcription of human 7SL RNA. *Gene* **86,** 217-225.

Bredow, S., Surig, D., Muller, J., Kleinert, H., Benecke, B. J. (1990b) Activating-transcription-factor (ATF) regulates human 7S L RNA transcription by RNA polymerase III *in vivo* and *in vitro*. *Nucleic Acids Res.* **18,** 6779-6784.

Ciliberto, G., Cortese, R., Costanzo, F., Dente, L., Raugei, G. (1983) Common and interchangeable elements in the promoters of genes transcribed by RNA polymerase III. *Cell* **32,** 725-733.

Dieci, G., Giuliodori, S., Catellani, M., Percudani, R., Ottonello, S. (2002) Intragenic promoter adaptation and facilitated RNA polymerase III recycling in the transcription of SCR1, the 7SL RNA gene of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **277,** 6903-6914.

Dignam, J. D., Lebowitz, R. M., Roeder, R. G. (1983) Accurate transcription initiation by polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11,** 1475-1489.

Galli, G., Hofstetter, H., Birnstiel, M. L. (1981) Two conserved sequence blocks within eukaryotic tRNA genes are major promoter elements. *Nature* **294,** 626-631.

Geiduschek, E. P., Kassavetis, G. A. (2001) The RNA polymerase III transcription apparatus. *J. Mol. Biol.* **310,** 1-26.

Hamada, M., Huang, Y., Lowe, T. M., Maraia, R. J. (2001) Widespread use of TATA elements in the core promoters for RNA polymerases III, II, and I in fission yeast. *Mol. Cell Biol.* **21,** 6870-6881.

Heard, D. J., Filipowicz, W., Marques, J. P., Palme, K., Gualberto, J. M. (1995) An upstream U-snRNA gene-like promoter is required for transcription of the *Arabidopsis thaliana* 7SL RNA gene. *Nucleic Acids Res.* **23,** 1970-1976.

Joazeiro, C. A. P., Kassavetis, G. A., Geiduschek, E. P. (1996) Alternative outcomes in assembly of promoter complexes: the roles of TBP and a flexible linker in placing TFIIIB on tRNA genes. *Genes Dev.* **10,** 725-739.

Kunkel, G. R., Pederson, T. (1988) Upstream elements required for efficient transcription of a human U6 RNA gene resemble those of U1 and U2 genes even though a different polymerase is used. *Genes Dev.* **2,** 196-204.

Lutcke, H. (1995) Signal recognition particle (SRP), a ubiquitous initiator of protein translocation. *Eur. J. Biochem.* **228,** 531-550.

Matoušek, J., Junker, V., Vrba, L., Schubert, J., Patzak, J., Steger, G. (1999) Molecular characterization and genome organization of 7SL RNA genes from hop (*Humulus lupulus L.*). *Gene* **239,** 173-183.

Murphy, S., DiLiegro, C., Melli, M. (1987) The *in vitro* transcription of the 7SK RNA gene by RNA polymerase III is dependent only on the presence of an upstream promoter. *Cell* **51,** 81-87.

Myslinski, E., Ame, J-C., Krol, A., Carbon, P. (2001) An unusually compact external promoter for RNA polymerase III transcription of the human H1RNA gene. *Nucleic Acids Res.* **29,** 2502-2509.

Ouyang, C., Martinez, M. J., Young, L. S., Sprague, K. U. (2000) TATA-binding protein-TATA interaction is a key determinant of differential transcription of silkworm constitutive and silk gland-specific $tRNA^{Ala}$ genes. *Mol. Cell Biol.* **20,** 1329-1343.

Pieler, T., Hamm, J., Roeder, R. G. (1987). The 5S gene internal control region is composed of three distinct sequence elements, organized as two functional domains with variable spacing. *Cell* **48,** 91-100.

Poritz, M. A., Siegel, V., Hansen, W., Walter, P. (1988). Small ribonucleoproteins in *Schizosaccharomyces pombe* and *Yarrowia lipolytica* homologous to signal recognition particle. *Proc. Natl. Acad. Sci. USA* **85,** 4315-4319.

Riedel, L., Putz, A., Hauser, M. T., Luckinger, R., Wassenegger, M., Sanger, H. L. (1995) Characterization of the signal recognition particle (SRP) RNA population of tomato (*Lycopersicon esculentum*). *Plant Mol. Biol.* **27,** 669-680.

Trivedi, A., Young, L. S., Ouyang, C., Johnson, D. L., Sprague, K. U. (1999) A TATA element is required for tRNA promoter activity and confers TATA-binding protein responsiveness in *Drosophila* Schneider-2 cells. *J. Biol. Chem.* **274,** 11369-11375.

Ullu, E., Weiner, A. M. (1985) Upstream sequences modulate the internal promoter of the human 7SL RNA gene. *Nature* **318,** 371-374.

White, R. J., Jackson, S. P. (1992) Mechanism of TATA-binding protein recruitment to a TATA-less class III promoter. *Cell* **71,** 1041-1053.

Yukawa, Y., Sugita, M., Choisne, N., Small, I., Sugiura, M. (2000) The TATA motif, the CAA motif and the poly(T) transcription termination motif are all important for transcription re-initiation on plant tRNA genes. *Plant J.* **22,** 439-447.