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Transcription of T-DNA in octopine and nopaline crown gall tumours is inhibited by low concentrations of α-amanitin

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ABSTRACT

Highly purified and physiologically active nuclei were isolated from four different octopine and nopaline crown gall lines. These nuclei exhibited a high endogenous RNA synthesizing activity involving all three RNA-polymerases I, II and III. Isolated nuclei were shown by Southern blotting to synthesize T-DNA specific RNA. This synthesis was shown to be sensitive to actinomycin D and therefore to be DNA-dependent. The transcription of the T-DNA was also inhibited for more than 90% by low concentrations of α-amanitin (0.7 µg/ml) indicating that the T-DNA, although from bacterial origin, is transcribed by the host RNA polymerase II.

INTRODUCTION

Crown gall is a neoplastic disease produced in most dicotyledonous plants by infection of the plant with the gram negative soil bacterium, *Agrobacterium tumefaciens*. The genetic determinants responsible for the synthesis of opines in the transformed plant cell as well as for their tumorous growth are carried by large plasmids (Ti-plasmids) present in the tumour-inducing bacteria (1-5).

Part of the Ti-plasmid, the so called T-region, is transferred to the transformed plant cell, a precise map of the transferred segment of the Ti-plasmid (the T-DNA) has been established for several octopine and nopaline crown gall lines (6-11).

The T-DNA is transcribed in a highly differentiated pattern (12-14) giving rise to several different poly-adenylated RNA species (14, unpublished results). As the T-DNA is present in the nucleus and absent from chloroplasts and mitochondria (15,16) the nature of the nuclear RNA polymerase providing the transcription of the T-DNA arises. In the case of DNA viruses transforming animal cells, such as SV 40, adenovirus and Herpes simplex, it has been shown that the transcription of the viral DNA within the nuclei of infected cells is performed by RNA polymerase II, the only exception known being the genes of adenovirus coding for the VA-RNA's which are transcribed by RNA polymerase III (17-23).

Using a newly developed procedure for the isolation of highly purified and physiologically active nuclei from plant cells (24) it was possible to study the transcription of T-DNA in isolated nuclei. To the best of our knowledge this represents the first detailed investigation of transcription of an integrated specified DNA-fragment in nuclei isolated from plant cells. Based on the inhibition of this transcription by low concentrations of α-amanitin the results indicate that more than 90% of the transcripts of the T-DNA are provided by RNA polymerase II.

MATERIALS AND METHODS

TISSUE CULTURES

The octopine crown gall tumours A6-S1 and B6S3-S1 and the nopaline tumours C58-S1 were induced on Nic.-Tab. var White Burley by *Agrobacterium tumefaciens* strains harbouring the Ti-plasmids pTiA6, pTiB6S3 and pTiC58 respectively. These three lines originated from the collection of Dr. Schilperoort, Leiden. The nopaline line BT-37 was induced on Nic.-Tab. var Havanna by *Agrobacterium tumefaciens* carrying the pTiT37. This line was kindly supplied by Dr. A. Braun.

Axenic cultures of these four lines were grown on hormone free medium according to Linsmeier and Skoog (25). All lines were kept in suspension culture with weekly transfer, except line BT-37 which was transferred every three weeks. Cultures were regularly analyzed and shown to synthesize either octopine or nopaline.

ISOLATION OF NUCLEI AND SUBSEQUENT INCUBATION FOR RNA SYNTHESIS AND ISOLATION OF RNA TRANSCRIPTS

Nuclei purified according to Willmitzer and Wagner (24) were washed three times with buffer B (60 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM mercaptoethanol. 0.1 mM EDTA, 15% glycerol (w/v), 0.5% bovine serum albumin). Reactions were started by adding the appropriate amount of a concentrated ammonium sulphate solution (240 mM in buffer B) to achieve the final ionic strength desired (40 mM $(NH_4)_2SO_4$ if not otherwise indicated), and nucleoside triphosphates to obtain a final concentration of 0.3 mM ATP, GTP and CTP and 0.02 mM UTP. The effect of certain inhibitors was studied by incubating the nuclei in their presence for 30 minutes on ice before adding the nucleoside triphosphates. A control without inhibitor was included. Reaction volumes of 250-500 µl were used containing $1\text{-}2\cdot10^7$ nuclei and 200-400 µCi α-^{32}P-UTP. α-amanitin and actinomycin D were used at concentrations of 0.7 µg/ml and 100 µg/ml respectively. The ionic strength was fixed at 40 mM ammonium sulphate.

After incubation for 20 minutes at 25°C, t-RNA and cold UTP were added at final concentrations of 100 µg/ml and 1 mM respectively. After incubation with purified DNase I at 37°C for 15 minutes, 1 volume of lysis buffer containing 7 M urea, 1% SDS, 0.3 M NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, was added and the reaction incubated with proteinase K (100 µg/ml) for 30 minutes at 37°C. After two extractions with phenol/chloroform/isoamylalcohol (25/24/1) RNA was precipitated with two volumes of ethanol overnight. After one more round of DNase and proteinase K treatment the aqueous phase was applied to a Sephadex G50 column equilibrated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% SDS, to separate RNA from non-incorporated UTP. The high molecular weight peak was collected and precipitated with ethanol after addition of 1/10th volume of 2 M sodium acetate pH 5.0.

ASSAY OF TOTAL RNA SYNTHESIS

In order to determine the activity of total RNA synthesis, about 10^6 nuclei were incubated in 100-200 µl of buffer B supplemented with the respective amount of $(NH_4)_2SO_4$ (cf. Fig. 1-3), the nucleoside triphosphates (ATP, GTP and CTP at a final concentration of 0.3 mM, UTP at a final concentration of 0.02 mM containing 10 µCi of α-^{32}P-UTP (350 Ci/mMol)) and the inhibitors as outlined in the results section.

After incubation for the time indicated in the results section, the reaction was stopped by the addition of 0.5 ml cold 10% trichloroacetic acid, precipitated RNA was collected on GF/C glass fibre filters and after washing twice with 10 ml of 10% and 10 ml of 5% trichloroacetic acid, the amount of radioactivity was determined by liquid scintillation counting.

PREPARATION OF DNA-FILTERS AND RNA-DNA HYBRIDIZATION

Plasmid DNA containing cloned fragments of octopine as well as nopaline T-regions (11) (G.De Vos *et al.*, in prep.) was isolated from *E.coli* cultures according to Clewell and Helinski (26). All manipulations were carried out under L1-conditions according to the Richtlinien zum Schutz vor Gefahren durch *in vitro* neukombinierte Nukleinsäuren.

Digestions with restriction endonucleases were performed according to the suppliers recommendations. After gel electrophoretic separation of the DNA fragments in 0.8% agarose gels in 40 mM Tris-acetate, 10 mM Na-acetate, 1 mM EDTA, 0.3 µg/ml ethidium bromide, pH 7.8, DNA was transferred to nitrocellulose filters according to Southern (27). Hybridization was performed in sealed plastic bags containing 4 ml of 3 x SSC, 1 mM EDTA, 0.3% SDS, 100 µg/ml t-RNA (*E.coli*), $40\text{-}80 \cdot 10^6$ cpm RNA at 72°C for 36-42 hours. After hybridization filters were

washed first with 3 x SSC, 1 mM EDTA, 0.5% SDS (3 times, 15 min each) and later with 3 x SSC, 1 mM EDTA (3 times, 15 min each) at 72°C, treated with RNase A (20 µg/ml) for 30 minutes at 37°C in 2 x SSC and washed again with 3 x SSC, 1 mM EDTA, 0.5% SDS and 3 x SSC, 1 mM EDTA at 72°C (three times each for 15 min). Subsequently filters were dried and autoradiographed for 2-14 days using Kodak X-R-omat films with Kodak intensifying screens.

RESULTS

CHARACTERISTICS OF THE RNA-SYNTHESIS IN ISOLATED NUCLEI

The α-amanitin sensitivity of the endogenous RNA polymerase activities in nuclei isolated from crown gall cells is demonstrated in Figure 1.

About 60% of the total activity was inhibited by an α-amanitin concentration of 0.5 µg/ml, the midpoint of the transition being at 0.01 µg/ml α-amanitin, thus matching the data published for RNA polymerase II (28,29). About 5% of the total activity was inhibited by concentrations of α-amanitin exceeding 5 µg/ml thus representing products of RNA polymerase III (28).

The residual activity of about 35%, being insensitive to α-amanitin concentrations up to 100 µg/ml, represents RNA polymerase I activity (28).

Previous studies performed in this laboratory have indicated that the total amount of poly A^+-polysomal RNA derived from T-DNA amounts to about 0.001%

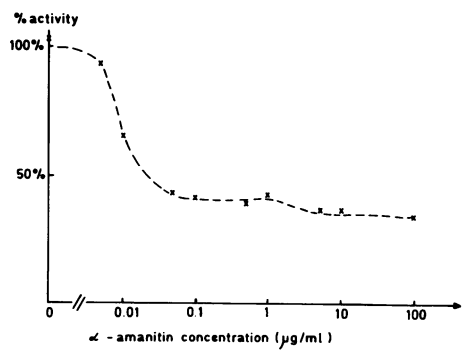

Figure 1
α-amanitin sensitivity of the endogenous RNA polymerase activities in nuclei isolated from crown gall cells. Reactions were performed at different concentrations of α-amanitin, the level of remaining synthesis is shown as percentage of the synthesis in the absence of α-amanitin. The ionic strength was kept at 40 mM ammonium sulphate, the samples were incubated for 20 minutes at 25°C. The assay was performed as described in MATERIALS AND METHODS.

of the total RNA. We therefore decided to optimize total RNA synthesis in our system in order to be able to detect T-DNA derived RNA synthesis. Fig. 2 shows the incorporation of α-^{32}P-UTP into acid precipitable material as a function of time. After an initial burst in the first ten minutes the reaction slows down reaching a maximum after 20 minutes and remaining on a plateau for the next 60 minutes. There is therefore no or little re-initiation taking place in isolated nuclei which is in accord with data published for animal systems (30,31). The nearly complete inhibition of RNA synthesis by actinomycin D rules out the possibility of RNA-dependent RNA synthesis establishing that the de novo RNA synthesis is DNA-derived (32,33). The inset in Fig. 2 demonstrates the linear relationship between the concentration of nuclei and the amount of RNA produced.

An important parameter concerning the activity of RNA polymerases is the ionic strength. Fig. 3 shows the RNA synthesizing activity of the nuclear system as a function of the ionic strength applied both in the presence and in the absence of 0.7 µg/ml of α-amanitin, a concentration which according to Fig. 1 is sufficient to specifically inhibit RNA polymerase II. The total RNA synthesis increased with ionic strength up to 60 mM $(NH_4)_2SO_4$ which is mainly due to an increase of the activity of RNA polymerase II. A ionic strength of

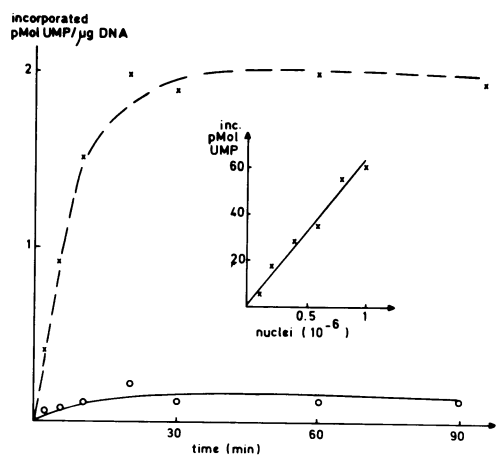

Figure 2
Time course of the synthesis of RNA in isolated nuclei from octopine crown gall cells in the absence (x) and presence (o) of 100 µg/ml actinomycin D. Ionic strength was kept at 40 mM ammonium sulphate. Inset: Effect of nuclei concentration on the amount of RNA synthesized. Increasing numbers of nuclei were incubated with a constant amount of α-^{32}P-UTP for 20 minutes at an ionic strength of 40 mM Ammonium sulphate.

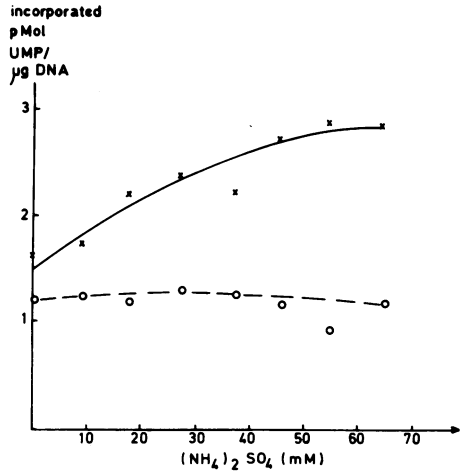

Figure 3
Influence of ammonium sulphate concentration on RNA synthesis in nuclei isolated from crown gall cells. A constant amount of nuclei was incubated for 20 minutes with varying ammonium sulphate concentrations in the absence (o) or presence (x) of 0.7 µg/ml of α-amanitin. Nuclei tend to lyse at ionic strength exceeding 70 mM $(NH_4)_2SO_4$.

40 mM ammonium sulphate was chosen for further experiments (total cation concentration: 150 mM) because this approaches physiological conditions. Under these circumstances the nuclei remain intact and the influence of α-amanitin on transcription is maximal.

ANALYSIS OF RNA SYNTHESIZED IN THE PRESENCE OF α-AMANITIN AND ACTINOMYCIN D FOR T-DNA DERIVED SEQUENCES

Preliminary studies indicated that due to the low concentration of T-DNA specific RNA's the approach used for similar studies concerning the transcription of several animal viruses (17-23) i.e. counting the RNA hybridized to filters containing viral DNA, could not be used for these studies. Thus the more sensitive technique of Southern hybridization was applied. In order to keep the sensitivity as high as possible the T-DNA clones used for hybridization were only cut once thus keeping the number of fragments offered for hybridization as small as possible. The conditions used in these experiments were taken from Fig. 1-3, i.e. the α-amanitin concentration was fixed to 0.7 µg/ml, the incubation time was 20 min, and the ionic strength applied was 40 mM ammonium sulphate.

The autoradiograms obtained after hybridization of the RNA synthesized in the presence of actinomycin D, α-amanitin or in the absence of any inhibitor by nuclei from the octopine crown gall line A6-S1 is presented in Figure 4a-d together with the corresponding data for total RNA synthesis. The same batch of nuclei was used to examine the effect of the different inhibitors on the synthesis of T-DNA specific transcripts. Total RNA synthesis was inhibited for 55% in the presence of 0.7 µg/ml α-amanitin and no hybridization was visible on the autoradiograms (lane 4c), on the other hand in the absence of both inhibitors clear hybridization was observed both to the left as well as to the right part of the T-DNA (lane 4b) The transcription of T-DNA is therefore specifically in-

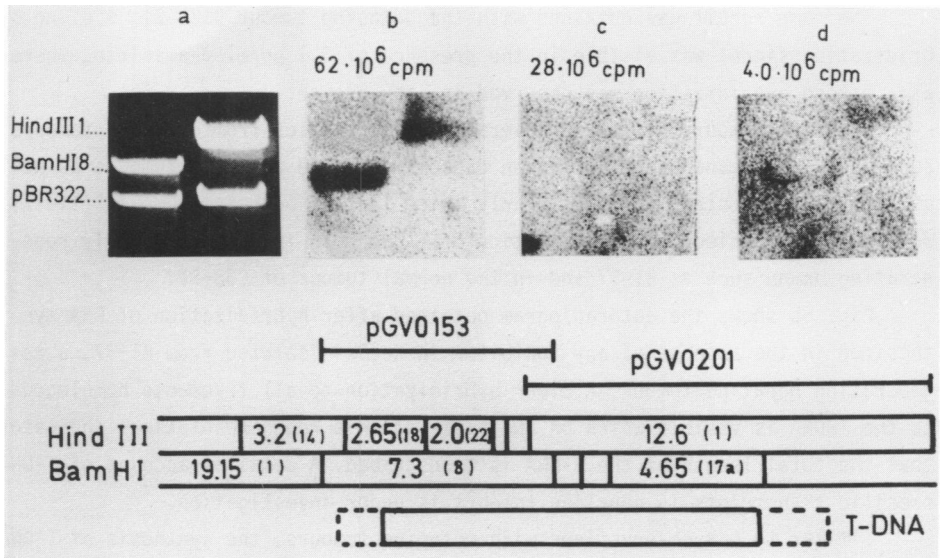

Figure 4
Hybridization of RNA synthesized in the absence or presence of various inhibitions by nuclei isolated from octopine tumour A6-S1, to blots of T-DNA. Lane a shows the ethidium bromide stain of DNA offered for hybridization. Lane b-d: hybridization of RNA synthesized in nuclei isolated from A6-S1 in the absence of any inhibitor (lane b), in the presence of 0.7 µg/ml of α-amanitin (lane c) and in the presence of 100 µg/ml actinomycin D (lane d). The amount of total RNA synthesis as determined by acid-precipitable counts is indicated above the corresponding lane. The same batch of nuclei has been used for the whole set of experiments (for example lane b-d), the same amount of nuclei was used in every single experiment (i.e. b,c,d). The lower part shows the physical map of T-DNA in the tobacco octopine crown gall tumours A6-S1 and B6S3-S1 as well as a partial map of the pTiACH5. The clones pGV 0153 (BamHI fragment 8 of pTiACH5) and pGV 0201 (HindIII fragment 1 of pTiACH5) were used for assessing transcription.

hibited by 0.7 µg/ml of α-amanitin. Lane 4d shows the autoradiograms obtained after hybridization of the RNA synthesized in the presence of 100 µg/ml of actinomycin D. The total RNA synthesis was inhibited for 94% and only a very weak hybridization signal was visible. Spectrophotometric scanning of the autoradiograms revealed that the hybridization intensity obtained in the presence of actinomycin D represented about 9% of the intensity of hybridization in the absence of any inhibitor which is in agreement with the observed residual activity of total RNA synthesis of 6%. This proves that the synthesis of T-DNA specific RNA was due to a de novo, DNA dependent process. Since a residual signal of about 6% gives rise to a hybridization visible on the autoradiogram, it can be concluded that α-amanitin inhibits the synthesis of T-DNA specific RNA for more than 90%.

The same result was obtained with the octopine tumour B6S3-S1, i.e. no hybridization signal was visible in the presence of 0.7 µg/ml α-amanitin, whereas a strong hybridization was observed in its absence.

Nopaline tumours differ in several characteristics from octopine tumours such as the spontaneous regeneration capacity of some nopaline tumours as well as their T-DNA content which is nearly twice that of octopine tumours (11,12). We therefore studied the transcription of T-DNA both in a spontaneously regenerating tumour such as BT-37 and in the normal tumour of C58-S1.

Fig. 5b shows the autoradiogram obtained after hybridization of RNA synthesized in the absence of any inhibitor in nuclei isolated from BT-37, a regenerating nopaline tumour. A clear hybridization to all fragments homologous to the T-DNA is visible which on the basis of this rough resolution indicates that the total length of the T-DNA is transcribed. A detailed account of T-DNA specific transcripts in nopaline tumours is under investigation.

Similar to our observations with octopine tumours, the synthesis of T-DNA specific RNA was found to be completely and specifically inhibited by α-amanitin. No signal was visible although the total incorporation was only reduced by a factor of 40% (Fig. 5, lane c).

A similar result was obtained for the nopaline tumour C58-S1, i.e. no T-DNA specific transcript could be detected in the presence of 0.7 µg/ml α-amanitin (data not shown).

It should be stated that the inhibition of the production of T-DNA derived RNA by low concentrations of α-amanitin (0.7 µg/ml) has been reproducible observed in 9 independent experiments performed for the octopine tumours A6-S1 and B6S3-S1 and in 3 independent experiments performed for the nopaline tumours C58-S1 and BT-37.

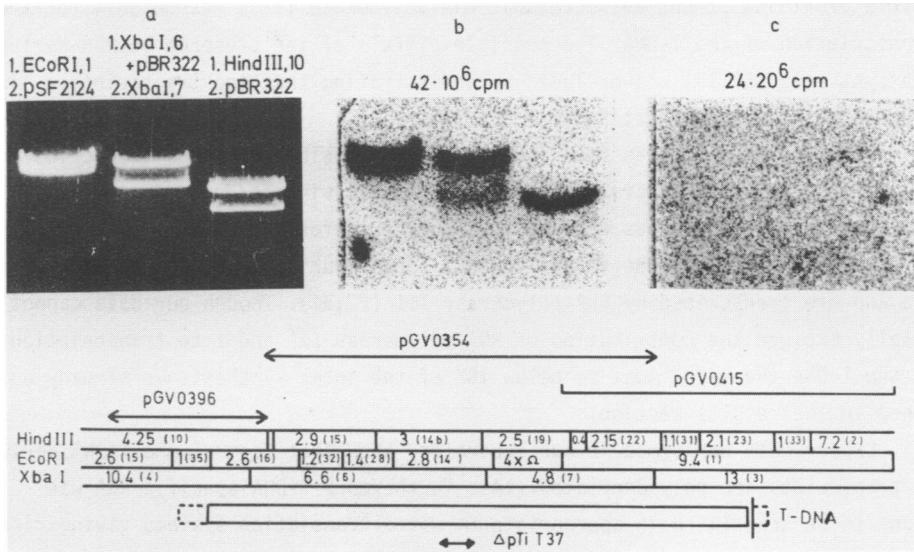

Figure 5
Hybridization of RNA synthesized in nuclei isolated from nopaline crown gall tumour BT-37 in the absence of any inhibitor (lane b) and in the presence of 0.7 µg/ml α-amanitin (lane c). Lane a shows the ethidium bromide stain of T-DNA fragments offered for hybridization, the numbers and abbreviations above lane a correspond to the bands seen in the ethidium bromide stained gel. - The lower part shows the physical map of T-DNA in the tobacco nopaline crown gall tumour BT-37 as well as a partial map of pTiC58 (9). The clones pGV 0396 (HindIII fragment 10 of pTiC58), pGV 0354 (HindIII fragments 15, 14b, 19, 41, and 22 of pTiC58) and pGV 0415 (EcoRI fragment 1 of pTiC58) have been offered for hybridization. - pGV 0396 and pGV 0354 are pBR 322 clones, pGV 0415 was constructed using pSF 2124 as a vector. Δ pTiT37 indicates a deletion in the Ti-plasmid pTiT37 compared to pTiC58 (9).

In 5 independent experiments performed with nuclei isolated from non-transformed, habituated tissue of tobacco, no hybridization to T-DNA probes was observed.

DISCUSSION

Due to a newly developed procedure for the isolation of highly purified and physiologically active nuclei from crown gall cells, the transcription of T-DNA in octopine and nopaline tumour lines could be studied in isolated nuclei. The nuclei were shown to contain a high endogenous activity of all three RNA polymerases. The transcription of the T-DNA was found to be inhibited by α-amanitin concentrations known to specifically inhibit RNA polymerase II, the-

refore providing strong evidence that RNA polymerase II is responsible for the transcription of the T-DNA. The possible pitfall of the presence of RNA-derived RNA synthesis (32,33) was excluded by demonstrating that the transcription of T-DNA was inhibited by actinomycin D.

RNA polymerase II has been shown to be responsible for the transcription of the majority of the viral genomes of oncogenic viruses during productive infection of animal cells as well as in stably transformed cells (17-23). The only exception known are the VA-RNA's which appear during infection by adenovirus and are transcribed by RNA polymerase III (22,31). Though our data cannot totally exclude the contribution of RNA polymerase III and I to transcription of the T-DNA the level must be below 10% of the total synthesis as already outlined in the results section.

Experiments performed in our laboratory show that most of the T-DNA derived transcripts are polyadenylated (14). Furthermore T-DNA specific RNA was found to be translated in appropriate *in vitro* translation systems giving rise to distinct protein bands (34,35). Both independent findings agree well with our observation that T-DNA is transcribed by RNA polymerase II since RNA polymerase II products are known to be polyadenylated (up to now there is no positive evidence for polyadenylation of products of RNA polymerase I and III) and to represent the precursors for mature translatable m-RNA (31). Results obtained recently in our laboratory have indicated, that initiation and termination of transcription takes place within the T-DNA. Therefore signals must be present on the T-DNA which are recognized by RNA polymerase II as termination and inititation signals.

An alternative but less likely explanation of our results would be that T-DNA codes for an α-amanitin sensitive RNA polymerase with properties similar to those of the plant RNA polymerase II.

One of the most challenging questions concerns the nature of this signals: how are the prokaryote-derived DNA sequences recognized by an eukaryotic RNA polymerase? Experiments are in progress with the aim to set up appropriate *in vitro* transcription systems using purified RNA polymerase II in order to get a more detailed insight into these processes.

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