A DNA-dependent RNA synthesis by wheat-germ RNA polymerase II insensitive to the fungal toxin α -amanitin

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Wheat-germ RNA polymerase II is able to catalyse a DNA-dependent reaction of RNA synthesis in the presence of a high concentration (1 mg/ml) of the fungal toxin α -amanitin. This anomalous reaction is specifically directed by single-stranded or double-stranded homopolymer templates, such as poly(dC) or poly(dC)·poly(dG), and occurs in the presence of either Mn²⁺ or Mg²⁺ as the bivalent metal cofactor. In contrast, the transcription of other synthetic templates, such as poly(dT), poly(dA)·poly(dT) or poly[d(A-T)] is completely abolished in the presence of 1 μ g of α -amanitin/ml, in agreement with well-established biochemical properties of class II RNA polymerases. Size analysis of reaction products resulting from transcription of (dC)_n templates of defined lengths suggests that polymerization of RNA chains proceeds through a slippage mechanism. The fact that α -amanitin does not impede this synthetic reaction implies that the amatoxin interferes with the translocation of wheat-germ RNA polymerase II along the DNA template.

INTRODUCTION

It is well established that in eukaryotic cells the transcription of nuclear genes is catalysed by three distinct classes of nuclear DNA-dependent RNA polymerases, called I, II and III. RNA polymerase I synthesizes rRNA precursors, RNA polymerase II synthesizes mRNA precursors and RNA polymerase III catalyses the synthesis of precursors of tRNA and 5 S RNA. A characteristic biochemical property of these enzyme classes is their differential sensitivity with respect to inhibition by α -amanitin, a powerful toxin from the toadstool *Amanita phalloides*. Thus RNA polymerases I are generally insensitive to α -amanitin, whereas RNA polymerases II are generally highly sensitive (50 % inhibition at 0.01–0.05 μ g/ml), and RNA polymerase III are inhibited only at relatively high concentrations of the toxin [50 % inhibition of wheat-germ RNA polymerase III by 2.5 μ g of α -amanitin/ml (Jendrisak, 1981)] [reviewed by Wieland & Faulstich (1978), Sentenac (1985) and Blair (1988)].

α -Amanitin inhibits RNA synthesis catalysed by wheat-germ RNA polymerase II both *in vivo* (Jendrisak, 1978) and *in vitro* (Guilfoyle & Jendrisak, 1978; Jendrisak & Guilfoyle, 1978). Cochet-Meilhac & Chambon (1974) have shown that the amatoxin binds very tightly to calf thymus RNA polymerase II ($K_d = 10^{-9}$ M) with a 1:1 stoichiometry, and acts to slow the rate of RNA chain elongation. Transcription studies employing poly[d(A-T)] as template, UpA as a primer and UTP as substrate revealed that synthesis of a single phosphodiester bond by calf thymus RNA polymerase II (Vaisius & Wieland, 1982) and wheat-germ RNA polymerase II (de Mercoyrol *et al.*, 1989) resists inhibition in the presence of excess α -amanitin. With this transcription system and under experimental conditions permitting RNA chain elongation (i.e. in the additional presence of ATP), we found that the productive elongation pathway leading to the synthesis of poly[r(A-U)] catalysed by wheat-germ RNA polymerase II was blocked after UpApU formation by α -amanitin (de Mercoyrol *et al.*, 1989). In agreement with the proposal by Vaisius & Wieland (1982), we suggested that the translocation step of the transcription complex along the DNA template is the main enzymic process blocked by the amatoxin (de Mercoyrol *et al.*, 1989). In the present paper we extend that previous study, and report that under some circumstances wheat-

germ RNA polymerase II can catalyse a DNA-directed reaction of polymerization of RNA chains in the presence of excess α -amanitin.

MATERIALS AND METHODS

Reagents

Ultrapure ribonucleoside triphosphates and the dinucleoside monophosphates were purchased from Pharmacia and Boehringer Mannheim. All the radioactive products were from Amersham International. α -Amanitin was from Boehringer Mannheim. Poly(dT), poly(dT)·poly(dA), poly[d(A-T)], poly(dC) and poly(dC)·poly(dG) were from P-L Biochemicals. *Ustilago sphaerogena* RNAase U1 was from Sigma. RNAase H was from Pharmacia.

dC_n ($n = 10, 20, 30, 40, 50$ and 60 bases) oligodeoxycytidylates were synthesized on a Biosearch 8750 automated synthesizer, by using β -cyanoethylphosphoramidite chemistry. They were purified by h.p.l.c. using a Zorbax BioSeries Oligo (8 cm $\times$ 6.2 mm internal diameter) column (DuPont). Elution was performed with a linear gradient of ammonium acetate (0.02–2 M) in 20 % (v/v) acetonitrile, developed in 40 min, at a flow rate of 1 ml/min. The products were freeze-dried to dryness, then re-freeze-dried several times from water. All polynucleotides were dissolved in 10 mM-Tris/HCl, pH 7.9.

Wheat-germ RNA polymerase II (essentially in the IIA form) was purified as described by Jendrisak & Burgess (1975) and by Teissère *et al.* (1990). Briefly, fraction 3 of Jendrisak & Burgess (1975) was purified by successive column chromatography on Fractogel TSK DEAE-650 (S) (Merck), Heparin-Ultrogel A4R (IBF), Fractogel TSK HW-55 (S) (Merck), Phospho-Ultrogel A6R (IBF), single-stranded DNA-Ultrogel A4R (IBF) and Mono Q HR 5/5 (f.p.l.c. system, Pharmacia). The enzyme preparation migrated as a single band on electrophoresis in native 7.5%-polyacrylamide gels and in native gels containing a continuous gradient of 8–25 % polyacrylamide. SDS/PAGE analyses disclosed the typical subunit pattern previously determined for wheat-germ RNA polymerase II (Jendrisak & Burgess, 1975; Jendrisak & Guilfoyle, 1978; Teissère *et al.*, 1990). Therefore the enzyme preparation is not contaminated by the other types of nuclear RNA polymerases. The specific activity

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of the enzyme was of the order of 2000 units/mg on poly[d(A-T)] as template and with 20 μ M-ATP, 5 μ M-UTP and 1.5 mM-MnCl₂, 1 unit being equivalent to the incorporation of 10 pmol of UMP into poly[r(A-U)] in 15 min at 35 °C. Control experiments were also carried out by using a commercial preparation of wheat-germ RNA polymerase II, free of contaminating RNA polymerases I and III, as obtained from Epicentre Technologies (Madison, WI, U.S.A.).

Reaction assays

The reaction mixtures contained 15 nM wheat-germ RNA polymerase II, 5 μ g of DNA/ml, 1.5 mM-MnCl₂ or 15 mM-MgCl₂, 330 μ M dinucleoside monophosphate primer, and appropriate amounts of nucleotide(s) and of α -amanitin, in transcription buffer consisting of 64 mM-Tris/HCl (pH 7.8), 12.5% (v/v) glycerol, 12.5 mM-2-mercaptoethanol, 5 mM- α -thioglycerol, 1.1 mM-dithiothreitol, 0.05 mM-EDTA, 0.05% Triton X-100 and 1.5 mM-NaF.

The protocol used for all reactions included a first 5 min incubation at 35 °C of all components, except the triphosphate substrates, allowing all components to equilibrate at that temperature. Then RNA synthesis was initiated by addition of labelled and unlabelled substrates, and assays were usually incubated for 100 min at 35 °C. Reactions were stopped by addition of 3 vol. of stop solution [1 mM-EDTA/80% (v/v) formamide/0.1% (w/v) xylene cyanol].

Activity measurements

Total RNA synthesis was measured by use of trichloroacetic acid precipitation of 10 μ l (reaction mixtures + stop solution) spotted on Whatman GF/C filters. After eight washing steps in cold 5% trichloroacetic acid containing 0.04 M-sodium pyrophosphate and two washing steps in ethanol for 5 min each, the filters were dried and the radioactivity was measured in a liquid-scintillation counter (Job *et al.*, 1984; Corda *et al.*, 1991). Transcription products were also analysed by high-resolution PAGE using 20% polyacrylamide [19:1 (w/w) acrylamide/bisacrylamide ratio]/8 M-urea/50 mM-Tris/borate (pH 8.0)/1 mM-EDTA gels (30 cm $\times$ 40 cm $\times$ 0.03 cm) (Maniatis *et al.*, 1982). Electrophoresis was conducted at a constant 2000 V until the marker dye, xylene cyanol, had migrated 10 cm. After electrophoresis, gels were covered with Saran wrap and exposed to Hyperfilm-MP films (Amersham) at -80 °C with a Cronex Li-Plus intensifying screen from DuPont. Labelling of (dC)_n chains with [γ -³²P]ATP and phage T4 polynucleotide kinase was performed as described (Maniatis *et al.*, 1982).

To evaluate the sensitivity of the synthesized RNA to various RNAases, RNA synthesis was carried out for 100 min at 35 °C, as described above. For RNAase H (which degrades specifically RNA-DNA hybrids), 10 μ l samples of these reaction assays were mixed with 30 μ l of a solution consisting of 40 mM-Tris/HCl (pH 7.8), 12 mM-NaCl, 18 mM-MgCl₂ or 8 mM-MnCl₂ and RNAase H (Job *et al.*, 1988). Digestion with RNAase U1 (an endonuclease that splits only the phosphodiester bonds of guanosine 3'-phosphates in single-stranded RNA) was performed as described by Arima *et al.* (1968). Incubations with RNAase H (1 unit) and RNAase U1 (1 unit) were for 1 h at 35 °C; then the reaction mixtures were analysed by trichloroacetic acid precipitation and high-resolution PAGE, as described above.

RESULTS AND DISCUSSION

Effect of α -amanitin on RNA synthesis

Fig. 1 shows the time course of typical experiments leading to RNA synthesis, with poly(dC) (Fig. 1a) or poly[d(A-T)] (Fig. 1b) as template. With poly(dC) as the template and GTP as the

substrate, about 30% of poly(rG) synthesis is resistant to inhibition by 100 μ g of α -amanitin/ml (Fig. 1a). Under these conditions, the extent of GMP incorporation into RNA considerably exceeds the amount of enzyme in the reaction assay, indicating that the α -amanitin-resistant reaction involves a catalytic reaction of nucleotide incorporation into RNA. For example, at 15 nM enzyme (0.15 pmol of enzyme/10 μ l of reaction mixture), the extent of RNA synthesis is 29.5 pmol of GMP

Fig. 1. Progress curves for RNA synthesis

Reactions were performed as described in the Materials and methods section, with 15 nM wheat-germ RNA polymerase II, 5 μ g of DNA template/ml, 1.5 mM-MnCl₂, 330 μ M-dinucleoside monophosphate primer, in the absence (●) or presence (○) of 100 μ g of α -amanitin/ml. After a 5 min preincubation at 35 °C, the reaction of labelled RNA synthesis was initiated by adding ³H-labelled triphosphate substrates. Levels of incorporation are expressed for 10 μ l portions of reaction mixtures. Conditions: (a) poly(rG) synthesis with poly(dC) as template, GpG as primer, and 10 μ M-[³H]GTP (1 pmol corresponded to 322 c.p.m.) as substrate; (b) poly[r(A-U)] synthesis with poly[d(A-T)] as template, UpA as primer, 1 μ M-[³H]UTP (1 pmol corresponded to 5250 c.p.m.) and 10 μ M-ATP as substrates.

Table 1. Reaction requirements for α -amanitin-resistant poly(rG) synthesis by wheat-germ RNA polymerase II with poly(dC) and poly(dC)·poly(dG) templates

Reactions were performed as described in the Materials and methods section, in the presence of 15 nM wheat-germ RNA polymerase II, 20 μ g of α -amanitin/ml, 5 μ g of DNA template/ml, 1.5 mM-MnCl₂ or 15 mM-MgCl₂, and 330 μ M-GpG primer. RNA synthesis was initiated by adding 10 μ M-[α -³²P]GTP (1 pmol corresponded to 2241 c.p.m.) or 1 μ M-[α -³²P]GTP (1 pmol corresponded to 21645 c.p.m.) to the assays containing poly(dC) or poly(dC)·poly(dG) as the template respectively. Levels of incorporation are expressed for 100 min incubations at 35 °C and for 10 μ l portions of reaction mixtures.

Template	Conditions for transcription assays	GMP incorporation (pmol)
poly(dC)	Complete assay	23.2
	minus enzyme	0.012
	minus template	0.02
	minus GpG primer	1.92
	minus Mn ²⁺	0.008
	minus Mn ²⁺ plus Mg ²⁺	3.5
poly(dC)·poly(dG)	Complete assay	0.64
	minus enzyme	0.003
	minus template	0.005
	minus GpG primer	0.16
	minus Mn ²⁺	0.003
	minus Mn ²⁺ plus Mg ²⁺	0.15

Fig. 2. Effect of α -amanitin on RNA synthesis

Reactions were run as those in Fig. 1 and as described in the Materials and methods section, with 15 nM wheat-germ RNA polymerase II, 5 μ g of DNA template/ml, 1.5 mM-MnCl₂, 330 μ M-dinucleoside monophosphate primer, and various concentrations of α -amanitin (0–1 mg/ml). After a 5 min preincubation at 35 °C, the reaction of labelled RNA synthesis was initiated by adding ³H-labelled triphosphate substrates. After incubation for 100 min at 35 °C, samples (10 μ l) were analysed and results (v) were normalized to the values in the absence of α -amanitin (V_0). Conditions: ●, poly(rG) synthesis with poly(dC) as template, GpG as primer, and 10 μ M-[³H]GTP as substrate [V_0 = 79 pmol of GMP incorporated into poly(rG)]; ○, poly[r(A-U)] synthesis with poly[d(A-T)] as template, UpA as primer, 1 μ M-[³H]UTP and 10 μ M-ATP as substrates [V_0 = 20.4 pmol of (AMP + UMP) incorporated into poly[r(A-U)]].

incorporated after incubation for 100 min at 35 °C for the assay carried out in the presence of 100 μ g of α -amanitin/ml. Yet, under the same experimental conditions, poly[r(A-U)] synthesis is totally inhibited (Fig. 1b). Similar results to those presented in Fig. 1(a) were obtained in the presence of poly(dC)·poly(dG) as template and GTP as substrate: here about 33% of poly(rG) synthesis resisted inhibition by 20 μ g of α -amanitin/ml. In contrast, when poly(dT) or poly(dA)·poly(dT) was transcribed in the presence of 10 μ M-ATP, over 98% inhibition of poly(rA) synthesis was obtained at 10 and 20 μ g of α -amanitin/ml (results not shown). These results show that α -amanitin-resistant RNA synthesis by wheat-germ RNA polymerase II displays a marked base-sequence specificity.

Reaction requirements

We characterized the reaction requirements for the synthesis of poly(rG) directed by the poly(dC) and poly(dC)·poly(dG) templates in the presence of excess α -amanitin. The results summarized in Table 1 show that the reaction is strictly dependent on the presence of enzyme, DNA template and bivalent cation, Mn²⁺ being a more effective bivalent metal cofactor than Mg²⁺. The presence of GpG dinucleoside monophosphate primer favours the reaction, indicating that free 3'-hydroxyl groups of the template molecules do not prime the α -amanitin-resistant RNA synthesis (Lewis & Burgess, 1980).

The influence of α -amanitin concentration on RNA synthesis was assessed by determination of the apparent inhibition constant, with poly[d(A-T)] and poly(dC) as templates. Fig. 2 shows plots of velocity (v) of RNA synthesis versus [α -amanitin] with data normalized to the corresponding V_0 values determined in the absence of α -amanitin. As observed in Fig. 1, poly[r(A-U)] synthesis is totally inhibited, whereas poly(rG) synthesis is

decreased but not completely suppressed, even at 1 mg of α -amanitin/ml. Likewise, the same pattern of behaviour as that presented in Fig. 2 was obtained by using a commercial preparation of wheat-germ RNA polymerase II (results not shown). The data in Fig. 2 also show that the value of the inhibition constant is not significantly different when poly[d(A-T)] or poly(dC) is used as template: in both cases, half-maximal inhibition is obtained at about 0.02 μ g of α -amanitin/ml, in agreement with previous determinations for plant RNA polymerases II (Jendrisak & Burgess, 1975; Jendrisak & Guilfoyle, 1978). The fact that poly[r(A-U)] synthesis is totally inhibited at a concentration of 1 μ g of α -amanitin/ml (Fig. 2) precludes the possibility that the enzyme preparation is contaminated by RNA polymerases I and III. Moreover, RNA synthesis by the wheat-germ enzyme was totally insensitive to 200 μ g of rifampicin/ml and to 10 μ M-taigetoxin (results not shown). The latter compound inhibits RNA synthesis directed by RNA polymerases from chloroplasts and *Escherichia coli* (Mathews & Durbin, 1990) and by RNA polymerase III (Steinberg *et al.*, 1990). We conclude that wheat-germ RNA polymerase II can catalyse a DNA-dependent reaction of RNA synthesis in the presence of excess α -amanitin, though to a smaller extent than in the absence of the fungal toxin.

Size distribution of transcription products

We characterized the size distributions of poly(rG) products by using oligo(dC) templates of defined lengths, referred to as (dC)_{*n*} (*n* = 10, 20, 30, 40, 50 or 60), in transcription assays conducted as described in the Materials and methods section, in the absence and presence of 20 μ g of α -amanitin/ml and with 10 μ M-[α -³²P]GTP as the labelled triphosphate substrate. After incubation for 100 min at 35 °C, the reactions were stopped and analysed by high-resolution urea/PAGE. (dC)_{*n*} oligomers, labelled with phage T₄ polynucleotide kinase and [γ -³²P]ATP, were used as size markers for these product length analyses.

For the assays carried out in the absence of α -amanitin, the autoradiograph in Fig. 3 shows that, whereas a chain length of 10 as in (dC)₁₀ proved too small to be an effective template for wheat-germ RNA polymerase II, all of the other oligomers, (dC)₂₀, (dC)₃₀, (dC)₄₀, (dC)₅₀ and (dC)₆₀, supported RNA synthesis. Template efficiency increases with template length: from a comparison of the intensities of the various bands in lanes 5 and 8 of the autoradiograph in Fig. 3, it is clear that (dC)₃₀ is a better template than (dC)₂₀, consistent with the fact that wheat-germ RNA polymerase II requires a stretch of about 30–40 bases in order to bind to DNA (Chandler & Gralla, 1980). In this respect the plant enzyme behaves similarly to *E. coli* RNA polymerase, although the bacterial enzyme appears capable of using homopolymer templates of smaller size than does wheat-germ RNA polymerase II. Thus Falaschi *et al.* (1963) reported that (dC)₉ and (dC)₁₀ exhibit significant template activity with the bacterial enzyme. Similarly, oligomers as small as (dT)₅ (Falaschi *et al.*, 1963) or (A)₈ (Hayes *et al.*, 1967) can serve as templates for RNA synthesis by *E. coli* RNA polymerase. Fig. 3 shows that RNA synthesis directed by (dC)₂₀, (dC)₃₀, (dC)₄₀, (dC)₅₀ and (dC)₆₀ is distributed as a ladder of species of increasing lengths, with all of the transcription products appearing substantially longer than the templates (compare lanes 5, 8, 11, 14, 17 and lanes 4, 7, 10, 13 and 16 in Fig. 3). In addition, the migration profiles for the different reaction mixtures exhibit a striking dependence of the product length on the template length: increasing the template length results in an increased length of the transcription product.

To understand better the structure of the various transcription products, we carried out the following experiments. When the radioactive products were submitted to alkaline hydrolysis in

Fig. 3. Product size distributions in transcription of $(dC)_n$ templates

Reactions were performed as described in the Materials and methods section, with 15 nM wheat-germ RNA polymerase II, 5 μ g of $(dC)_n$ template/ml, 1.5 mM-MnCl₂, 330 μ M-GpG primer, in the absence or presence of 20 μ g of α -amanitin/ml. After a 5 min incubation at 35 °C, labelled RNA synthesis was initiated by adding 10 μ M-[α -³²P]GTP. After 100 min incubation at 35 °C, 5 μ l samples (reaction mixtures + stop solution) were analysed by urea/PAGE. Key: OR, origin of the polyacrylamide gel; unin., unincorporated nucleotides. Conditions: lanes 2, 5, 8, 11, 14, 17, complete with $(dC)_{10}$, $(dC)_{20}$, $(dC)_{30}$, $(dC)_{40}$, $(dC)_{50}$ and $(dC)_{60}$ as the template, respectively; lanes 3, 6, 9, 12, 15, 18, as for lanes 2, 5, 8, 11, 14, 17 respectively, except that the reaction mixtures contained 20 μ g of α -amanitin/ml; lanes 1, 4, 7, 10, 13, 16, oligomers $(dC)_{10}$, $(dC)_{20}$, $(dC)_{30}$, $(dC)_{40}$, $(dC)_{50}$, and $(dC)_{60}$, labelled with [γ -³²P]ATP and phage T4 polynucleotide kinase respectively.

either 0.1 M-KOH for 20 min at 100 °C or 0.3 M-KOH for 20 h at 35 °C, the amount of trichloroacetic acid-precipitable radioactivity decreased to background values; moreover, the ladders of radioactive transcription products shown in Fig. 3 (lanes 5, 8, 11, 14 and 17) were not detected upon urea/PAGE analysis of the hydrolysis products. This confirmed that the labelled transcription products do not correspond to DNA chains labelled with [α -³²P]GTP. We also observed that the radioactive transcription products synthesized with the $(dC)_n$, poly(dC) and poly(dC)·poly(dG) templates resisted the action of RNAase U₁, by using the experimental procedures described by Arima *et al.* (1968). This behaviour suggested that the RNA product remains associated with the template strand, as previously reported by Dez *et al.* (1974) for poly(rG) synthesis catalysed by yeast RNA polymerase II with a poly(dC) template. The experiments

in Fig. 4(a) further illustrate this finding. Two identical reaction assays were prepared in the presence of enzyme, Mn²⁺ and GpG. Before incubation for 100 min at 35 °C, one of them received unlabelled $(dC)_{40}$ as template and [α -³²P]GTP as substrate, while the other received ³²P-labelled $(dC)_{40}$ as template and unlabelled GTP as substrate. Urea/PAGE analysis of these reaction mixtures (lanes 2 and 3 of Fig. 4a) clearly shows that the RNA synthesized is annealed to the template strand, as, with both types of transcription assays, the radioactive transcription products display the same electrophoretic mobilities and the same retardation of gel mobility compared with ³²P-labelled $(dC)_{40}$. Furthermore, transcription of the $(dC)_{40}$ template under the experimental conditions of lane 2 in Fig. 4(a) gave rise to a product that was fully sensitive to RNAase H (results not shown). In agreement with this finding, Fig. 4(b) shows the sensitivity to RNAase H of transcription products synthesized under the conditions of Fig. 4(a) (lane 3), i.e. in the presence of enzyme, ³²P-labelled $(dC)_{40}$ as template and unlabelled GTP as substrate (Fig. 4b, lanes 2 and 4). Clearly, after RNAase digestion in the presence of either Mn²⁺ (Fig. 4b, lane 3) or Mg²⁺ (Fig. 4b, lanes 5), the labelled template recovers its original electrophoretic mobility (compare lanes 1, 3, 5 and 6 of Fig. 4b), supporting the findings of Dez *et al.* (1974) for yeast RNA polymerase II.

Concerning α -amanitin sensitivity, as with the poly(dC) polymer template, the fungal toxin does not completely inhibit RNA synthesis directed by the oligomers $(dC)_{20}$, $(dC)_{30}$, $(dC)_{40}$, $(dC)_{50}$ and $(dC)_{60}$, even at a concentration of 20 μ g/ml (Fig. 3, lanes 6, 9, 12, 15 and 18). Furthermore, the transcription products obtained from assays incubated in the presence of excess of the amatoxin are longer than the template molecules, although the patterns of product-size distributions are somewhat simpler in the presence than in the absence of α -amanitin. Similar results were obtained with incubations in 15 mM-MgCl₂ (results not shown).

A slippage model to account for α -amanitin-resistant poly(rG) synthesis

Previous experiments showed that, with a poly[d(A-T)] template, the single-step addition reaction of UTP substrate to UpA dinucleoside monophosphate primer by wheat-germ RNA polymerase II (de Mercoyrol *et al.*, 1989) and calf thymus RNA polymerase II (Vaisius & Wieland, 1982) resists inhibition by excess α -amanitin. Nevertheless, under the same experimental conditions, but in the additional presence of ATP, the synthesis of poly[r(A-U)] was completely abolished, a finding that is consistent with the results in Fig. 1(b). From these results, we (de Mercoyrol *et al.*, 1989) and others (Vaisius & Wieland, 1982) have proposed that the main enzymic process disrupted by α -amanitin in RNA synthesis by RNA polymerases II is the translocation step of the transcriptional complexes along the DNA template. According to this model, we can attribute the observed α -amanitin-resistant poly(rG) synthesis by wheat-germ RNA polymerase II to a polymerization mode that does not require translocation of the transcriptional complex after each extension step. This suggests a slippage mechanism, as has been well documented for *E. coli* polymerase in the transcription of homopolymer templates (Chamberlin & Berg, 1962, 1964; Berg & Chamberlin, 1964). In this mechanism, the RNA polymerase undergoes a reiterative incorporation of a single ribonucleoside triphosphate, by repeated melting cycles of the RNA·DNA hybrid, slippage of the two strands relative to each other, rehybridization, and subsequent elongation at the 3' end of the growing RNA. Transcriptional slippage has also been shown to occur in non-enzymic template-directed synthesis of oligoguanylates catalysed by Mg²⁺ (Chen *et al.*, 1985). In that study, using $(dC)_5$, $(dC)_6$ and $(dC)_7$ as templates, Chen *et al.* (1985) observed

Fig. 4. Formation of DNA-RNA hybrids in transcription of (dC)₄₀

Reactions of transcription were run in the absence of α -amanitin as those in Fig. 3 (lane 11) and as described in the Materials and methods section. The template was (dC)₄₀. Assays were analysed by urea/PAGE. Key: OR, origin of the polyacrylamide gels; unin., unincorporated nucleotides. (a) Size distribution of transcription products. Conditions: lane 2, complete reaction mixture with (dC)₄₀ as template and 10 μ M-[γ -³²P]GTP as substrate; lane 3, as in lane 2, except that the assay contained (dC)₄₀ labelled with [γ -³²P]ATP and phage T4 polynucleotide kinase as template, and 10 μ M unlabelled GTP as substrate; lanes 1 and 4, (dC)₄₀ labelled with [γ -³²P]ATP and phage T4 polynucleotide kinase. Conditions: lanes 2 and 4, as for lane 3 in (a); lanes 3 and 5, reaction assays conducted as those shown in lanes 2 and 4 received 1 unit of RNAase H for 60 min in the presence of 6 mM-MnCl₂ and 12 mM-MgCl₂ respectively; lanes 1 and 6, (dC)₄₀ labelled with [γ -³²P]ATP and phage T4 polynucleotide kinase. (c) Influence of template concentration on product-size distributions. The template was (dC)₄₀. Transcription was carried out in the absence of α -amanitin, as for lane 11 in Fig. 3, except that the template concentration was 0.13, 0.43, 1.48 and 5 μ g/ml for lanes 2-5, respectively; lanes 1 and 6, (dC)₄₀ labelled with [γ -³²P]ATP and phage T4 polynucleotide kinase.

the synthesis of a series of reaction products corresponding to oligomers from $n+2$ to $2n+1$ in length, where n is the length of the template. To account for this peculiar feature, these authors proposed a sliding mechanism for polymerization, and suggested that once the number of G bases incorporated into nascent RNA exceeds the number of dC bases present in the template, dangling G bases are produced, which may then bridge two templates; then synthesis continues on the 'double template', as it occurred on a single template. If this holds true for wheat-germ RNA polymerase II, a tentative explanation for the complex product-length distributions observed in transcription of the (dC)_{*n*} templates (Fig. 3) might be that, owing to a bridging process, the reaction products correspond to DNA-RNA hybrids containing different amounts of RNA transcript and template molecules. One expects from such a mechanism that the bridging phenomenon would be favoured upon increasing the free DNA concentration. Consistent with this mechanism, Fig. 4(c) shows that the higher the free template concentration the more complex the distribution pattern of transcription products. The pattern is disrupted by RNAase H treatment, freeing the single-stranded DNA (Fig. 4b). In addition, the presence of multi-helical complexes in the transcription products cannot be excluded. Poly(G) has a strong propensity for forming double- and quadruple-helical structures, depending on ionic conditions [for a review, see Saenger (1984)]. For example, recent experiments showed that a RNA oligonucleotide containing a terminal

GGGG sequence forms a highly stable tetrameric aggregate that survives largely intact in polyacrylamide gels containing 8 M-urea (Kim *et al.*, 1991).

Conclusion

The results presented in this paper demonstrate that the inhibition pattern of wheat-germ RNA polymerase II by α -amanitin exhibits distinct features, depending on the sequence of the template polymer. We found that when poly(dC) and poly(dC)·poly(dG) are used as templates, a substantial proportion of poly(rG) synthesis resists inhibition by a high concentration of α -amanitin (1 mg/ml). Otherwise, under these experimental conditions, other synthetic polymers, such as alternating poly[d(A-T)], or homopolymers, such as poly(dT) or poly(dT)·poly(dA), do not support RNA synthesis, in agreement with well-characterized biochemical properties of class II RNA polymerases (Sentenac, 1985). We suggest that the observed behaviour of wheat-germ RNA polymerase II is related to slippage events promoted by templates containing a homopolymeric stretch of dC bases. This suggestion is supported by previous results showing that α -amanitin does not prevent phosphodiester bond formation by class II RNA polymerases, and interferes with the translocation step (Vaisius & Wieland, 1982; de Mercoyrol *et al.*, 1989).

Reiterative copying has also been reported for other DNA-dependent RNA polymerases, such as *E. coli* RNA polymerase

(Chamberlin & Berg, 1962, 1964; Berg & Chamberlin, 1964; Heyden *et al.*, 1975; Nusslein & Schaller, 1975; Machida *et al.*, 1984; Kassavetis *et al.*, 1986), phage T7 RNA polymerase (Martin *et al.*, 1988) and yeast RNA polymerase B (II) (Dezélée & Sentenac, 1973). This suggests that reiterative copying is a common feature of transcription by RNA polymerases of all organisms. For prokaryotic systems, there has been renewed interest in transcriptional slippage, since this peculiar synthetic mode is not restricted only to the case of artificial single-stranded homopolymer templates, but may also occur during initiation and elongation of RNA synthesis directed by natural double-stranded templates, provided that the latter contain a homonucleotide stretch (Guo & Roberts, 1990; Harley *et al.*, 1990; Wagner *et al.*, 1990).

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