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Faithful In Vitro Transcription by Fission Yeast RNA Polymerase III Reveals Unique α-Amanitin Sensitivity

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Transcription with fission yeast (*Schizosaccharomyces pombe*) RNA polymerase III (pol III) was studied in two different in vitro systems. Reactions performed with isolated nuclei gave rise to 5S and pre-tRNA molecules. Because the α-amanitin sensitivity of that reaction clearly differed from what has been observed with pol III enzymes of other eukaryotes, a cell-free *S. pombe* transcription extract was developed and analyzed with the homologous 7S L RNA (srp RNA; signal recognition particle RNA) gene. Synthesis of 7S L RNA was found to be sensitive to high concentrations of α-amanitin, with 50% reduction seen at 400 μg/ml of the toxin. However, even with very high α-amanitin concentrations, exceeding 1 mg/ml, no full inhibition of the *S. pombe* pol III enzyme could be obtained. Together, these results demonstrate that in contrast to the yeast *Saccharomyces cerevisiae*, pol III from *S. pombe* is sensitive to high concentrations of α-amanitin, yet with a clearly different dose response than that observed with the corresponding RNA polymerase of higher eukaryotes. Furthermore, while the *S. pombe* 7S L RNA gene was efficiently transcribed in HeLa cell extracts, the human 7S L RNA gene was not actively transcribed in the *S. pombe* system. This finding of divergent promoter structures of both genes was verified by the analysis of 5′ deletion mutants of the *S. pombe* 7S L RNA gene.

Schizosaccharomyces pombe Cell-free extract RNA polymerase III transcription
α-Amanitin sensitivity 75 L RNA

EUKARYOTIC cells contain three distinct RNA polymerases, each responsible for the transcription of specific subclasses of nuclear genes [reviewed in (25)]. These three RNA polymerases can be differentiated with respect to their sensitivity towards the fungal toxin α-amanitin (3). RNA polymerase I is totally resistant against this inhibitor, whereas RNA polymerase II has been found to be extremely sensitive. In addition, RNA polymerase III reveals an intermediate sensitivity towards α-amanitin with total inhibition only at higher concentrations (150 μg/ml) (18). The latter enzyme is responsible for the synthesis of a number of low molecular weight RNA species [reviewed in (6)], including 5S and tRNA. In contrast to those other two transcription systems, pol III genes (genes transcribed by RNA polymerase III) reveal a remarkable heterogeneity in promotor structures that in turn have been grouped into four different classes (23). In addition, a recent study revealed an interesting switch in pol III promoter class during evolution of the 7S L RNA gene from *Arabidopsis thaliana* (7). This heterogeneity of the pol III transcription system was also reflected by the finding that RNA polymerase III from yeast (*S. cerevisiae*) was found resistant to α-amanitin (15,20). In contrast, pol III enzymes of a variety of higher eukaryotes revealed the same intermediate sensitivity towards α-amanitin as that originally described by Seifart et al. (18) for the rat liver enzyme.

In the present work we have studied in vitro transcription by RNA polymerase III from the fission yeast *S. pombe*. By using either isolated nuclei or cell-free extracts and the homologous 7S L RNA gene, we show that, in accordance with other eukary-

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otic cells, RNA polymerase III of fission yeast is clearly sensitive towards α-amanitin, yet with different degrees of inhibition. This finding supports the notion that the genetic system of fission yeast more closely resembles that of higher eukaryotes than that of *S. cerevisiae*. Furthermore, the y7S L promoter structure, as analyzed here, clearly distinguishes the *S. pombe* 7S L RNA gene from its counterparts in plants and mammals.

MATERIALS AND METHODS

Templates and In Vitro Transcription

The *S. pombe* 7S L RNA gene with 1012 bp of upstream and 669 bp downstream sequences, respectively, was cloned from a genomic DNA fragment by the inverse polymerase chain reaction (13) and corresponds essentially to the sequences published previously (2,14).

In vitro transcription in HeLa cell S100 extracts (21) of the human 7S L RNA gene has been described in detail previously (1,9,19). Briefly, 0.5 μg of the human or the *S. pombe* 7S L plasmid DNA was transcribed in 10 μl of HeLa extract for 60 min at 37°C. After incubation with proteinase K (0.5 mg/ml with 0.5% SDS for 30 min at 37°C) and phenol extraction, transcripts were analyzed in denaturating (8 M urea) 6% polyacrylamide gels as described (4). Autoradiography of dried gels at −70°C was for 8 h using a Cronex intensifier screen.

In vitro transcription with 20 μl of *S. pombe* extracts was in the presence of 16 mM HEPES/KOH (pH 7.9), 70 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.6 mM each of ATP, CTP, and GTP, and 10 μCi of [α-^{32}P]UTP (800 Ci/mmol) in a total volume of 50 μl. Reactions were incubated for 1 h at 30°C, followed by proteinase K digestion (0.5 mg/ml in the presence of 0.5% SDS and 0.2 mg/ml of tRNA) for 30 min at 37°C. Transcripts were analyzed as above, except that exposure of the dried gels to X-ray films was for 16 h.

S. pombe *Cell Culture and Isolation of Nuclei*

S. pombe SA2 cells were grown in suspension at 30°C in YEL1/6 medium (0.5% yeast extract, 0.5% glucose) to $OD_{578} = 0.8–2.0$. For isolation of nuclei [all steps at room temperature (RT)], cells were harvested by centrifugation (4200 × *g*; 5 min), washed twice, and resuspended (1.4 M sorbitol) to $1–2 \times 10^9$ cells/ml. Spheroplasts, obtained by addition of Novozym (Sigma) to 2 mg/ml and incubation for 30 min at 30°C (5), were collected (2000 × *g*; 5 min), washed with SLP (1.2 M sorbitol, 50 mM Tris-acetate, pH 7.9, 10 mM $MgAc_2$), resuspended in the same buffer, and lysed with 0.5% Nonidet P40 and 20 strokes of a pestal A douncer. The nuclear suspension was cleared from debris by centrifugation (370 × *g*; 2 min) and nuclei collected for 15 min at 1950 × *g*, followed by resuspension (4×10^6 nuclei/μl) in SLP. In vitro transcription reactions (50 μl) were with 20 μl of nuclear suspension in the presence of 0.6 M sorbitol, 25 mM Tris-acetate (pH 7.9), 80 mM KCl, 5 mM $MgCl_2$, 0.8 mM each of ATP, GTP, and CTP, 10 μM of UTP, and 2.5 μCi of [α-^{32}P]UTP.

Preparation of S. pombe *S100 Extracts*

Cells were grown as above, harvested by centrifugation (3400 × *g*; 4 min; all steps at 4°C), and washed once with water and a second time with extraction buffer [100 mM HEPES/KOH (pH 7.9), 245 mM KCl, 5 mM EGTA, 1 mM EDTA, 2.5 mM DTT]. The cell pellet (1 g) was resuspended in 1.3 ml of extraction buffer containing protease inhibitors (0.2 mM PMSF, 3.5 μg/ml pepstatin A, 5 μg/ml leupeptin, 5 μg/ml antipain, 100 μg/ml chymostatin), recollected by centrifugation, and shock-frozen in liquid nitrogen. For extract preparation (12,17), frozen cells were broken under liquid nitrogen by extensive manual grinding in a porcelain mortar and pestel, followed by resuspension in extraction buffer (1.3 ml/g) containing protease inhibitors. After ultracentrifugation (100,000 × *g*; 2 h) the supernatant was dialyzed against 50 vol. of buffer D100 [20 mM HEPES/KOH (pH 7.9), 100 mM KCl, 5 mM EGTA, 0.05 mM EDTA, 2.5 mM DTT, 0.2 mM PMSF, and 0.1 μg/ml leupeptin], four times for 1 h each. Proteins precipitated during dialysis were removed by centrifugation (12,000 × *g*; 10 min) and final extracts (15–20 mg/ml protein) shock-frozen and stored in aliquots at −70°C.

RESULTS

In Vitro Transcription in Nuclei Isolated From S. pombe *Spheroplasts*

Because we were interested to study pol III transcription in the *S. pombe* system in vitro, we first decided to start with a more native system. For this, nuclei were isolated from *S. pombe* cells and used for in vitro transcription in the presence of increasing concentrations of α-amanitin. The results shown in Fig. 1 demonstrate that under optimized conditions a complex spectrum of transcripts is observed. Synthesis of all these RNA molecules was resistant to low concentrations of α-amanitin (compare lanes 1 and 2) but, on the other hand, was totally suppressed by 12 μg/ml of actinomycin D (lane 11), indicating that all products detected were indeed transcribed RNA. The

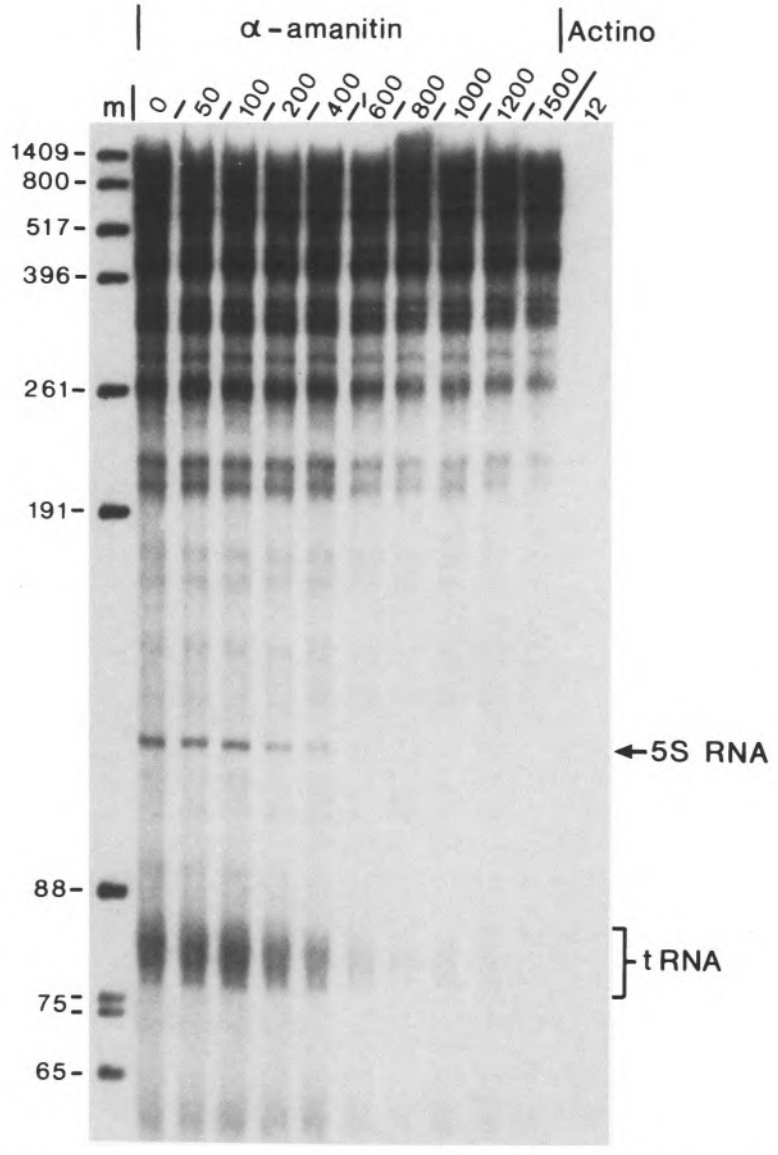


FIG. 1. In vitro transcription reactions performed with nuclei isolated from *S. pombe* spheroplasts. Reactions in a total volume of 50 µl were with 20 µl of nuclear suspension in presence of the α-amanitin concentrations indicated above each lane. The control in lane 11 contained no α-amanitin; instead, 12 µg/ml of actinomycin D (Actino) was applied. m = size marker DNA fragments.

vast majority of labeled molecules was larger than 200 nt in length. Very likely, these larger RNA transcripts represent immature ribosomal RNA products, because their synthesis is virtually resistant against α-amanitin. This conclusion is supported by the α-amanitin titration experiments described below and by the previously described very high sensitivity towards this inhibitor of pol II transcription in the *S. pombe* system (24).

However, two distinct classes of low molecular weight RNA species, designated as 5S and tRNA, were also obtained. Their spectrum revealed the well-established pattern of 5S and pre-tRNA synthesis observed, for example, with isolated HeLa cell nuclei (22,26). Furthermore, the position of the *S. pombe* 5S RNA was verified by Northern blot hybridization (data not shown). In contrast to most of the predominant high molecular weight RNA seen on top, synthesis of these known pol III transcripts (5S and tRNA) appeared sensitive to very high concentrations of α-amanitin, although no full suppression was obtained even at inhibitor concentrations exceeding 1 mg/ml.

Active pol III Transcription in S100 Extracts Isolated From S. pombe *Cells*

The inhibition by α-amanitin of pol III transcription in vitro within these *S. pombe* nuclei was divergent from that observed with both *S. cerevisiae* and

higher eukaryotes. Therefore, we wanted to know whether this unique α-amanitin sensitivity of the fission yeast pol III enzyme could also be observed in a more reconstituted system. For this, we have prepared an *S. pombe* whole-cell S100 extract system suitable for pol III-catalyzed transcription of the homologous 7S L RNA gene. As is shown in the left panel of Fig. 2, the S100 extract isolated as described under Materials and Methods supported active transcription of the *S. pombe* 7S L (y7S L) RNA gene in vitro. The size of transcripts observed corresponded exactly to the length of 254 nt of the *S. pombe* 7S L RNA (2,13,14). Furthermore, dialysis of the extract obtained after ultracentrifugation significantly increased the in vitro transcription efficiency (compare lanes 2–5 with 7–10). In addition, both extracts revealed a template optimum between 300 and 600 ng of plasmid DNA per assay. The significant inhibition of in vitro transcription by higher template concentrations of 1 μg and more (lanes 4, 5 and 9, 10) seems to be a 7S L RNA-specific phenomenon, because it has also been observed with the human 7S L RNA gene in HeLa S100 extracts (unpublished observation).

In Vitro Transcription of 7S L RNA Genes in Homologous and Heterologous Extracts

To verify the specificity of in vitro trancription observed with the *S. pombe* gene/extract system before, we performed comparative analyses with the human system. For this the human 7S L RNA gene (h7SL) (1,9,19) was transcribed in HeLa S100 extracts, in comparison with the *S. pombe* 7S L RNA gene (y7SL) described before. As is evident from a comparison of lanes 1 to 3 and 4 to 6 of Fig. 3A, respectively, transcription of both 7S L RNA genes is equally efficient in this extract sytem and reveals identical responses towards the specific inhibitor α-amanitin, that is, resistance against low concentrations (1 μg/ml) but full inhibition by high amounts (150 μg/ml) of the toxin. This result is clearly indicative for specific pol III transcription in vitro (3). The different lengths of the transcripts obtained in vitro exactly corresponded to the size of the authentic 7S L RNA species (h7SL = 299 nt; y7SL = 254 nt), respectively. However, a very different picture emerged, if both genes were transcribed in parallel in the *S. pombe* S 100 extract (Fig. 3B). First, no transcription at all could be observed with the h7SL RNA gene (lanes 1–3), indicating that the promoter structure of the human gene requires the presence of factors not available in the fission yeast extract. Furthermore, although a clear reduction could be observed, no full suppression of in vitro transcription of the yeast 7S L RNA gene was obtained by an α-amanitin concentration of 150 μg/ml. PhosphoImager quantification of the signal in lane 6 (not shown) re-

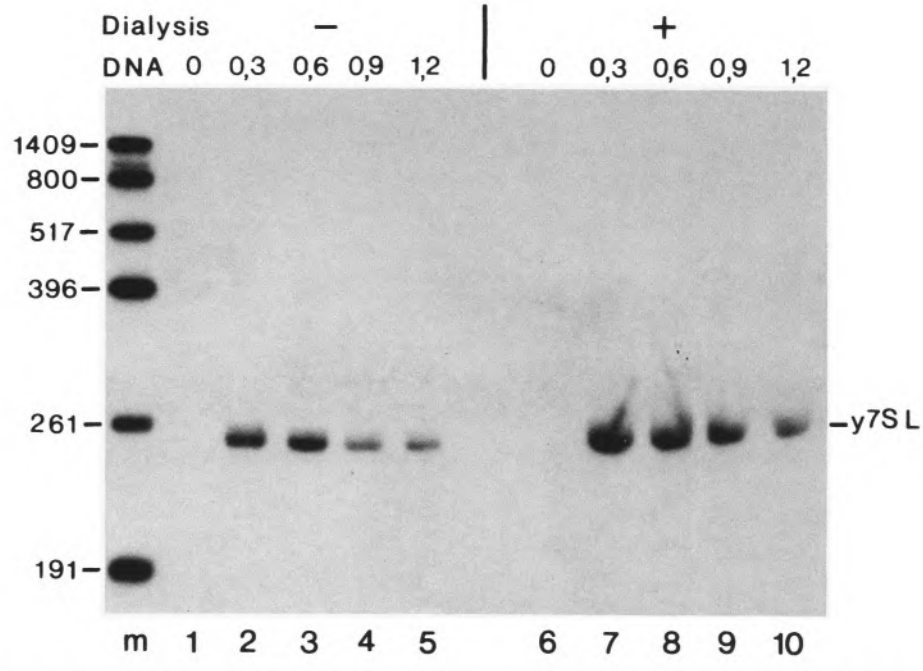


FIG. 2. In vitro transcription of the *S. pombe* 7S L RNA gene in homologous S100 extracts. Reactions with 20 μl of extract and varying amounts of plasmid DNA (μg indicated above each lane) were performed in the presence of 1 μg/ml of α-amanitin in a total volume of 50 μl. Prior to use, the extract was either not dialyzed (lanes 1–5) or dialyzed against buffer D100 (lanes 6–10), as described for the preparation of extracts. The analysis of transcripts was by electrophoresis in 6% polyacrylamide gels containing 8 M urea, as outlined under Materials and Methods. Autoradiography of the dried gel was for 16 h at –70°C, using a Cronex intensifier screen. y7SL = *S.pombe* 7S L RNA; m = marker fragments as in Fig. 1.

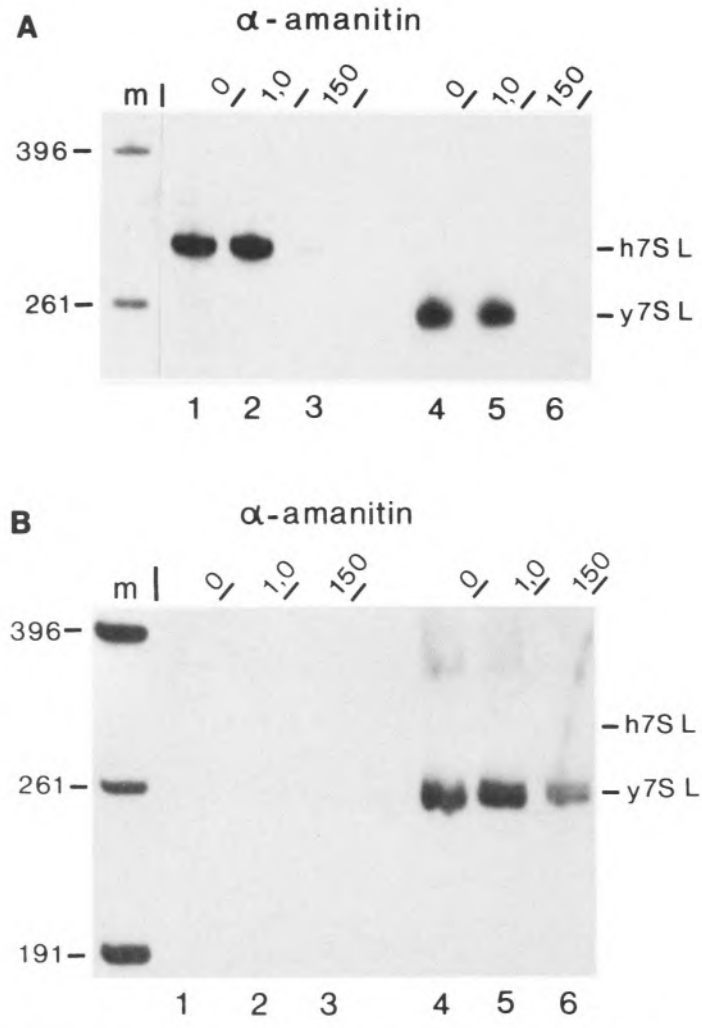


FIG. 3. In vitro transcription of 7S L RNA genes in homologous or heterologous extracts. (A) In vitro transcription in HeLa S100 extract (10 μl) was with 0.5 μg of plasmid DNA of the human 7S L RNA gene (h7SL; lanes 1–3) or the *S. pombe* 7S L RNA gene (y7SL; lanes 4–6) in the presence of varying amounts of α-amanitin as indicated above each lane. Analysis of transcripts by gel electrophoresis was as in Fig. 2. m = marker fragments. (B) Same analysis as in (A); however, in this case 20 μl of *S. pombe* extract was used for in vitro transcription.

vealed that the level of synthesis was reduced to 64% of the values obtained in the absence or presence of low amounts of α-amanitin (lanes 4 and 5), respectively. This result is in clear contrast to what was observed with the HeLa cell extracts in Fig. 3A, but is in full agreement with the results obtained for pol III transcription in isolated *S. pombe* nuclei (see Fig. 1).

Unique Dose Dependence of Inhibition of y7SL RNA Gene Transcription by Increasing Amounts of α-Amanitin

In view of the in vitro pol III transcription results obtained with isolated nuclei or S100 extracts from the *S. pombe* cells, a detailed titration assay was performed with increasing amounts of α-amanitin (Fig. 4). As also evident from the bands in the upper panels of Fig. 4, in vitro transcription reactions tend to reveal some variation between individual assays. Therefore, in addition to the two gel autoradiograms shown in Fig. 4, the mean values obtained by PhosphoImager quantification of those two separate experiments are summarized in the lower section of Fig. 4. As observed before, no inhibitory effect was obtained with low amounts (up to 50 μg/ml) of the inhibitor (Fig. 4, lane 2). Furthermore, at inhibitor concentrations of 150 μg/ml of α-amanitin, that are known to inactivate mammalian RNA polymerase III completely [(18); Fig. 3A], transcription of the y7S L gene in *S. pombe* extracts was only reduced by about 31.5% (lane 4). Further increase in the amount of inhibitor steadily reduced the pol III transcription capacity and it appeared that the inhibition curve be-

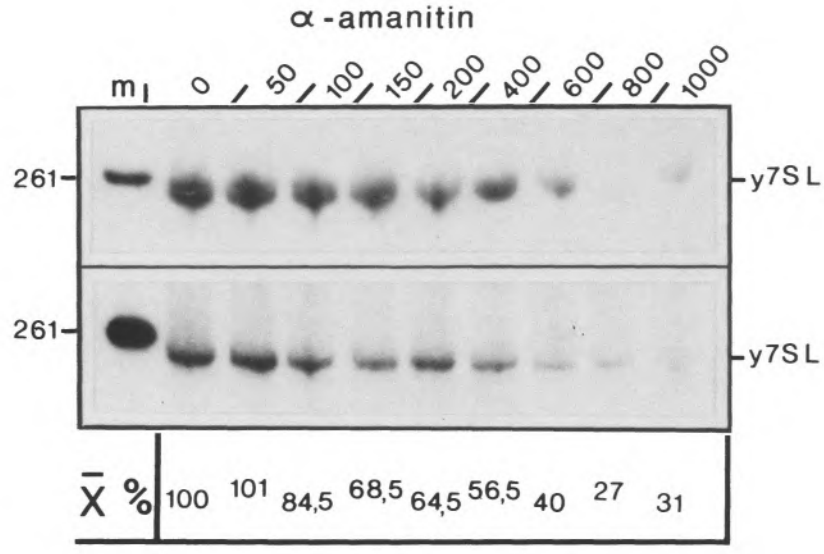
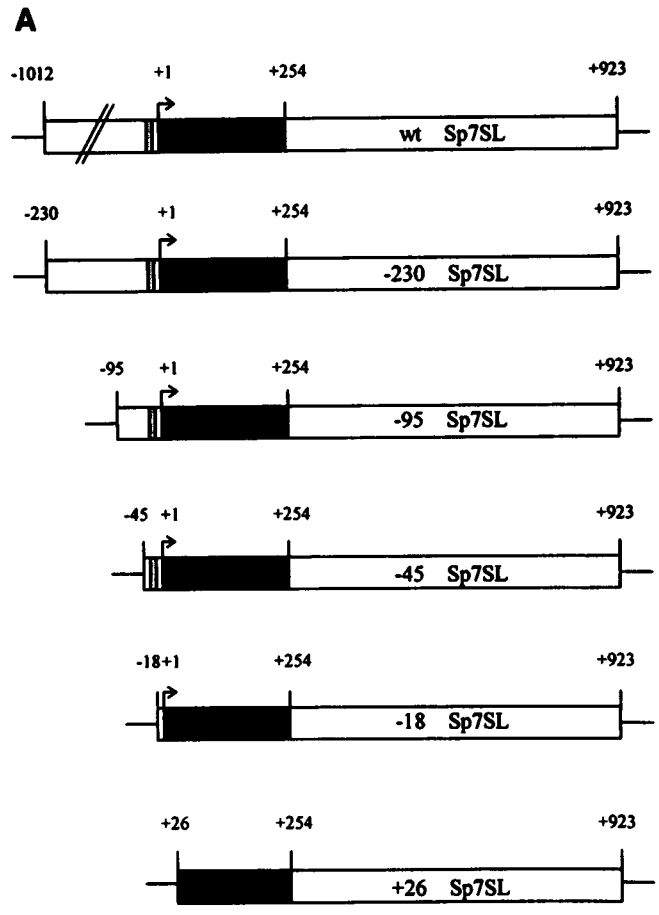


FIG. 4. Dose response to α-amanitin of 7S L RNA synthesis in *S. pombe* extracts. Upper panel: gel autoradiogram of two separate in vitro transcription experiments. Reactions in the presence of increasing amounts of α-amanitin (indicated on top) and analysis of labeled transcripts were as before. m = marker band of 261 nt in length. Lower panel: the intensity of the signals (y7SL) on top was quantified using a Fuji PhosphoImager (program TINA 2.09) and the mean values (X) of both analyses expressed as relative activity (%), in comparison to the minus α-amanitin controls (100%).

gan to level off at α-amanitin concentrations approaching 1 mg/ml (Fig. 4, lanes 8 and 9). Indeed, even inhibitor concentrations exceeding 1.5 mg/ml allowed low, albeit clearly detectable, in vitro transcription activities of 10–15% (data not shown). Together with an apparent 50% value for *S. pombe* pol III transcription between 400 and 600 μg/ml of α-amanitin, these titration curves clearly differentiate the fission yeast RNA polymerase III from the corresponding enzymes of both *S. cerevisiae* and vertebrates. In contrast to the situation in *S. pombe*, RNA polymerase III from *S. cerevisiae* was found to be resistant against α-amanitin while the mammalian enzyme can be fully inactivated and at clearly lower concentrations than those applied in the last lanes of Fig. 4.

Analysis of Promoter Mutants of the S. pombe *7S L RNA Gene*

The previous observation that the *S. pombe* 7S L RNA gene was efficiently transcribed in extracts isolated from HeLa cells but, vice versa, not the human gene in *S. pombe* extracts seemed to indicate that both genes reflect a different promoter structure. Because at least the gene external part of the human 7S L promoter has been analyzed in great detail (1,19), a series of 5′ deletion mutants of the y7SL gene was generated. A schematic presentation of these constructs in comparison to the wild-type clone is shown in Fig. 5A. Of these deletion mutants, 500 ng was analyzed by in vitro transcription (Fig. 6), in both the heterologous (human) and the homologous (yeast) extract systems. The results obtained by in vitro transcription in HeLa S100 extracts revealed that no essential promoter element is located between position −1012 and −45 of the *S. pombe* 7S L RNA gene (Fig. 6A). In fact, compared to the wild-type gene, in these extracts a slight increase in template activity was reproducibly observed with the −95 (plus 10%) and the −45 (plus 32%) mutants, possibly indicating that same inhibitory element be present further upstream. However, further deletion of 5′ sequences down to position −18 resulted in a significant decrease in transcriptional activity. Quantification of three individual in vitro transcription experiments (including the one presented in Fig. 6A) gave a mean value of 55% (±9) for the −18 deletion mutant. Furthermore, that clone constantly revealed a quite heterogenous pattern of transcripts, indicating that the specificity of the transcription initiation reaction was affected and that additional minor start sites were utilized. This interpretation is in good agreement with the fact that, by deleting the sequences between −45 and −18, a canonical TATA-box (TATATAAT) was removed that is located around position −28 of the gene (Fig. 5B). Surprisingly, however, further 5′ deletion into the transcribed sequence of the gene (down to position +26) resulted in a pronounced recovery of template activity (95% compared, for example, to the −45 construct). In addition, this recovery was accompanied by an almost complete loss of use of the cryptic start



B

	-41	-31	-21	-11	-1
-230:	GAATTAACTT	ATTTATTTTC	TATATAATTT	ATTAATACAT	ATATGTATTC
-95:	GAATTAACTT	ATTTATTTTC	TATATAATTT	ATTAATACAT	ATATGTATTC
-45:	AGCACAACTT	ATTTATTTTC	TATATAATTT	ATTAATACAT	ATATGTATTC
-18:	TTGGAGCTCC	ACCGCCGTGG	CGGCCGCTCT	AGTAATACAT	ATATGTATTC
+26:	AAACGACGGC	CAGTGAGCGC	GCGTAATACG	ACTCACTATA	GGGCGAATTG

	+1	+10	+20	+30	+40	+50
-230:	GCTGTAATGG		CTTGGTCGAA	GTGTTTAGTA	CTCCCAATAG	TGCATGTTCG
-95:	GCTGTAATGG		CTTGGTCGAA	GTGTTTAGTA	CTCCCAATAG	TGCATGTTCG
-45:	GCTGTAATGG		CTTGGTCGAA	GTGTTTAGTA	CTCCCAATAG	TGCATGTTCG
-18:	GCTGTAATGG		CTTGGTCGAA	GTGTTTAGTA	CTCCCAATAG	TGCATGTTCG
+26:	GAGCTCCACC		GCGGTGGCGG	CCGCTCAGTA	CTCCCAATAG	TGCATGTTCG

FIG. 5. Schematic presentation of 5′ deletion mutants of the *S. pombe* 7S L RNA gene. (A) The filled box depicts the transcribed region of the wt gene, with positions +1 and +254 indicating the transcription start site and the termination signal, respectively. The small open box upstream of +1 (see upper four constructs) represents the TATA element located around position −28 of the gene. Sp7SL = *S.pombe* 7S L RNA gene. (B) Comparison of sequences around the transcription start site (+1) of the five 5′ deletion constructs of part A. Wild-type y7SL RNA gene sequences are given in bold and putative TATA elements are underlined.

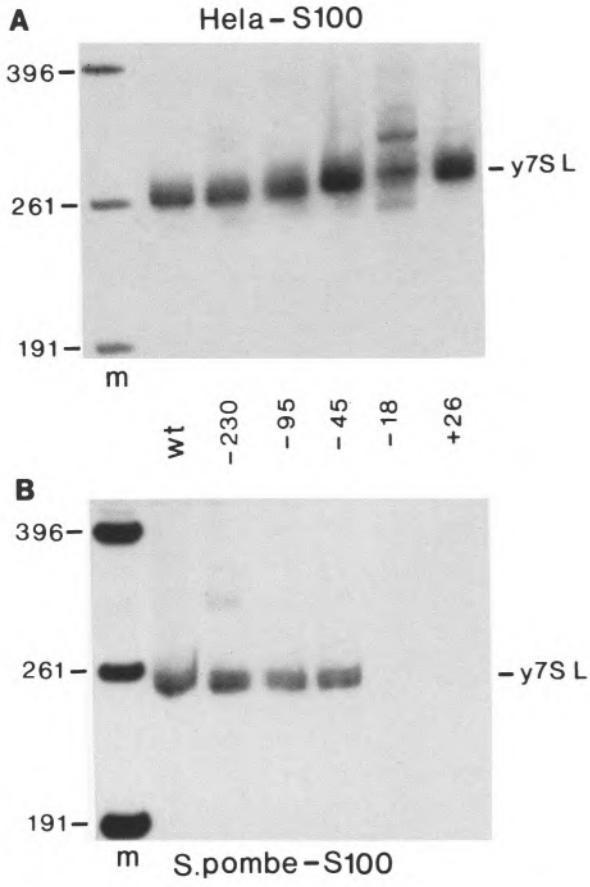


FIG. 6. Analysis of 5′ deletion mutants by in vitro transcription. (A) 0.5 μg of plasmid DNA of the 5′ deletion constructs shown in Fig. 5 were each transcribed in 10 μl of HeLa extract and the RNA (y7SL) analyzed as before. (B) Same as (A); however, in this case in vitro transcription was with 20 μl of *S. pombe* S100 extract.

sites, observed with the −18 deletion construct before. Very likely, these unexpected results are explained by the structure of the vector sequences brought into the vicinity of the truncated gene during clone construction. Indeed, a close inspection of the +26 construct revealed an A/T-rich sequence (TAATA) of the multiple cloning site that was located at position −27 again (Fig. 5B), as extrapolated from the transcribed sequence of the 7S L RNA gene. We conclude that this TATA-like vector element of the construct somehow is able to replace the authentic TATA-box of the y7SL gene, functionally.

The analysis of these 5′ deletion constructs in the homologous *S. pombe* extract (Fig. 6B) revealed clearly different results. Deletion of 5′-flanking sequences from the wild-type clone (−1012) down to −45 resulted in a slight but reproducible reduction in activity to 92%. It should be noted that in this extract system no increased activity (as in HeLa extracts) was associated with the −95 and −45 deletion mutants, compared to the wt gene. Furthermore, in contrast to what was observed in HeLa cell extracts, the homologous extract system did not support detectable transcription of constructs truncated further downstream (−18) and into the gene (+26). Thus, it appears that the homologous yeast system was unable to initiate in vitro transcription in the absence of the authentic TATA-box of the y7SL gene. In contrast, the mammalian extract system seemed to be flexible enough to initiate accurately and efficiently, provided that some kind of external TATA element was present.

In summary, the results presented here establish clear differences between lower and higher eukaryotes in the characteristics of enzymes, promoter elements, and presumably transcription factors involved in RNA polymerase III-catalyzed transcription of 7S L RNA genes.

DISCUSSION

The availability of efficient in vitro systems has greatly facilitated the analysis of the mechanisms of eukaryotic gene transcription. Up to now, accurate in vitro transcription by RNA polymerase III in yeast was largely restricted to *S. cerevisiae*, using isolated nuclei (16) or whole cell extracts (10). In agreement with the results obtained with isolated RNA polymerases III (8), these analyses revealed a remarkable resistance of yeast RNA polymerase III transcription, even against highest concentrations of α-amanitin. That observation was in contrast to the sensitivity towards this inhibitor of mammalian pol III transcription performed with purified enzymes (18) or in isolated nuclei (22,26). Here we present the first description of in vitro systems suitable for accurate transcription by RNA polymerase III from *S. pombe*. Both systems, isolated nuclei as well as cell-free extracts, supported specific transcription of genes known to be transcribed by RNA polymerase III. Interestingly, both in vitro systems revealed that pol III transcription of *S. pombe* genes was sensitive to high concentrations of α-amanitin. However, this sensitivity was not identical to that observed in vertebrates.

Another unexpected feature of the yeast extracts analyzed here seems to substantiate this unique position of the *S. pombe* RNA polymerase III among the corresponding eukaryotic enzymes. In our hands, the S100 extracts isolated from *S. pombe* cells revealed considerable inactivation when stored for longer periods at −70°C. Typically, pol III transcriptional activity decreased to about 50% within 3 weeks and was almost completely gone after 2–3 months (F. Rödicker, unpublished). In contrast, under the same conditions HeLa cell extracts retained full activity for several months. Of course, at present we cannot exclude that this instability of the *S. pombe* extract sys-

tem may not be related to the pol III enzyme itself, but rather reflects an unusually labile transcription factor or other component of the system. However, that fact that up to now no report exists in literature that describes the isolation and characterization of an isolated RNA polymerase III of *S. pombe* cells may point into the same direction.

In addition to the pol III enzymes, the 7S L RNA (srp RNA) genes too seem to have undergone significant changes during evolution. While the promoter of the mammalian 7S L RNA gene clearly belongs to the type 4 group of pol III genes with mixed intragenic and upstream promoters (23), the plant (*Arabidopsis thaliana*) gene—like the vertebrate 7S K and U6 RNA genes—is under control of a type 3 promoter, with two highly conserved (USE and TATA) elements located upstream of the coding region. These two essential elements account for ≥90% of the transcriptional activity (7). Within the coding region of the plant gene, no A-box homology is detectable but a sequence element with a 10/11 match to the consensus B-box of tRNA genes is present further downstream, between +60 and +70 of the gene. This element, however, was not important for transcription (7). At first glance, the y7SL promoter seems to share these characteristics of the type 3 promoter of the *A. thaliana* gene: no internal A-box, but a B-box located at the very same position. Furthermore, both genes share the TATA element between position −24 and −30. However, there is a significant difference that might argue against such an assignment. In comparison to the plant gene, no upstream sequence element (USE) can be detected within the *S. pombe* 7S L RNA promoter. With a spacing of about 25 bp, such an element should be located upstream of the TATA-box around position −60. Yet, the −45 deletion mutant revealed almost full template activity for in vitro transcription in *S. pombe* extracts and in HeLa extracts was even more active than the wt gene. Therefore, it appears that, aside from the TATA-box, the y7SL promoter lacks any functional element located further upstream. In principle, the lack of effect of upstream sequences could reflect nonoptimized conditions (e.g., too high amounts of template). However, the template concentrations used for our optimizations in Fig. 2 revealed optimal amounts of 300–500 ng of DNA per assay, which is moderate for such experiments. Nevertheless, we cannot exclude the possibility that a similar optimization performed with the 5′ deletion mutants might change these results slightly, yet without affecting our major conclusion that the structure of the y7SL RNA gene promoter certainly is different from those of the vertebrate genes and very likely also from those of lower eukaryotes.

Therefore, we conclude that the *S. pombe* 7S L RNA gene represents some kind of a "minimal" pol III promoter. It is quite conceivable that such a "minimal" promoter is efficiently utilized by the complex system of mammalian transcription factors, whereas the factors available in *S. pombe* cells in turn are not sufficient for the sophisticated human 7S L RNA gene promoter. Therefore, the system described here may be very useful to dissect the promoter organization of an ancient pol III gene and by that to learn more about the evolutionary pathway of the pol III transcription system in general. From the data here it appears that the pol III transcription in *S. pombe* somehow is intermediate between yeast (*S. cerevisiae*) and higher eukaryotes. Similar conclusions originated from the analysis of other components of the pol III system, for example, the sequence comparison of 5S RNA genes (11).

In summary, the pol III transcription system in general (6), and in particular that of the 7S L (srp) RNA gene (1,7,9,19), was found to be surprisingly complex, with distinct features observed at different levels of evolution. With the many advantages at hand that *S. pombe* offers for biochemical and genetic studies, we feel confident that a detailed analysis of that system undoubtedly will provide valuable insights into the mechanism of transcriptional regulation of eukaryotic pol III genes.

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