
Sequence and regulatory responses of a ribosomal protein gene from the fission yeast *Schizosaccharomyces pombe*

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ABSTRACT

We have determined the nucleotide sequence and mapped the 5' and 3' termini of a ribosomal protein gene. The gene is transcribed into a RNA molecule of about 770 nt and appears to initiate at multiple sites, as judged by S1 nuclease analysis. Gene dosage experiments with a plasmid born gene leads to a proportional increase of the messenger RNA, but not to an overproduction of the protein, suggesting a posttranscriptional control mechanism. However, the heat shock response of this gene indicates that there is also a potential for transcriptional control. Comparison of the 5' flanking region of this gene with the ribosomal protein gene S 6 from *Schizosaccharomyces pombe* and with ribosomal protein genes from *Saccharomyces cerevisiae* revealed homologous sequences, which may be involved in the regulation of ribosomal protein genes.

INTRODUCTION

Under all physiological conditions transcription of ribosomal protein (rp) genes in eukaryotes has been found to be constitutive. However, ribosome formation is proportional to the growth rate depending on growth conditions or developmental stages (1,2). This indicates an underlying control mechanism for 70-80 rp genes, which is involved in ribosome synthesis. It has been shown, particularly for the lower eukaryote *Saccharomyces cerevisiae*, that the ribosomal protein synthesis is coordinated and that the synthesis of the rp proteins is well balanced. In fact, none of the proteins is produced in vast excess, if not assembled into the ribosome (1, 3). During the past few years several regulatory levels of this control mechanism have been identified. The control mechanism of ribosomal protein genes operate on a transcriptional - as well as on a translational - and splicing level (4, 5, 6). It has also

been suggested that messenger stability and degradation of rp proteins may play an important role in this control process (7, 8, 9, 10). A computer homology search of the promoter regions of the ribosomal protein genes from Saccharomyces cerevisiae has identified two common sequences, called Homol I and RPG-box, respectively (11, 12). Both elements seem to be involved in the modulation of transcription of these genes (13). It could be shown that the RPG-box acts in transcription activation and can bind protein factor(s) (14, 15). Although none of these factors have yet been identified, it seems plausible that most of the ribosomal protein genes can be identified by the yeast cell through these specific sequences and probably act together with factor(s) in recognition, transcription activation and modulation of these genes.

Recently we isolated and characterized two ribosomal protein genes from the fission yeast Schizosaccharomyces pombe (16, 17). We are studying the regulation mechanism of ribosomal protein genes in S. pombe. It has already been discussed that basic molecular mechanism in the yeasts S. cerevisiae and S. pombe have considerably diverged (18). For example, S.pombe splices out a higher eukaryotic intron (19) but does not recognize the introns of the rp genes from S.cerevisiae (T. Digel and N. Kaeufer, unpublished). Furthermore, S.pombe recognizes promoter regions from S.cerevisiae, but uses different transcriptional start sites (20).

Therefore we would expect that regulatory mechanisms have also diverged. In this paper we examine the response of S. pombe, exploiting gene dosage effects. We describe the response of S.pombe when cells are transformed with a ribosomal protein gene and shifted to higher temperatures. Furthermore, we demonstrate that additional copies of the rp gene K 37 (16) do not lead to an overproduction of its protein. We will discuss the sequence of this gene and compare the promoter regions of K 37 and the ribosomal protein gene S 6 (17) from S.pombe with the promoter regions of ribosomal protein genes from S.cerevisiae.

MATERIALS AND METHODS

Strains, Plasmids and Phages

S.pombe strains 972 h⁻ wildtype and leu I.32 h⁻ were used as a source of RNA and DNA. The plasmids pRNI carrying the ribosomal protein gene K 37 and pTGR carrying the ribosomal protein gene S6 were described previously (16, 17). The phages M13 mp 18 and mp19 were used to transform the *E.coli* strain K 12 JM 101.

Preparation of DNA and RNA

DNA was isolated essentially as described by Adachi et al. (21) and blotted onto nitrocellulose according to Southern (22). RNA was prepared as described previously (16). Cell cultures were grown until an E₅₉₅ = 0.5 was reached. Cells of 10 ml aliquots were broken with glassbeads and extracted with Phenol, Chloroform, Isoamylalcohol. Samples of total RNA were fractionated on 1.2 % Agarosegels and blotted onto nitrocellulose paper (23) and hybridized to [³²P]-labelled M13 phage DNA probes.

Estimation of specific RNA and DNA concentrations

The densities of the bands on autoradiographs were quantified with a Laser densitometer and the area under the peaks calculated by triangulation. To verify the linear range we repeated this procedure at various exposures.

In vivo labelling of proteins

Cells were grown in glucose EMM medium (21) at 28^oC. At a mid-log phase a 5 ml portion was pulse-labelled for 45 seconds with [³⁵S] methionine (30 µCi/ml, 800 Ci/mmol, NEN). The cells were stopped by pouring into ice-cold methionine (500 µg/ml), harvested and total protein was extracted with 67 % acetic acid according to Hardy et al (24). Analysis of newly synthesized protein was carried out using the 2 D electrophoresis system developed by Kaltschmidt and Wittman (25). The gels were dried and autoradiographed.

DNA sequence determination

The 3.9 kb Hind III fragment containing the ribosomal protein gene K 37 was isolated and mapped by standard methods (26). Fragments were subcloned in M13 mp 18 and mp 19 either

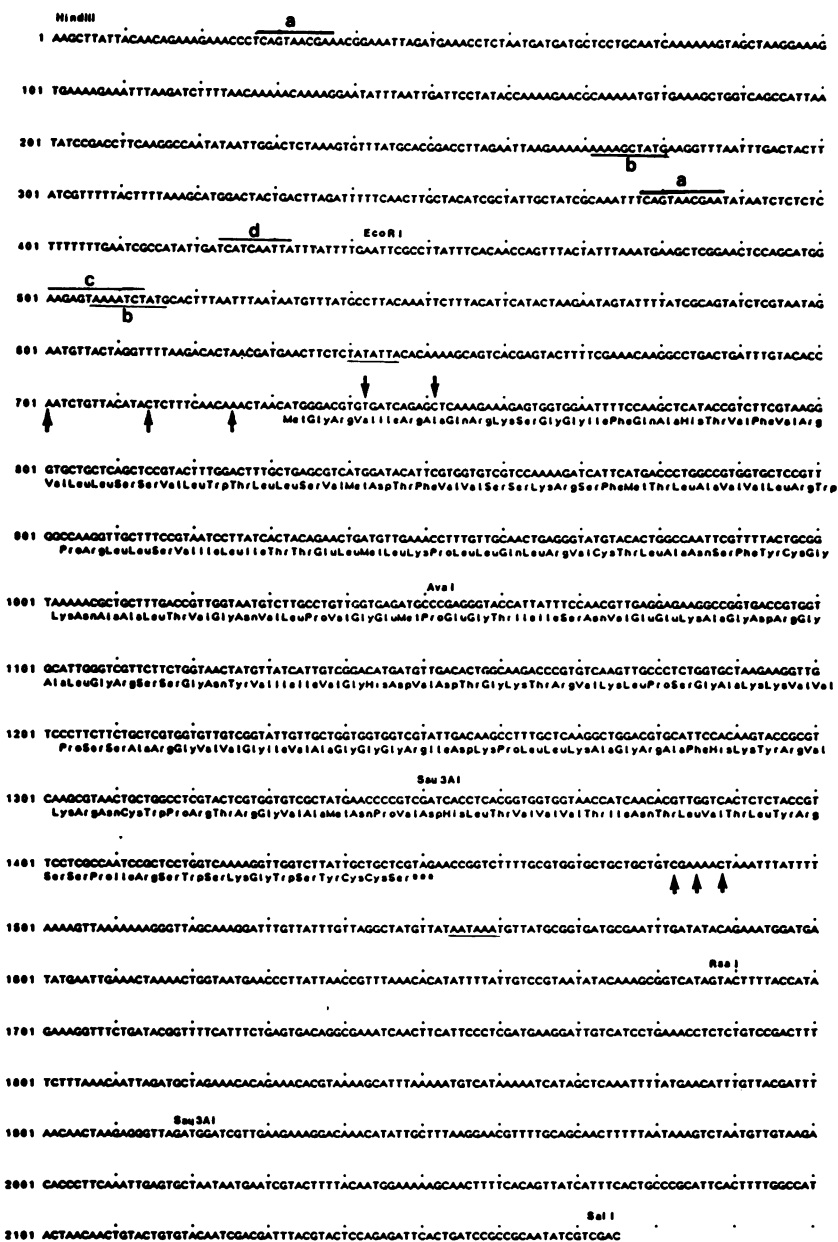


Fig. 1. Nucleotide and amino acid sequence of the ribosomal protein gene K 37. The DNA sequence containing the derived amino acid sequence and its 5' and 3' flanking region is shown. The arrows indicate the transcriptional start and stop sites (see

text). The underlined sequences a, b, c, and d are discussed in the text and table 1. The TATA-like box is underlined. The AAUAAA sequence in the 3' flanking region is also underlined. The restriction sites used for isolating the probes to map transcriptional start- and termination sites are indicated.

as defined isolated fragments or by shotgun techniques (27), and the sequence determined by the dideoxy chain termination method (28).

S₁-Nuclease mapping

was performed exactly as previously described (29). 30 - 100 µg total RNA was hybridized with DNA fragments in vitro labelled at one 5' or at a 3' end. The hybridization conditions were 80 %, 70 % or 65 % v/v formamide in 40 mM PIPES pH 5, 400 mM NaCl and 1 mM EDTA at 48°C for 3 h. Nuclease digestion for 30 min at different temperatures (21°C - 37°C) or with different S₁ nuclease concentrations (200 U/ml - 800 U/ml). The nuclease protected RNA were analyzed on 8 % or 6 % polyacrylamide/Urea sequencing gels.

RESULTS

Nucleotide sequence of the ribosomal protein gene K 37

The 2.2 kb Hind III/Sal I fragment of pRN I (16), which contains the ribosomal protein gene, was cut with several restriction enzymes, subcloned into the appropriate sites of M13 mp 18 and M13 mp 19, and sequenced by the dideoxy technique (28). The sequence of this Hind III/Sal I fragment containing a single open reading frame of 239 amino acids for a protein of 26 kD including the 5' and 3' flanking sequences is shown in Fig. 1. As expected, this ribosomal protein is basic. We find 34 Arg, Lys residues compared to 11 Asp, Glu residues, respectively. The predicted molecular weight is consistent with our electrophoretic estimates of the molecular weight (16).

Transcriptional start sites

We have undertaken S₁ nuclease mapping experiments in order to determine the transcriptional start of the transcript. A 600 base pair (bp) EcoR I/Ava I fragment was 5'-end labelled at the Ava I site and hybridized to total RNA or poly (A)⁺RNA isolated from both wildtype and cells transformed with pRNI.

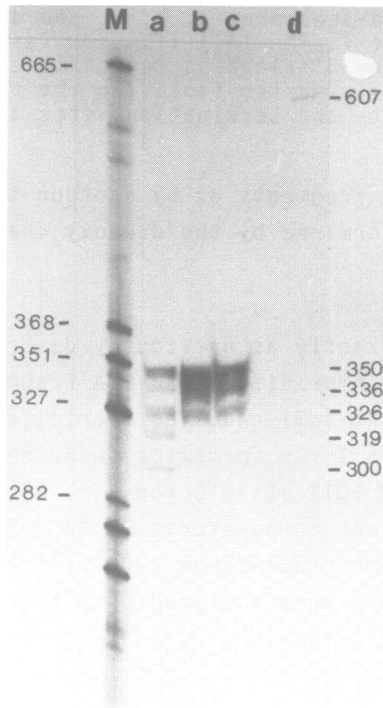


Fig. 2. 5' mapping of RNA transcripts. Total RNA (30 μ g) was hybridized to completion with an excess of a 600 bp K 37 probe endlabeled with 32 p at the Ava I site. The reaction products were treated with S 1 nuclease and subjected to electrophoresis in a 6 % polyacrylamid/Urea gel. Lane a contains RNA from a wildtype strain, and it was hybridized in 65 % formamide. In lanes b and c RNA from transformed cells were prepared and hybridized in b 65 % formamide and c 80 % formamide. Lane d contains the probe which was used. M contains 32 p-labelled defined pBR322 sequences as a Marker.

Both, wildtype RNA and RNA from pRNI transformed cells revealed three S1 nuclease protected bands at position 350, 336 and 326, respectively (Fig. 2, lane a, b, c). Densitometric tracing of the autoradiograph (Fig. 2) revealed that the amount of RNA

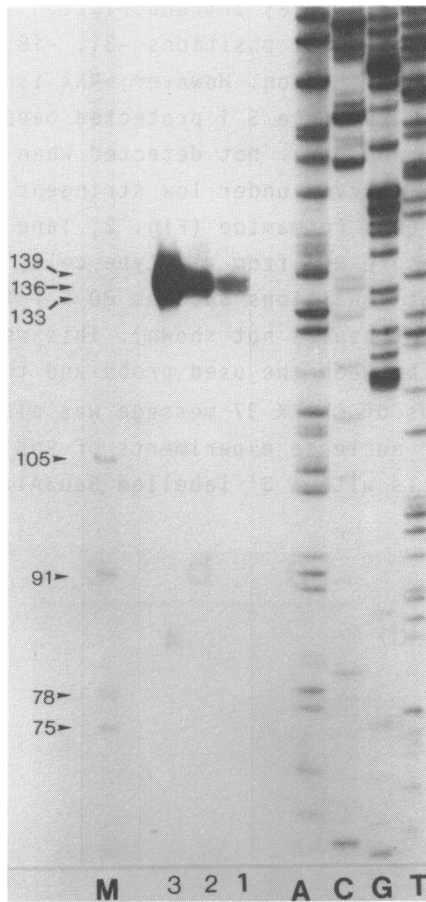


Fig. 3. 3' mapping of RNA transcripts. Total RNA was hybridized with an excess of a end labelled Sau3AI/RsaI fragment using 65 % formamide, treated with S 1 nuclease and subjected to electrophoresis in a 8 % polyacrylamid/Urea gel. Lane 1 RNA from transformed cells (20 μ g). Lane 2 RNA from transformed cells (40 μ g). Lane 3 RNA from wildtype cells (80 μ g). A, C, G, T is the ladder of a known sequence, M contains defined 32 p labelled fragments.

from transformed cells protected from our probe is always considerably higher than the amount of the protected RNA from wildtype cells. This indicates that there are plasmid derived transcripts which initiate at the same transcriptional start sites as the wildtype RNA. Therefore we suggest that wild type

and transformed celles display 3 transcription initiation sites for the K 37 gene mapping at positions -31, -18, and -7 with respect to the AUG start codon. However, RNA isolated from wildtype cells yield two more S 1 protected bands (Fig. 2, lane a). These two bands are not detected when we use RNA from transformed cells even under low stringent hybridization conditions such as 65 % formamide (Fig. 2, lane c). On the other hand, when we use RNA from wildtype cells and hybridize under high stringent conditions such as 80 % formamide, these two bands disappear (results not shown). This result indicates a limited homology between the used probe and this RNA.

The 3' terminus of the K 37 message was also mapped using the S1 technique. S1 nuclease experiments of RNA from wildtype and transformed cells with a 3' labelled Sau3AI/RsaI fragment

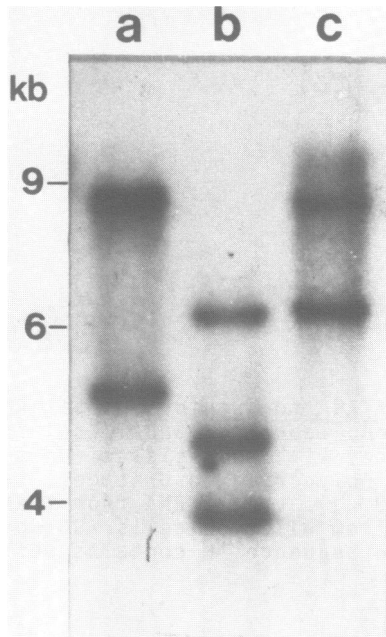


Fig. 4. Genomic DNA blot-hybridization analysis. DNA from a wildtype strain was digested with a Clal, b Hind III, and c Sal I. Fragments were separated on a 0.7 % agarose gel, transferred to nitrocellulose, and hybridized to radio-labelled single stranded 400 bp KpnI/TaqI probe, spanning the second half of the K 37 gene.

labelled at the *Sau3AI* site revealed no obvious difference (Fig. 3). Under all stringency conditions used we detected one major transcriptional stop site at position 1485 (Fig. 1 and Fig. 3).

These results let us suggest, i) there might be two expressed copies of this gene in the genome, which probably differ in their 5' flanking regions, ii) an increase of the copy number of the ribosomal gene K 37 affects the transcription of the second genomic copy.

In order to probe for the presence of a second copy in the genome, we performed a Southern analysis using DNA from wild-type cells. The genomic DNA was digested with restriction enzymes *Hind III*, *Sal I* and *Cla I*, which do not cut in the DNA fragment containing the characterized gene of K 37 (16). As a probe we used single stranded DNA containing a 400 bp *Kpn I*/*Taq I* sequence of the isolated structural ribosomal protein gene. As shown in the Southern hybridization patterns (Fig. 4) the *Hind III* digestion reveals 3 bands. The 3.9 kb band contains as expected the K 37 gene (16). The *Sal I* digested DNA and the *Cla I* digest, respectively yield 2 bands. This clearly indicates that there is at least one closely related fragment of this gene in the genome.

Regulatory response of the plasmid borne ribosomal protein gene

In order to investigate regulatory responses when *S.pombe* cells are transformed with extra copies of the ribosomal protein gene K 37, we introduced the plasmid pRN I (16) into cells and determined by quantitative Southern analysis the copy number of this plasmid. We revealed a copy number of 8-10 per cell (data not shown). Analysis of total RNA by Northern analysis of the transformed cells and densitometric tracing of the specific transcript revealed that the level of the ribosomal protein transcript in transformed cells is approximately 8 times higher than in wildtype cells (Fig. 5). These results indicate that the gene dosage is roughly proportional to the amount of the transcribed messenger as reported for several other genes from *S.pombe* and *S.cerevisiae* (30). We are aware that in wildtype cells these transcripts could stem from 2 genes, but this will not affect the following approach.

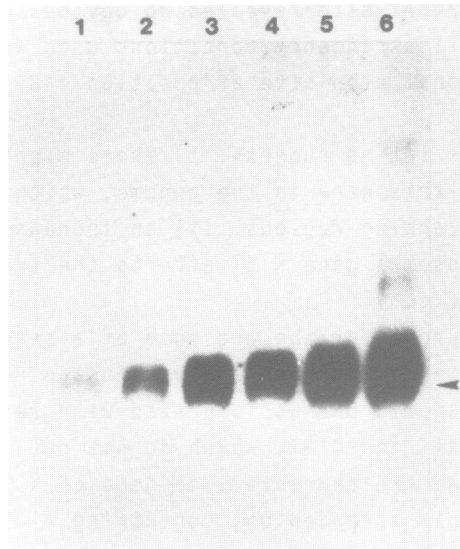


Fig. 5. Northern blot-hybridization analysis. Total RNA was isolated, separated on a 1.2 % agarose gel, transferred to nitrocellulose and hybridized to ^{32}p labelled Hind III/Sal I probe containing the whole gene. Lanes 1-3 contain 2, 4 and 8 μg RNA isolated from wildtype cells. Lanes 4-6 contain 2, 4 and 8 μg RNA isolated from cells transformed with the plasmid containing the K 37 gene.

Next we examined the effect of this elevated level of messenger RNA on the in vivo synthesis of the encoded ribosomal protein. Wildtype cells and cells transformed with pRNI were pulse labelled for 45 sec with [^{35}S] methionine. Total protein was isolated and analyzed using the Kaltschmidt/Wittmann 2D-electrophoresis system (25). The results shown in Fig. 6 suggest that elevated amounts of this transcript do not lead to the accumulation of newly synthesized ribosomal protein. We have chosen such a short pulse labelling time, because it is known from *S.cerevisiae* (31) and higher eukaryotes (2) that ribosomal proteins have a rapid turnover. In fact, it has been suggested that cells regulate ribosomal protein synthesis by rapidly degrading ribosomal proteins, which are not assembled into ribosomes (8, 10).

We did not find an accumulation of this protein even after this short labelling time, however, all the transcripts accumu-

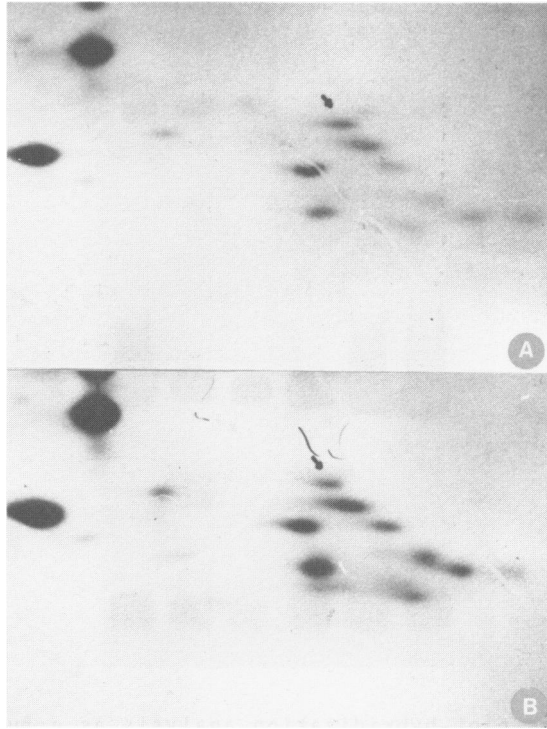


Fig. 6 A, B. In vivo synthesis of the ribosomal protein K 37 in wildtype and transformed cells. Cells were labelled with ^{35}S methionine for 45 s as described in Materials and Methods. Total protein was extracted and run on a 2-D gel according to Kaltschmidt and Wittmann (25). A Autoradiograph of proteins from wildtype cells. B Autoradiograph of proteins from cells transformed with pRNI containing the K 37 gene. Arrows indicate the product of the K 37 gene.

lated in the polysome fraction, no free messenger could be detected (data not shown). Therefore we suggest that this indicates a translational control mechanism. It is not yet clear whether this is due to inefficient translation of the excess messenger RNA or to a stop of translation after initiation. In any case, plasmid borne additional copies of this gene cause a response of the cell on the translational level.

It is well known that ribosomal protein genes of Saccharomyces cerevisiae show a coordinated transient shut-off in transcription when shifted to higher temperatures. Plasmid

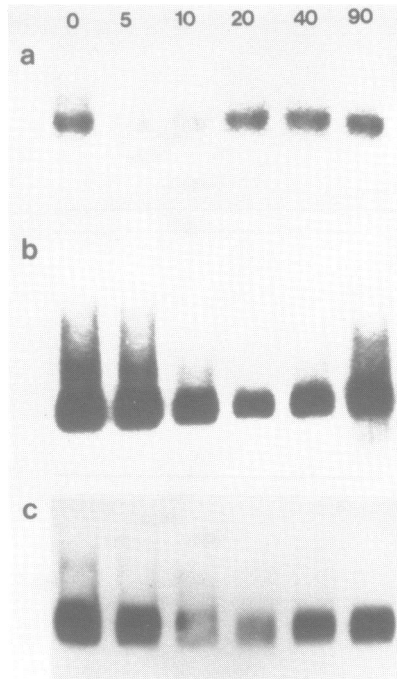


Fig. 7. Northern blot-hybridization analysis as a function of time after temperature upshift. At various times (indicated at top in minutes) after upshift (28°C - 37°C) total RNA was prepared. Equal amounts (5 µg) were fractionated on a 1.2 % agarose gel and transferred to nitrocellulose. A total RNA from wildtype cells probed with a radiolabelled fragment containing the URA 4 gene, b total RNA transformed with the plasmid pTGR containing the ribosomal protein gene S 6 (17) and probed with a fragment from this gene, c total RNA transformed with the plasmid pRNI containing the K 37 gene and probed with a Hind III/Sal I fragment shown in Fig. 1.

encoded ribosomal protein genes from S.cerevisiae reveal the same heat inducible response (4).

To determine whether ribosomal protein genes from S.pombe are heat repressible and show a similar response as reported for S.cerevisiae, we shifted cells transformed with pRNI and cells transformed with a plasmid pTGR containing the ribosomal protein S 6 (17) from 28°C to 37°C. At different time points between 5 min and 90 min after the shift, we harvested aliquots of the cultures and prepared total RNA. The ribosomal protein

Table I. Common upstream sequence elements in 2 ribosomal protein genes

rp-proteins	homol a	homol b	homol c	homol d
K37	TCAGTAACGAAT(-355)	AAAATCTATGCA(-224)	AAGAGTAAAATC(-231)	GATCATCAATTA(-311)
	TCAGTAACGAAT(-704)	AAAAGCTATGAA(-459)		
S6	CTCGTAACGAAT(-359)	CACATCTATGGA(-339)	AACAGTAAAATC(-222)	CTCCATAAATTA(-292)
Homol1 consensus ^a		AACATCTATGCA CG A		
RPG-box consensus ^b				ACCCATACATTA CT

a, b Data for upstream sequence elements from *Saccharomyces cerevisiae* from Teem et al. (11) and Leer et al. (12).

transcripts at the indicated time points are shown in Fig. 7 (b, c) after blotting and hybridizing with a [³²P] labelled specific probe. Both ribosomal protein transcripts show the same response. After 20 min a minimum of the messenger level is reached, whereas with in the next 60 min the concentration of both transcripts increases, after 90 min we measured a concentration almost comparable to cells grown at 28^oC.

The URA 4 gene coding for orotidine monophosphate decarboxylase in *S.pombe* was used as a probe to measure the level of a non ribosomal protein transcript during temperature upshift in a wildtype strain. We observed a similar pattern (Fig. 7 a) as shown for the ribosomal protein transcripts.

In all cases the drop of messenger concentration is indeed due to a transient transcriptional shut-off within the first 10 min as determined by pulse labelling with [³H] Uracil (data not shown). The concentration of the URA 4 transcript decreases significantly within the first 5 min, which is probably due to a less stable message of this protein and to the lower gene dosage compared to the ribosomal protein genes.

These results clearly indicate that in response to a heat shock ribosomal protein genes from *S.pombe* are affected on the level of transcription as reported for ribosomal protein genes from *S.cerevisiae* (4). With respect to *S.pombe*, however, this response is not limited to ribosomal protein genes.

DISCUSSION

The results of S 1 nuclease mapping (Fig. 2) indicate heterogeneous 5' termini of the isolated ribosomal protein gene K 37. At position -91 of the sequence we find a TATATTA sequence, which is located 60 bp upstream of the most abundant trans-

criptional start site (position -31). It is not yet known in S.pombe whether this sequence functions in regulation or in transcription site determination (32). S 1 mapping of the termination site revealed one strong terminus 33nt down-stream of the TAA stop codon (Fig. 1). At position 1554 we find the sequence AAUAAA, which is believed to be required for cleavage and polyadenylation at the 3' termini of eukaryotic mRNA precursors and is located 6 to 26 bp upstream of the poly (A) addition site (33). Interestingly, we find this sequence down-stream of the mapped 3' termini. This is also the case in the sequence of the ribosomal protein S 6 from S.pombe (T. Groß and N. Kaeufer, unpublished data).

A comparison of the 5' flanking sequences of the genes K 37 (Fig. 1) and S 6 (Groß and Kaeufer, unpublished data) revealed 4 common sequences (Table 1). All these sequences are located in upstream regions between position -200 and -700 with respect to the AUG start codon. Notabene, the sequence element b and the sequence element d show a considerable homology with Homol 1 and the RPG box, respectively (Table 1). These two sequences are found in most 5' upstream regions of the ribosomal protein genes from Saccharomyces cerevisiae (11, 12). It has been shown that Homol 1, together with the RPG box, can play a role in the modulation of the transcription of ribosomal protein genes in S.cerevisiae. The RPG box acts as a binding site for protein factor(s) (15) and could clearly be identified as an element which is involved in activating transcription of ribosomal protein genes in S.cerevisiae (14). We are now determining by promoter scanning deletions of the K 37 and S 6 genes whether these common sequences (Table I) have any regulatory or functional significance for transcription of ribosomal protein genes of S.pombe. It is interesting to note that no significant homology to these sequences could be found in 6 promoters of different S.pombe genes. Our report here demonstrates that both ribosomal protein genes can respond on a transcriptional level. The heat shock response of both rp genes from S.pombe is similar to that reported for ribosomal protein genes from S.cerevisiae (4). After temperature upshift, a transient stop of transcription is observed, however,

it remains to be shown whether transcriptional control mechanisms play a general role in the expression of ribosomal protein genes in S.pombe. The Southern analysis (Fig. 4) demonstrates the presence of a closely related sequence of the rp gene K 37. From the set of S 1 experiments mapping the 5' termini of this gene (Fig. 2) we conclude that it is a second copy of this gene which is expressed. By increasing the copy number of the isolated gene the transcript of the second copy disappears. This result indicates that the transcription of this gene can be modulated. Modulation of transcription within -tubulin gene family in S.pombe has been reported by Adachi et al (21). However, further studies such as isolating and sequencing the second copy are necessary to confirm our conclusions.

Increasing the copy number of the gene K 37 by about 10 copies did not result in a measurable overproduction of the ribosomal protein, although we used a very short labelling time (45 s). This suggests that translational control is involved.

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