Biochem. J. (1985) 232, 205–209 (Printed in Great Britain)

Upstream activation of ribosomal RNA biosynthesis in *Saccharomyces cerevisiae*

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Yeast was transformed with eight recombinants that contained an rRNA minigene and upstream elements of rDNA in different orientations in the multi-copy yeast–*Escherichia coli* shuttle vector, pJDB207. The effect of these elements of upstream rDNA on the initiation of transcription of the minigene at the site for rRNA biosynthesis was determined by using an S_1 nuclease mapping procedure to measure the abundance of the minigene transcript in RNA from the yeast transformants. Transcription of the minigene was enhanced 3-fold by DNA within a 2.2 kb element more than 1.5 kb upstream from the initiation site. Inversion of the 2.2 kb element decreased expression of the minigene by 40%. This 2.2 kb element contained approx. 500 bp from the 25S rRNA coding region at the 3′ end of the preceding rRNA gene and 1 kb of adjacent nontranscribed spacer rDNA. The enhancing activity was independent of interference from readthrough that might have contributed to the 7-fold decrease in minigene expression caused by removing all rDNA upstream from −209 bp.

INTRODUCTION

Eukaryotes contain tandemly repeated copies of the rRNA gene that are separated from each other by several kb of nontranscribed spacer rDNA. Atypically, the nontranscribed spacer in yeast contains the gene for 5S rRNA. In several organisms, sequences approx. ±50 bp from the initiation site (+1) of the rRNA gene are sufficient for transcription to begin at the correct place in cell extracts and in intact cells containing manipulated copies of these genes (Kohorn & Rae, 1983; Yamamoto *et al.*, 1984; Sollner-Webb *et al.*, 1983). In two of these organisms, enhancing effects of further upstream sequences have been detected under particular experimental conditions. Factors that affect the response are the topology of the DNA, the nature of the transcription assay and the presence/absence of a competitor DNA template. In the mouse, DNA extending to −145 bp has been implicated (Grummt, 1982), whilst in *Xenopus laevis*, transcription of the rRNA gene may be affected by DNA between −7 bp and −142 bp and by DNA several kb upstream in the nontranscribed spacer (Sollner-Webb *et al.*, 1983; Labhart & Reeder, 1984).

Previously, we described the construction in an *Escherichia coli*–yeast shuttle vector of a yeast rRNA minigene that consisted of the initiation and termination sites for rRNA biosynthesis separated by approx. 700 bp of vector DNA (Quincey & Arnold, 1984). This minigene in yeast yielded transcripts that could be detected by S_1 nuclease mapping using a [^{32}P]DNA probe labelled at a site in the foreign DNA. These transcripts originated from the initiation site for rRNA biosynthesis and were less abundant in yeast transformed with a derivative lacking 2.8 kb of upstream rDNA. The interpretation of the cause of this effect was complicated by the appearance of a readthrough transcript that might have interfered with transcription of the minigene. The results of the present work, in which the effect on expression of manipulating upstream rDNA was measured in eight recombinants, showed that an element of rDNA more than 1.5 kb upstream from the minigene increased transcription 3-fold in a manner that was dependent on orientation and was independent of interference from readthrough.

EXPERIMENTAL

Plasmids

All recombinants were derived from Rp6 (Fig. 1) that was described previously (Quincey & Arnold, 1984). This plasmid contains a yeast rRNA minigene and 3 kb of yeast rDNA upstream from the initiation site of rRNA biosynthesis cloned into pJDB207. The plasmid, pJDB207, is an *E. coli*–yeast shuttle vector that consists of pAT153, the yeast *leu*2 gene and part of the yeast 2 μ DNA (Beggs, 1981). Plasmids were propagated in *E. coli* JA221 (*rec*A1,*leu*B6,*trp*E5,*hsd*R$^-$,*hsd*M$^+$,*lac*Y,C600).

Yeast transformation, growth and the extraction of RNA

Saccharomyces cerevisiae LL20 (α,*leu*2.3,*leu*2.112, *his*3.11, *his*3.15) was transformed with plasmid DNA as described by Beggs (1978). Transformants were selected on agar plates containing minimal medium (0.67% Difco nitrogen base without amino acids, 2% glucose) and 40 μg of histidine/ml. To obtain RNA, yeast was grown with shaking at 30 °C in the same medium in liquid culture and was harvested during exponential growth ($A_{650}^{1\,cm} \sim 1$). Yeast was disrupted by vortex-mixing with glass beads in buffer containing 1 mg of heparin/ml and 10 mM-vanadyl–ribonucleoside complexes, extracted with phenol, phenol/chloroform (1:1, v/v) and chloroform and precipitated with ethanol (Quincey & Arnold, 1984). The concentration of RNA was measured by the

Abbreviations and definitions used: bp, base pairs; kb, kilobases and kilobase pairs; rRNA gene, the gene encoding the precursor RNA for 17–18S rRNA, 5.8S rRNA and 25–28S rRNA; rDNA, DNA of the rRNA gene, 5S rRNA gene and the nontranscribed spacer DNA.

Schmidt–Tannhauser method as described by Munro & Fleck (1966).

Estimation of the relative abundance of the minigene transcript in different yeast transformants

These estimates were obtained by hybridizing RNA from yeast transformants with excess [^{32}P]DNA probe, treating with S_1 nuclease and measuring the density of the appropriate band on autoradiograms of 5% polyacrylamide/urea gels (Quincey & Arnold, 1984). The DNA probe was the 682 bp *Bam*HI–*Hin*dIII fragment of Rp8 that extended from −208 bp to the *Hin*dIII site at +474 bp (Fig. 1). The probe was labelled with ^{32}P in the 5′ position at the *Hin*dIII site. The probe was made by firstly labelling the dephosphorylated 5′ ends of *Hin*dIII, *Sal* I double digest of approx. 35 μg of Rp8 DNA using approx. 100 μCi of [γ-^{32}P]ATP (> 3000 Ci/mmol from Amersham International) diluted with 40 pmol of unlabelled ATP. After labelling, the DNA was digested with *Bam*HI and the *Bam*HI–*Hin*dIII probe was purified on a 5% polyacrylamide gel. The density on autoradiograms of the band of 474 nucleotide residues that represented the minigene transcript was measured by scanning two sections on a Joyce–Loebl double-beam spectrophotometer.

To form hybrids, the [^{32}P]DNA probe was incubated at 52 °C with 25 μg of RNA (yeast RNA with wheat-germ tRNA as required) in 30 μl of 40 mM-Pipes (pH 6.4)/1 mM-EDTA/0.4 M-NaCl/80% (v/v) formamide. Then 0.3 ml of ice-cold 0.28 M-NaCl/0.05 M-sodium acetate (pH 4.6)/4.5 mM-$ZnSO_4$ containing 20 μg of denatured calf thymus DNA/ml and 150 units of S_1 nuclease (Bethesda Research Laboratories, Cambridge, U.K.)/ml was added. After incubating for 1 h at 25 °C, 20 μg of tRNA was added and each sample was extracted with phenol/chloroform (1:1, v/v), precipitated with propan-2-ol and dissolved in 10 μl of freshly prepared 0.1 M-NaOH/10 M-urea containing 0.3% Xylene Cyanol and 0.3% Bromophenol Blue. Samples (7 μl) were loaded onto a 5% polyacrylamide/urea gel (300 mm × 400 mm × 0.4 mm) and run at 1500 V and 45 mA for approx. 5 h (Quincey & Arnold, 1984). Gels were exposed to Fuji RX X-ray film for several days at −70 °C using an enhancer screen.

Two preparations of probe were used. Preliminary experiments with each were performed to determine conditions of DNA excess in which the densities of the bands were proportional to the concentration of RNA in the hybridization reaction. The probe used for experiments A, C and D (Table 1) and for that shown in Fig. 1 was calibrated by hybridizing approx. 9000 d.p.m. of the probe with 2, 4, 8 and 16 μg of RNA from two independent isolates of yeast transformed with Rp6 (in which the transcript was most abundant). The average densities of the bands representing the minigene transcript (expressed in arbitrary units, with the RNA input in parentheses) were 0.23 (2 μg), 0.57 (4 μg), 1.1 (8 μg) and 1.0 (16 μg). In the experiments to measure abundance, 1.20 times this amount of probe was hybridized with 5 μg of yeast RNA. The other preparation of probe was calibrated similarly. Densities of 0.33, 0.66 and 1.0 were obtained after hybridizing 4, 8 and 16 μg of RNA from an Rp6 yeast transformant with approx. 9000 d.p.m. of the probe. In the experiments to measure abundance, 1.28 times this amount of probe was hybridized with 5 μg of yeast RNA.

RESULTS

Recombinants

A family of eight recombinants was obtained from Rp6 that differed in the amount and orientation of rDNA upstream from the minigene (Fig. 1). Two recombinants, in addition to Rp6, retained all of the upstream rDNA. In Rp7, *Bam*HI linkers had been added to Rp6 at −206 bp and in Rp14 the 3.5 kb *Bam*HI fragment containing the upstream rDNA had been inverted. Four other recombinants retained part of the upstream rDNA and were obtained after adding *Bam*HI linkers at the *Pvu*II site at −1475 bp, which divided the upstream 3.5 kb *Bam*HI fragment of Rp7 into a promoter-distal 2.2 kb fragment and a promoter-proximal 1.3 kb fragment. These smaller *Bam*HI fragments were correctly orientated in Rp16 and Rp18, and were inverted in Rp15 and Rp17. In Rp14 and Rp15, inversion resulted in approx. 700 bp of vector DNA being interposed between the *Bam*HI site at −208 bp and the further upstream element of rDNA. The recombinant Rp8, described previously (Quincey & Arnold, 1984), retained no rDNA upstream from −209 bp.

The eight recombinants contained the same minigene, which meant that the relative abundance of the minigene transcript in different transformants could be measured with the same hybridization probe.

Abundance of the minigene transcript in transformed yeast

Yeast was transformed with recombinant plasmids containing the minigene. The relative abundance of the minigene transcript in these transformants was measured from the relative abundance of the transcript in RNA from independent isolates of each type of transformant in four experiments similar to that shown in Fig. 2. The data were expressed relative to Rp6 and were combined (Table 1). The abundance of the minigene transcript in Rp6 transformants was not significantly different from that in Rp7 transformants and was 7-fold greater than in Rp8 transformants. In yeast transformed with Rp16, the minigene transcript was about two-thirds as abundant as in Rp6 or Rp7 transformants and was 4-fold more abundant than in Rp8 transformants. Inversion of upstream DNA in Rp7 to form Rp14 and in Rp16 to form Rp15 decreased the abundance of the minigene transcript by about 40%. Additional data supporting the relation between the abundance of the transcript in Rp15 and Rp16 transformants came from the results of another experiment in which the abundance relative to Rp7 = 100 ± 4.8 (S.E.M.) (n = 3) was 40 ± 2.9 (4) for Rp15 and 71 ± 5.1 (4) for Rp16. In yeast transformed with Rp18, the minigene transcript was one-third as abundant as in Rp6 or Rp7 transformants and half as abundant as in Rp16 transformants. Inversion of the upstream rDNA of Rp18 to form Rp17 decreased the abundance of the transcript 10-fold to a level significantly lower than in Rp8 transformants.

These measurements of the relative steady-state concentrations of the minigene transcript provided estimates of relative rates of expression because the only variable that might have affected the rate constant for degradation of the transcript was its own concentration.

The prominent band corresponding to the full-length probe in analyses of RNA from Rp17 and Rp18 transformants, but not in analyses of RNA from any

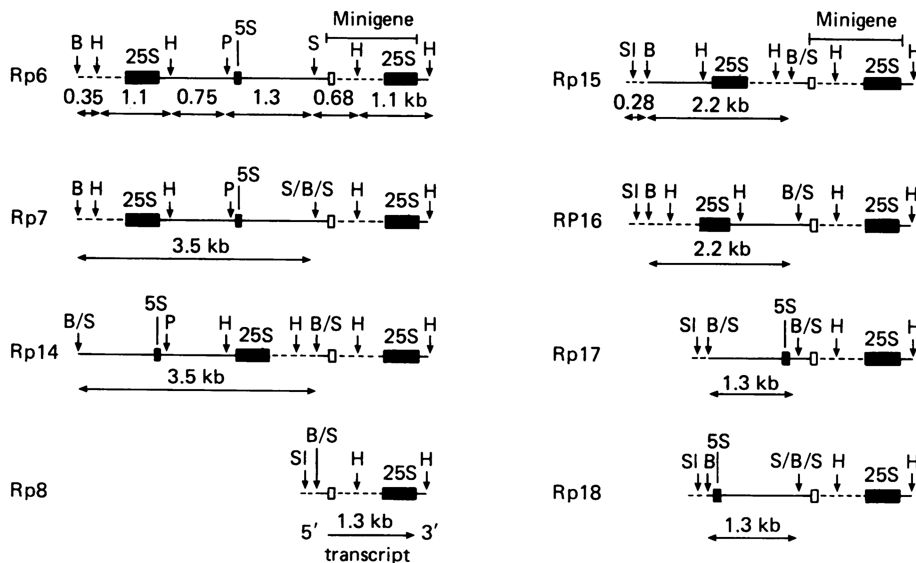

Fig. 1. Structure of recombinants containing a yeast rRNA minigene

All of the recombinants were derived from Rp6 that contains 3 kb of yeast rDNA, including the 3′ end of the preceding rRNA gene and all of the nontranscribed spacer, upstream from an rRNA minigene. The minigene consists of 128 bp from the 5′ end of the rRNA gene (open box) and 0.5 kb from the 3′ end that codes for 25S rRNA (shaded box, labelled 25S), separated by about 700 bp of vector DNA. Downstream from the minigene are about 270 bp of rDNA. The expected 1.3 kb transcript of the minigene is indicated for Rp8. The box marked 5S represents the 5S rRNA gene. ----, vector DNA; ——, yeast nontranscribed spacer. Restriction sites are shown for *Bam*HI (B), *Pvu*II (P), *Sma*I (S), *Hin*dIII (H) and *Sal* I (SI). Details of structure and derivation are as follows. Rp7: derived from Rp6 by adding *Bam*HI linkers at the *Sma*I site at −206 bp. Several *Bam*HI linkers may have been added in creating the *Bam*HI site at −208 bp. The yeast rDNA sequence was maintained to −209 bp and the *Sma*I site was duplicated. Rp14: obtained by inverting the 3.5 kb *Bam*HI fragment of Rp7. This caused the loss of a 2.7 kb *Hin*dIII fragment and appearance of a 1.03 kb *Hin*dIII fragment. Rp8: obtained by eliminating the 3.5 kb *Bam*HI fragment of Rp7. Rp15, Rp16, Rp17, Rp18: obtained after adding *Bam*HI linkers at the *Pvu*II site of Rp7 and subcloning to randomize the insertion of the 2.2 kb and 1.3 kb *Bam*HI fragments. The promoter distal 2.2 kb fragment is in Rp15 and Rp16. The promoter-proximal 1.3 kb fragment is in Rp17 and Rp18. These elements of upstream DNA are correctly orientated in Rp16 and Rp18 and are inverted in Rp15 and Rp17. The orientation of the 2.2 kb *Bam*HI fragment was determined from the size of the *Hin*dIII–*Sal*I fragment which is 0.63 kb in Rp16 and 1.03 kb in Rp15. The orientation of the 1.3 kb *Bam*HI fragment was determined from the size of the *Sma*I fragment which is negligible in Rp18 and 1.3 kb in Rp17.

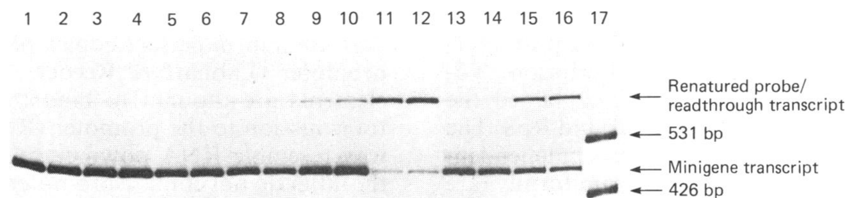

Fig. 2 Nuclease S_1 mapping of RNA from yeast transformed with recombinants containing an rRNA minigene

RNA (5 μg) from growing yeast plus 20 μg of tRNA from wheat-germ was hybridized for 3 h at 52 °C with approx. 10000 d.p.m. of the 682 bp *Bam*HI–*Hin*dIII fragment of Rp8 labelled with ^{32}P in the 5′ position at the *Hin*dIII site (Fig. 1). Fragments protected from digestion with 150 units of S_1 nuclease/ml for 1 h at 25 °C were separated on a 5% polyacrylamide/urea gel and detected by autoradiography. RNA was from independent isolates of Rp6 (lanes 1 and 2), Rp7 (lanes 3 and 4), Rp14 (lanes 5 and 6), Rp15 (lanes 7 and 8), Rp16 (lanes 9 and 10), Rp17 (lanes 11 and 12), Rp18 (lanes 13 and 14) and Rp8 (lanes 15 and 16). Lane 17, ^{32}P-labelled DNA fragments of the sizes indicated.

other type, with the partial exception of Rp16 (Fig. 2), was interpreted to mean that readthrough occurred in those recombinants. This explanation was preferred to the alternative that these bands represented predominantly renatured probe, for three reasons. First, there was evidence for a readthrough transcript in Rp8 but not in Rp6 (Quincey & Arnold, 1984). Second, the probe was always in excess in the hybridization reaction, but was barely detected after treating with S_1 nuclease in all other cases. Third, the inverse correlation between the densities

Table 1. Relative abundance of the minigene transcript in yeast transformants

Estimates of abundance were obtained by hybridizing 5 μg of yeast RNA with approx. 10000 d.p.m. of the [^{32}P]DNA probe, treating with S_1 nuclease and measuring the density of the band representing the minigene transcript on autoradiograms of 5% polyacrylamide/urea gels. RNA from three to six independent isolates of two or three types of yeast transformant was compared with RNA from four independent isolates of Rp6 transformants in each of four experiments. These data ($\pm$S.E.M.) are given relative to Rp6 = 100 and were combined after showing by analysis of variance of the data for Rp16, Rp18 and Rp8 that variation between experiments was not significantly greater than variation within samples. The values in parentheses indicate the numbers of observations and of different RNA preparations analysed. The letters in parentheses indicate from which of the four experiments, A, B, C or D, the data were obtained. The errors (S.E.M.) for Rp6 were: A$\pm$7.2; B$\pm$8.1; C$\pm$4.8; D$\pm$12.

Yeast transformant	Abundance of minigene transcript relative to Rp6 = 100
Rp7	90$\pm$7.2 (6, 6, A)
Rp14	57$\pm$2.1 (6, 6, A)
Rp16	63$\pm$3.9 (8, 4, C, D)
Rp15	37$\pm$6.9 (4, 4, C)
Rp18	33$\pm$2.6 (12, 4, B, C, D)
Rp17	3$\pm$0.9 (3, 3, B)
Rp8	15$\pm$2.2 (8, 5, B, D)

of the bands of 682 and 474 nucleotide residues evident in Rp8 and Rp17 was not general (compare Rp6, Rp7 with Rp15, Rp16, Rp18).

DISCUSSION

Comparisons of minigene expression between recombinants containing correctly orientated elements of upstream rDNA and with Rp8 showed that at least two factors contributed to the 7-fold difference in expression between Rp6, Rp7 and Rp8. The results for Rp6 and Rp7 indicated that no sequence important for transcription of the minigene spanned -209 bp. By contrast, comparison of Rp6 and Rp7 with Rp18 showed that DNA within a 2.2 kb element more than 1.5 kb upstream from the minigene enhanced transcription 3-fold. Similarly, differences in expression between Rp6, Rp7 and Rp16, and between Rp18 and Rp8, suggested that a promoter-proximal 1.3 kb element enhanced transcription. Together, the product of these two effects explained the difference between expression in Rp6, Rp7 and Rp8. The enhancing activity of the 2.2 kb element was independent of readthrough which was absent from, or uniformly rare in, Rp6, Rp7 and Rp18. Readthrough, however, occurred in Rp8 and to a lesser extent in Rp16 and may have caused, or contributed to, the apparent enhancing activity of the 1.3 kb fragment.

The magnitude of the enhancing effect of the 2.2 kb element was smaller, but not dissimilar to the 5- to 10-fold effect of the nontranscribed spacer of *X. laevis* on the transcription of rRNA genes injected into *Xenopus* embryos (Busby & Reeder, 1983).

Inversion of elements within upstream DNA changed their distance from the minigene as well as their orientation. In Rp7, inversion to form Rp14 moved most of the DNA in the 2.2 kb element closer to the minigene, yet caused a 40% decrease in expression. Inversion of the 2.2 kb element in Rp16 decreased transcription of the minigene by 40%. These changes, which were not accompanied by readthrough, suggested that orientation was important for the proper functioning of the enhancing activity in the 2.2 kb element. In this respect, the activity that enhanced transcription of the minigene differed from the mechanism by which rRNA biosynthesis is enhanced in *X. laevis* (Labhart & Reeder, 1984).

Most known enhancers increase the expression of viral and cellular genes coding for proteins in a way that is relatively independent of their position and orientation with respect to the gene (Khoury & Gruss, 1983). Upstream activator sequences in yeast also act relatively independently of orientation, but may not function when placed downstream from the promoter (Guarente & Hoar, 1984; Struhl, 1984). These various enhancing elements of DNA are commonly thought to act as entry points for transcription factors and/or RNA polymerase II. Enhancement of rRNA biosynthesis has been demonstrated in *X. laevis* and in yeast. In *X. laevis*, rRNA biosynthesis is enhanced independently of orientation by 60/81 bp repeated elements in the nontranscribed spacer that contain imperfect copies of a 42 bp region of the promoter (Labhart & Reeder, 1984). These 60/81 bp elements are thought to bind transcription factors for transmission to the promoter (Reeder, 1984) and in this way resemble RNA polymerase II enhancers, although the latter do not contain promoter duplications. A similar mechanism for enhancing rRNA biosynthesis may operate in *Drosophila melanogaster* where promoter duplications also occur in the nontranscribed spacer (Coen & Dover, 1982). In yeast, promoter duplications are absent from the nontranscribed spacer (Skryabin *et al.*, 1984), but within the 2.2 kb *Bam*HI fragment that enhances transcription of the minigene is a 15 bp sequence (AGGCA–TTGCCGCGAA) from nucleotide 3070 of 25S rRNA (Georgiev *et al.*, 1981) that is homologous with a 16 bp sequence (AGGTACTTCAT-GCGAA) from -9 to $+7$ bp at the 5′ end of the rRNA gene that is conserved in several yeasts and is part of the putative promoter sequence (Verbeet *et al.*, 1984). The need for this upstream sequence will be determined. Transmission of factors or RNA polymerase from this region through the termination site for rRNA biosynthesis and the entire 5S rRNA gene might not be required for activity. The two widely separated homologous sequences could be bound together by the interaction of specific

DNA binding proteins to form a looped structure, as has been proposed for the rDNA of the rat (Mroczka *et al.*, 1984) and for the operator region of the L-arabinose operon of *E. coli* (Dunn *et al.*, 1984).

After completing this work we learned of the results of Elion & Warner (1984), who investigated the effect of the nontranscribed spacer on the expression of a yeast rRNA minigene containing T7 DNA, mainly using Northern blots and RNA dot blots to analyse RNA transcripts. Transcription of the minigene was enhanced approx. 15-fold by DNA within a 190 bp *Eco*RI–*Hin*dIII fragment 2 kb upstream from the initiation site in an orientation-dependent manner. This 190 bp fragment lies within the 2.2 kb *Bam*HI element that had a 3-fold effect on transcription of the minigene constructed by ourselves. The difference in the extent of enhancement may have been caused by limitation of some component of the transcription apparatus in yeast containing the minigene in pJDB207, but not in yeast containing YCp50 which carried the minigene of Elion & Warner (1984). The copy number of pJDB207 in yeast is similar to the 100 host rRNA genes (Broach, 1983), whereas YCp50 is present at about one copy per cell. Alternatively, the difference in enhancing activity might have been due to differences in the rDNA component of the constructions. Elion & Warner (1984) also noted that elimination of all rDNA upstream from −210 bp caused transcription to start occasionally at −10 bp and −40 bp. In our experiments, a minor transcript originating at approx. −25 bp was detected in Rp6 and Rp7 that retained all of the nontranscribed spacer, but a transcript originating at −10 bp was not observed in any transformant (Fig. 2).

One explanation of our results that has not been excluded is that the changes in abundance of the minigene transcript were caused by changes in the copy number of the recombinant plasmids in yeast. However, this explanation seems unlikely given the general correspondence between our results and those of Elion & Warner (1984), who used a different type of vector that contained a yeast centromere.

Received 23 May 1985/10 July 1985; accepted 16 July 1985

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