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An RNA Polymerase I Enhancer in *Saccharomyces cerevisiae*

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By the use of an artificial gene coding for rRNA (rDNA gene), we found that transcription of the major precursor rRNA in *Saccharomyces cerevisiae* cells is stimulated 15-fold by a positive control element located 2 kilobases upstream of the transcription initiation site. Analysis of in vitro runon transcripts suggests that this promoter element increases the frequency of initiation by RNA polymerase I molecules. A 190-base-pair fragment encompassing the promoter element can stimulate transcription on a centromere plasmid in either orientation, upstream or downstream of the transcription initiation site, suggesting that it is an enhancer element. Integration of artificial rDNA genes into a nonribosomal locus in the genome demonstrates that the rDNA enhancer functions either 5′ or 3′ to an rRNA transcription unit, suggesting it may operate in both directions within the rDNA tandem array. This is the first observation in *S. cerevisiae* of the stimulation of transcription by an element placed downstream. Finally, enhancer activity is dependent upon sequences that lie at both boundaries of the 190-base-pair fragment. In particular, a 5-base-pair deletion at the extreme 3′ boundary of the 190-base-pair fragment greatly reduces the activation of transcription and implicates a set of inverted repeats.

The transcription of many eucaryotic genes is regulated by a variety of *cis*-acting positive control elements that can stimulate transcription 10- to 200-fold (for reviews, see references 12, 13, and 55). Transcriptional stimulation can be brought about in a variety of ways. Some positive control elements respond to hormones, e.g., the mouse mammary tumor virus long terminal repeat enhancer and the sequences upstream of the chicken vitellogenin gene (3, 21). Others, such as those in the immunoglobulin genes, are expressed only in a specific cell type (11, 38). Many positive control elements respond to specific metabolic or environmental stimuli, such as heme (15), galactose (16), heavy metals (5), or temperature shift (19).

Enhancer elements comprise one class of positive control elements. They are distinguished from other positive control elements by their great flexibility in spacing relative to a transcription initiation site. Usually short regions of DNA (100 to 200 base pairs [bp]), enhancer elements can operate in either orientation, both 5′ and 3′ to a transcription initiation site, as well as over distances of several kilobase pairs (1, 4, 31). On the basis of these criteria, enhancer elements include positive control elements of many viral promoters, including simian virus 40 (1, 31), Moloney murine sarcoma virus (26), and adenovirus (17), and of cellular genes (11a), including the immunoglobulin mu and kappa genes (11, 38).

Until recently, positive control elements had been characterized exclusively for RNA polymerase II genes. However, RNA polymerase I upstream positive control elements have now been described for the rRNA genes of *Xenopus* spp. (32, 40) and *Saccharomyces cerevisiae* (6). The upstream positive control elements in *Xenopus* spp. genes encoding rRNA (rDNA) share similarities with enhancer elements (25, 40). We have shown that the transcription of the *S. cerevisiae* precursor rRNA is stimulated 15-fold by the presence of a 190-bp region of ribosomal spacer DNA (termed the E-H fragment) located 2.2 kilobases (kb) upstream of the transcription initiation site (6). The E-H fragment can stimulate the rate of rRNA transcription whether located at its native position or placed 0.2 kb upstream of the transcription initiation site.

The work presented herein demonstrates that the E-H fragment is able to stimulate transcription of rRNA by RNA polymerase I when placed in either orientation, upstream or downstream of the transcription start site. Its function, however, is strongly influenced by adjacent sequences. Finally, a 5-bp deletion at the extreme 3′ boundary of the 190-bp fragment greatly reduces the activation of transcription. This result implicates a set of inverted repeats.

MATERIALS AND METHODS

Strains and media. The host strains of *S. cerevisiae* used in this study are J400 [*MATa ura3-52 his3 his7 ade1,2 tyr1 rna2*(Ts)] and W303-a, a haploid derivative of W303 (*Mata ade2-1 leu2-3,112 his3-11 trp1-1 ura3-1 can 1-100*; a generous gift from R. Rothstein). Cultures of J400 were grown at the permissive temperature of 25°C. W303-a was grown at 30°C. J400 and W303-a transformants harboring *URA3* plasmids were grown in SC minimal medium (51) without uracil that was supplemented with the appropriate nutritional requirements. W303-a integrants were grown in the same media. Yeast strains tested for segregation of the *URA3* marker were grown in YEPD medium (10 g of yeast extract, 20 g of peptone, and 20 g of glucose per liter). *Escherichia coli* C600 (F^- *thi-1 thr-1 leuB6 lacY1 tonA21 supE44* λ^- *hsdR*$^-$ *hsdM*$^+$) was used for cloning purposes.

Plasmids. (i) YCprR30, YCprR31. The 190-bp *Eco*RI-*Hin*dIII fragment with a *Cla*I linker inserted next to the *Eco*RI site was blunt-end ligated into the *Pvu*II site of YCprR10 (6) after filling in the recessed 3′ ends of the *Cla*I and *Hin*dIII sites with Klenow fragment.

(ii) YCprR32, YCprR33. The 190-bp *Eco*RI-*Hin*dIII fragment was blunt-end ligated into the *Bam*HI site of YCprR10 after filling in all recessed 3′ ends with Klenow fragment.

(iii) YCprR37, YCprR38. YCprR10 and YCprR14 were digested with *Pst*I and *Pvu*II. The 1.07-kb *Pst*I-*Pvu*II fragment encompassing the 190-bp *Eco*RI-*Hin*dIII fragment was purified from low-melting-temperature agarose as described previously (29). The protruding 3′ terminus of the *Pst*I site was rendered flush with T4 DNA polymerase. The fragment

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was then blunt-end ligated into the *Hin*dIII and *Pvu*II sites of YCprR8 after filling in the *Hin*dIII-recessed 3′ end with Klenow fragment.

(iv) YCprR41 through YCprR50. Three *Cla*I fragments of yeast DNA (a 0.5-kg fragment of the actin gene (10, 35) and 2.0- and 0.9-kb fragments of the ribosomal protein S10B gene [24]) were each ligated into the *Cla*I site of YCprR10 and YCprR14.

(v) YCprR51. A 5-μg sample of YCprR10 was linearized with *Hin*dIII, and the protruding 5′ ends were removed exonucleolytically by treating for 15 min with 5 U of mung bean nuclease (New England Biolabs) at 37°C in 30 mM sodium acetate (pH 4.6)–50 mM NaCl–1 mM $ZnCl_2$. The mixture was then made 0.4% sodium dodecyl sulfate, neutralized with 1 M Tris hydrochloride (pH 9.5), and then extracted once with phenol-chloroform-isoamyl alcohol (50:48:2, vol/vol/vol) and ethanol precipitated. The DNA was ligated and then digested with *Hin*dIII to linearize any residual starting plasmid before transformation of *E. coli*. A plasmid lacking the *Hin*dIII site was sequenced directly as follows. A 28-pmol sample of a 20-mer oligonucleotide complementary to rDNA 30 bp downstream of the *Hin*dIII site (of the sequence 5′ CTG CTC CAT GAA GCA AAC TG) was mixed with 0.2 pmol of plasmid digested with *Pst*I (which yields a 5-kb fragment encompassing the mutation) in 50 mM Tris hydrochloride (pH 7.5)–50 mM KCl–10 mM $MgCl_2$. The oligonucleotide primer was annealed to the plasmid by heating the mixture for 5 min at 90°C and then chilling on ice. Sequencing was performed by the dideoxy method as described previously (30).

(vi) YCprR52, YCprR53. A 1.4-kb *Cla*I-*Sph*I fragment of rDNA that overlaps the 190-bp *Eco*RI-*Hin*dIII fragment was isolated from YCprR10 by electrolution from a 4% polyacrylamide gel essentially as described previously (29). A 1-μg sample of purified fragment was digested with *Fok*I and *Scr*FI, generating five fragments of 13, 60, 140, 600, and 580 bp. The protruding 5′ ends were filled in with Klenow fragment, and the mixture of fragments was blunt-end ligated into the *Pvu*II site of YCprR10. Recombinants containing fragments overlapping the 190-bp E-H fragment were identified by colony hybridization, as described previously (29), using nick-translated 190-bp E-H fragment as a probe.

DNA analysis of hybridization positive clones was done to identify recombinants containing the 140-bp *Fok*I-*Scr*FI fragment at the *Pvu*II sites. The orientation of the insert was determined by the asymmetric position of the *Hin*dIII site within the fragment.

RNA isolation and quantitation. RNA was extracted from logarithmically growing cells (40 to 60 Klett units, red filter) exactly as described previously (6). Steady-state levels of T7rRNA (see description in Results) were quantitated by probing slot blots (in a Schleicher & Schuell Minifold II slot blotter) of different amounts of total RNA with a partially duplex M13 probe containing a single-stranded insert of *E. coli* bacteriophage T7 DNA (6). Input was normalized by reprobing the same filter with either nick-translated ribosomal protein gene *TCM1* or *CYH2* DNA (6). A Joyce-Loebl densitometer and a Quantomat Image Analysis system were both used to scan autoradiograms. The level of T7rRNA represented in Fig. 2 through 6 is measured in arbitrary units relative to the amount of T7 rRNA synthesized from a construct containing 200 bp of spacer DNA upstream of the 35S transcription start site (6). The experimental values presented are the mean of at least three independent quantitations of RNA with slot blots. (A certain amount of variation in signal was found to occur among different slot blots; the standard deviation for all of the values given is ±0.5 for values less than 10 and ±1 to 2 for values greater than 10.)

Run-on transcription. A culture containing 2×10^7 to 3×10^7 log-phase cells was chilled by pouring over sterile ice. All subsequent steps except the incubation were carried out at 4°C or in an ice bucket. Cells were collected by centrifugation at 5,000 rpm (Sorvall RC-5B centrifuge) for 6 min and washed with 5 ml of TMN (10 mM Tris hydrochloride [pH 7.4], 100 mM NaCl, 5 mM Mg_2Cl_2). Cells were suspended in 0.95 ml of cold H_2O, to which was added 0.05 ml of 10% (wt/wt) sodium *N*-lauroyl sarcosine (Sigma Chemical Co.). After transfer to an Eppendorf tube, the suspension was left on ice for 15 min and then centrifuged, and the supernatant was removed with a drawn-out Pasteur pipette. The cells were suspended in 100 μl of reaction mix containing 50 mM Tris hydrochloride (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM dithiothreitol, 0.5 mM ATP, 0.25 mM each GTP and CTP, 10 mM phosphocreatine, 1.2 μg of creatine phosphokinase, and 100 μCi of [α-^{32}P]UTP (3,000 Ci/mmol; Amersham Corp.). After incubation for 7.5 min at 25°C, 1 ml of cold TMN containing 1 mM nonradioactive UTP was added, the cells were collected by centrifugation, and RNA was prepared after breaking the cells with glass beads as previously described (6). Yields of 0.5 to 1 cpm per cell were obtained routinely.

Isolation of yeast DNA. A 10-ml culture of stationary-phase yeast cells grown in YEPD was pelleted and suspended in 1 ml of 0.9 M sorbitol–0.1 M EDTA–0.1% β-mercaptoethanol. A 50-μl sample of 5.0-mg/ml zymolyase 5,000 (Miles Laboratories, Inc.) dissolved in the same buffer was added, and the cells were digested for 1 h at 37°C. The spheroplasts were collected by centrifugation at 6,000 rpm (Sorvall RC-5B centrifuge) for 5 min and then lysed in 1 ml of TE (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA) to which 0.5 ml of 0.5 M EDTA (pH 8.5), 0.02 ml of 2M Tris, and 0.07 ml of 10% sodium dodecyl sulfate were added. The suspension was heated for 30 min at 65°C and then incubated for 1 h on ice after the addition of 0.06 ml of 10 M potassium acetate. The precipitated material was removed by centrifugation for 15 min in a microfuge. The yeast DNA was then precipitated by the addition of 0.7 ml of isopropanol, collected by centrifugation, and dissolved in 0.6 ml of TE. The DNA was then treated with RNase A and proteinase K as described previously (29). The treated DNA was reprecipitated by the addition of 0.1 volume of 3 M sodium acetate and 0.54 volume of isopropanol. Samples were dissolved in 0.2 ml of TE; 30 to 40 μl was routinely used for Southern analysis. Yeast DNA was digested with *Hin*dIII, electrophoresed on 0.8% agarose gels, and blotted to nitrocellulose by the method of Southern (46). Nitrocellulose filters were probed with nick-translated (29), replicative-form M13mp8 DNA containing an insert of *E. coli* bacteriophage T7 DNA (6).

RESULTS

rDNA enhancer stimulates RNA polymerase I transcription. Analysis of the sequences specifying and regulating transcription of 35S rRNA has been possible through the use of an artificial rDNA gene, rR8 (Fig. 1b). The details of this gene have been described elsewhere (6); it consists of rDNA sequences allowing proper initiation and termination of 35S rRNA transcription in vivo and a fragment of unique DNA from *E. coli* bacteriophage T7 that serves as a reporter sequence for measurement of transcription from the gene. The rDNA constructs are maintained in yeast cells on the centromere plasmid YCp50, which contains the yeast *URA3*

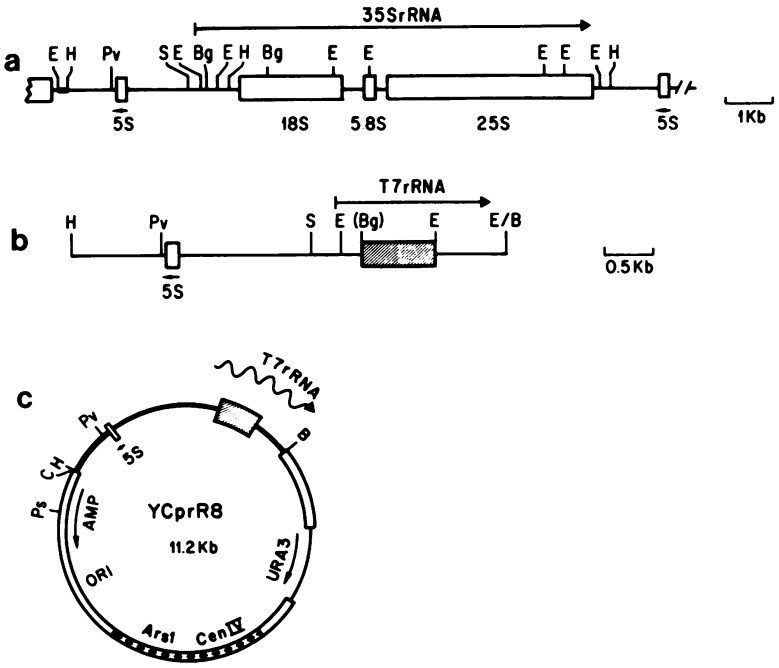

FIG. 1. a, rDNA repeat unit which encodes two divergently transcribed rRNAs, 5S and 35S. The 5′ terminus of 35S is located 48 bp to the left of the *Eco*RI site (22), and the 3′ terminus lies within a 0.6-kb *Eco*RI fragment (2) as shown. The 190-bp *Eco*RI-*Hin*dIII fragment is indicated with a bolder line. b, Construct rR8. It contains a 2.35-kb *Hin*dIII-*Bgl*II fragment of rDNA which includes 130 bp of 35S 5′-terminal coding sequences adjacent to a 0.6-kb fragment of *E. coli* bacteriophage T7 DNA (shown as a hatched box) and a 0.6-kb EcoRI fragment of rDNA encompassing the 3′ terminus of 35S. c, Vector YCprR8 contains the ribosomal construct rR8 and is a derivative of YCp50 which contains the yeast *URA3* gene, ARS1, and centromere IV sequences. All constructs described in this paper were maintained in *S. cerevisiae* on the plasmid YCp50. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II.

gene, an autonomously replicating sequence (ARS), and centromere sequences (Fig. 1c). The advantages of using a centromere plasmid for transcription studies have been discussed (6). The artificial rDNA gene generates a 1.2-kb transcript (termed T7rRNA) whose 5′ terminus is the same as that of 35S rRNA (6). A 190-bp segment (the E-H fragment) located 2.2 kb upstream of the 5′ terminus of 35S rRNA stimulates 15-fold the accumulation of T7 rRNA (compare rR8 and rR10 in Fig. 2) (6). Measurement of transcription of the artificial rDNA genes by pulse-labeling cells with [^{3}H]uracil demonstrated that the E-H fragment stimulates the rate of transcription of T7 rRNA (6). To determine whether transcription of the artificial rDNA gene is carried out by RNA polymerase I, we developed a yeast run-on transcription system to measure the relative resistance of T7 rRNA transcription to α-amanitin, to which *S. cerevisiae* cells are normally impermeable (see Materials and Methods). Cells bearing rR8 or rR10 were prepared for run-on transcription by briefly permeabilizing their cell membranes with sodium *N*-lauroyl sarcosine and then incubating them with ^{32}P-labeled ribonucleoside triphosphates. Under the conditions used the transcription continued for less than 5 min, yet yielded radiolabeled transcription products with an average length of ~3,000 nucleotides (data not shown). This result suggests that most labeled transcripts represent elongation (run-on) of previously initiated RNA chains. The amount of RNA synthesized was measured by hybridization of the radiolabeled RNA to an excess of single-stranded DNA affixed to nitrocellulose (see Materials and Methods). Figure 2a shows that the run-on transcription of rRNA is relatively insensitive to α-amanitin as expected for RNA polymerase I, whereas transcription of the ribosomal protein gene *CYH2*, presumably by RNA polymerase II, is very sensitive to the toxin. Figure 2b shows not only that the enhanced transcription of rR10 compared with rR8 is reproduced in the run-on system but also that the enhanced transcription of rR10 is relatively resistant to 300 μg of

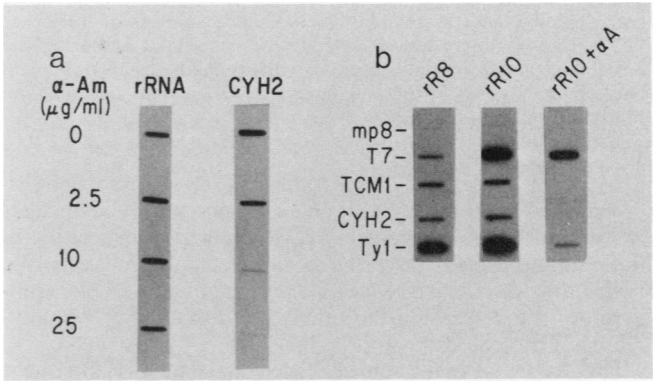

FIG. 2. a, Effect of α-amanitin on RNA polymerase I and RNA polymerase II genes. Yeast cells were prepared for run-on transcription with [α-^{32}P]-labeled nucleoside triphosphates as described in Materials and Methods and incubated in the presence of 0, 2.5, 10, or 25 μg of α-amanitin per ml. The resultant labeled RNA was extracted and hybridized to single-stranded DNA probes containing either a fragment of rDNA complementary to the 5′ transcribed spacer region of 35S rRNA or *CYH2* DNA. The film for the rRNA slots was exposed for 0.25 h; that for the *CYH2* slots was exposed for 22 h. b, Effect of α-amanitin on transcription of T7 rRNA. Cells carrying plasmids YCprR8 or YCprR10 were prepared for run-on transcription and incubated in the presence or absence of 300 μg of α-amanitin per ml. The resultant labeled RNA was hybridized to slot blots loaded with excess single-stranded DNA carrying the indicated genes.

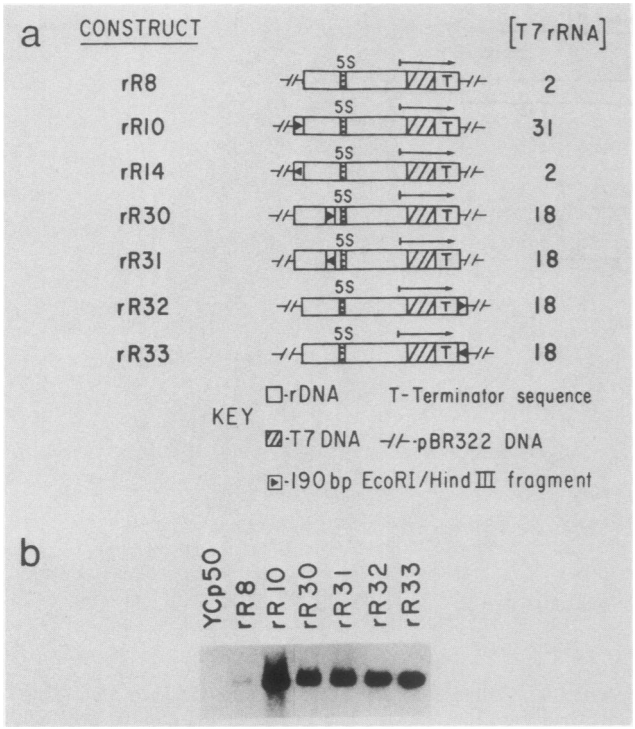

FIG. 3. Effect of orientation and position on function of the 190-bp *Eco*RI-*Hin*dIII fragment. a, Left column indicates the names of the various ribosomal constructs, the center column shows their diagram, and the right column shows the amount of T7rRNA observed. The numbers shown here are derived from densitometry tracings of autoradiograms of RNA slot blots probed with *E. coli* bacteriophage T7 DNA as described in Materials and Methods. The amounts are normalized by reprobing the slot blots with the ribosomal protein gene *TCM1* or *CYH2*. All numbers are relative to the amount of T7rRNA produced from a construct containing only 200 bp of 5′ flanking spacer (rR9) (6). The values given represent the averages of at least three independent determinations of RNA concentration with a slot blot. b. Northern analysis of T7rRNA. Total RNA was isolated from cells of strain J400 harboring the designated constructs and probed with a single strand of *E. coli* bacteriophage T7 DNA homologous to T7rRNA as described in Materials and Methods.

α-amanitin per ml, whereas transcription of three protein-coding genes, *CYH2*, *TCM1*, and Ty1, was nearly abolished. A role for RNA polymerase III, which is also insensitive to α-amanitin, cannot be ruled out but seems unlikely. We conclude that the rDNA enhancer augments rRNA transcription by increasing the number of initiating RNA polymerase I molecules.

E-H fragment is an enhancer element. The E-H fragment was found initially to function in only one orientation at its native position within the rDNA repeat (rR10 versus rR14 in Fig. 3) (6), suggesting that it might not be an enhancer element in the strict sense. To determine more conclusively whether the E-H fragment has the properties of an enhancer element, we made four constructs that vary the position and orientation of the element relative to the artificial rDNA gene rR8 (Fig. 3a). The 190-bp *Eco*RI-*Hin*dIII fragment was ligated into either the *Pvu*II or *Bam*HI sites of YCprR10 (Fig. 1c) after filling in all recessed 3′ ends with Klenow fragment (see Materials and Methods) (Fig. 3a). The *Eco*RI-*Hin*dIII fragment is 1.46 kb 5′ to the transcription start site in rR30 and rR31 (and 110 bp 3′ to the 5S gene) and 1.3 kb 3′ to the transcription start site in rR32 and rR33, immediately adjacent to the *Eco*RI E fragment as normally found in the rDNA tandem repeat. Total RNA from cells bearing the artificial rDNA genes was isolated, and the steady-state level of T7rRNA was quantitated by dot-blot analysis with a single stranded T7-specific probe and densitometric scanning of the resulting autoradiograms. Amounts of T7rRNA were normalized by reprobing the blots with the ribosomal protein gene *CYH2* or *TCM1* (see Materials and Methods). The size of the transcripts generated from the various constructs was analyzed by Northern blot hybridization (Fig 3b). It is clear from Fig. 3b that the E-H fragment enhances transcription when inserted in either orientation at a *Pvu*II site 1.1 kb upstream of the 35S rRNA initiation site (rR30 and rR31), as well as when inserted in either orientation at a *Bam*HI site 1.5 kb downstream of the 35S initiation site (rR32 and rR33 in Fig. 3). Thus, the E-H fragment has many of the properties of a RNA polymerase II enhancer.

Integrated T7rDNA genes. The interpretation of the results obtained with rR32 and rR33 is not, however, unambiguous. Since the T7 rDNA gene is on a circular plasmid, the E-H element could be acting from far upstream, around the circle. This seems unlikely because the element is separated from the 5′ end of the artificial rDNA gene by 8 kb of DNA that includes the *URA3* gene, yeast ARS and centromere sequences, and actively transcribed pBR322 DNA. It is worth noting that the criteria used to define most enhancer elements have been based on experiments utilizing plasmids rather than chromosomes (1, 11, 31, 38).

A more definitive experiment, clearly, would be to integrate the artificial rDNA genes into the genome. To avoid adjacent E-H fragments in the rDNA repeats on chromosome XII, we integrated the constructs rR8, rR10, and rR32 into the *ura3-1* locus of yeast strain W303-a by using the yeast integrative vector YIp5, which carries the *URA3* gene (Fig. 4). This plasmid differs from the centromere plasmid YCp50 used in our other studies only by the lack of centromere IV and ARS1 sequences. Thus, the integrated constructs are flanked by the same vector sequences previously tested on a circular plasmid. Any differences found in the transcription of the constructs cannot, therefore, be attributed to the influence of immedately adjacent sequences.

For the integrative transformation, plasmids were linearized within *URA3* sequences with the endonuclease *Nco*I. Southern analysis of mitotically stable Ura$^+$ transformants showed that proper integration at the *ura3* locus occurred in 15 out of 59 transformants, whereas 39 of the transformants arose from integration into the rDNA locus on chromosome XII. Of these, 23 had plasmid sequences at both the *ura3-1* and rDNA loci. A representative Southern analysis of 12 Ura$^+$ transformants is shown in Fig. 4b. Proper integration of the plasmid into the *ura3-1* locus yields a 5.7kb *Hin*dIII fragment containing T7 sequences (Fig. 4b, lanes 1, 2, 4, 6, and 11). Integration of the plasmid into the rDNA repeat generates a fragment of ~9 kb (Fig. 4b, lanes 3, 7, 9, and 10), whereas integration of the plasmid into the *ura3* and rDNA loci yields both fragments (Fig. 4b, lanes 5 and 12). One Ura$^+$ strain lacks T7 and pBR322 sequences, suggesting that proper integration may have been followed by a recombination event that left behind an intact *URA3* gene (Fig. 4b, lane 8).

Transcription of the integrated constructs was determined as described in Materials and Methods. RNA was isolated from two independent integrants for each construct (Fig. 4). In strain W303-a, the effect of the enhancer on the plasmid was 7-fold rather than the 15-fold found in J400. Neverthe-

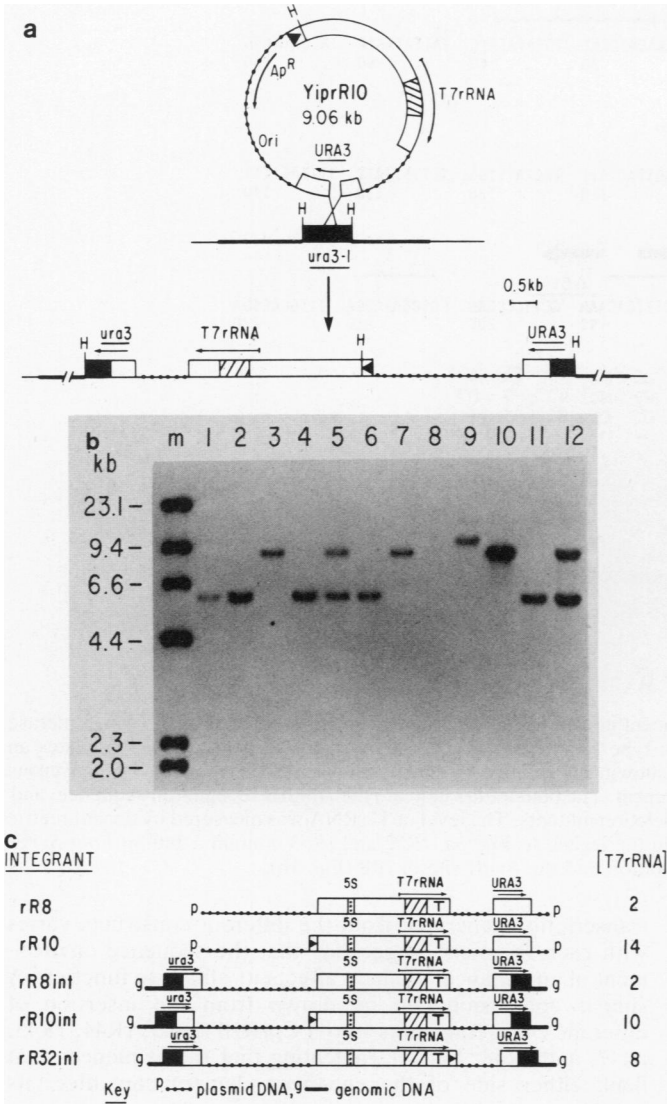

FIG. 4. a, Integration of ribosomal constructs into the *ura3-1* gene of strain W303-a. Ribosomal constructs were cloned into the *Hin*dIII-*Bam*HI sites of YIp5. To direct homologous recombination into the host *ura3-1* gene the resultant vectors were linearized with NcoI which cuts once within *URA3* sequences and used to transform the Ura$^-$ yeast strain W303-a by the method of Ito et al. (20). Mitotically stable Ura$^+$ transformants were analyzed by Southern analysis. b, Southern analyses of Ura$^+$ transformants. Total yeast DNA was digested with *Hin*dIII, electrophoresed on 0.8% agarose gels, transferred to nitrocellulose, and hybridized to nick-translated, replicative-form M13mp8 containing a fragment of *E. coli* bacteriophage T7 DNA. Proper integration of the vector into the *ura3-1* locus generates a 5.7-kb *Hin*dIII fragment that contains T7 sequences, whereas vector integration into the rDNA repeat on chromosome XII generates a 9-kb fragment homologous to T7 DNA. Lanes 1 through 12 contain total yeast DNA from 12 representative mitotically stable Ura$^+$ transformants from strain W303-a transformed with YIprR32. The 5.7-kb fragment (lanes 3, 7, 9, and 10) indicates integration of YIprR32 into the *ura3* locus as shown in panel a. The ~9-kb fragment indicates integration of the plasmid into the rDNA repeat. c, Diagram of T7 rDNA integrants at the *URA3* locus. The left column indicates the name of the integrant and can be compared with the constructs in Fig. 3a. The center column is a diagram of the integrants and shows that the sequences immediately flanking the constructs are the same whether on plasmids or integrated. Levels of T7rRNA were determined as described in the legend to Fig. 3a and Materials and Methods.

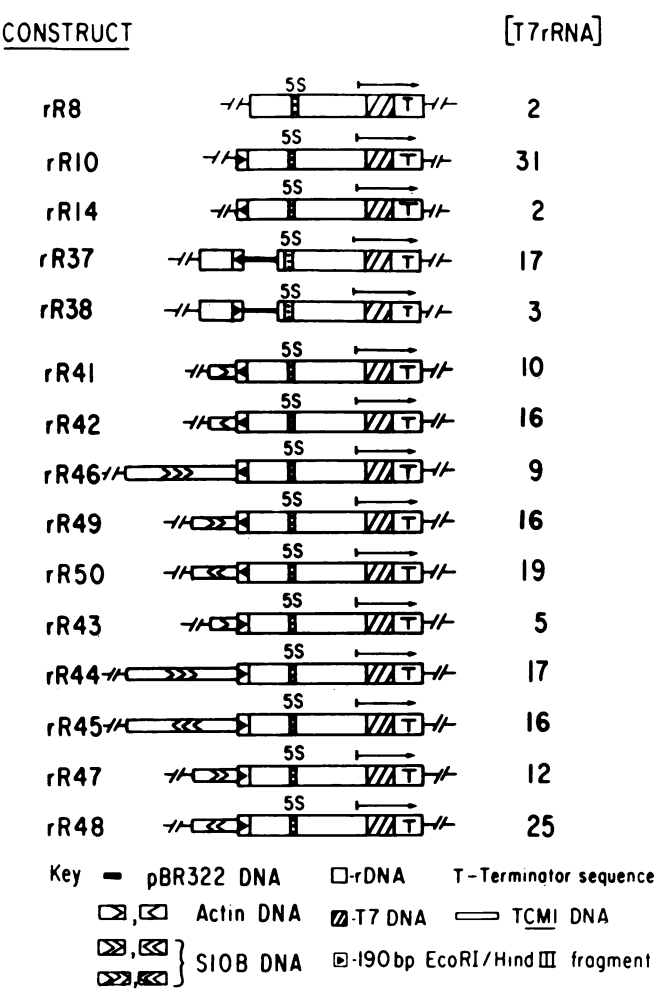

FIG. 5. Effect of flanking sequences on T7rRNA transcription. Three *Cla*I fragments of yeast DNA were cloned into the *Cla*I site of YCprR14 and YCprR10 as shown. The arrow-heads indicate the orientation of the fragments shown in the key. Levels of T7rRNA were determined as described in Materials and Methods and the legend to Fig. 3a.

less, the same relative amounts of T7rRNA are produced from rR8, rR10, and rR32 when they reside within the *URA3* locus as when they are on a centromere plasmid (Fig. 3 and 4c). This result demonstrates that the enhancer is effective within the genome both upstream and downstream of the site of initiation of transcription. We conclude that the effect of the enhancer does not depend on the unique properties of circular plasmids.

Flanking sequences affect the function of the rDNA enhancer. That the E-H fragment can effectively stimulate transcription in an orientation- and position-independent manner satisfies the definition that it is an enhancer element. Nevertheless, its effectiveness is not invariable. In particular, the E-H fragment does not function at all in rR14, which differs from rR10 only in the orientation of the rDNA enhancer (Fig. 3). One obvious explanation for the asymmetry of the effect of the rDNA enhancer at its native position is that flanking sequences could inhibit its function. To test this possibility, a 1.7-kb *Pst*I-*Pvu*II fragment encompassing the rDNA enhancer flanked by 0.75 kb of pBR322 DNA on the left and 0.74 kb of rDNA on the right was inverted in rR10 and rR14 to generate rR37 and rR38, respectively (see

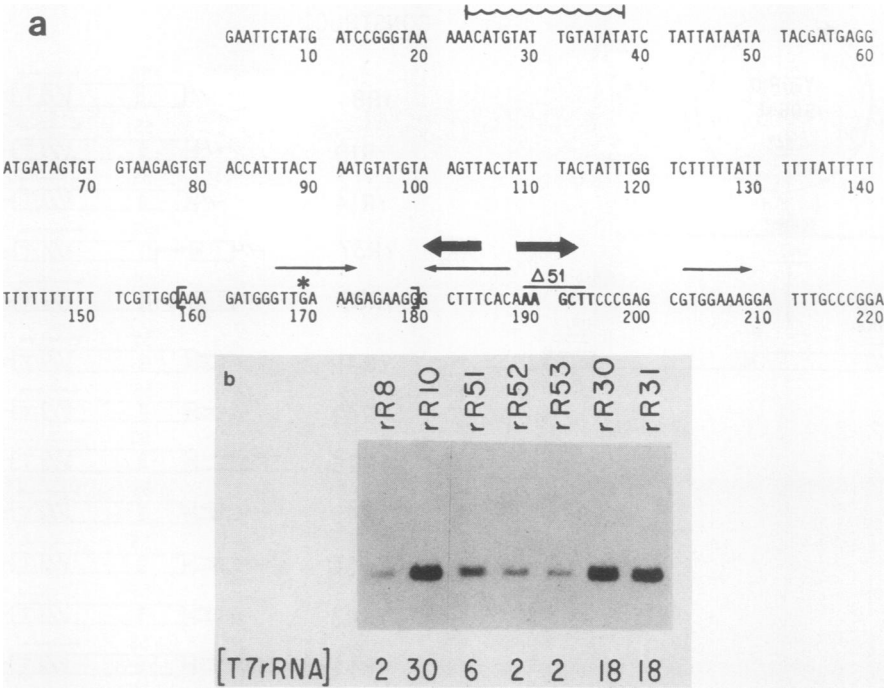

FIG. 6. a, Sequence of the 190-bp *Eco*RI-*Hin*dIII fragment and adjacent nucleotides. The brackets indicate the in vitro RNA polymerase I promoter (48), the asterisk indicates a nucleotide difference between type I and type II rDNA (49; see text), the wavy line indicates an alternating purine pyrimidine tract, the thin arrows indicate repeats showing homology to the simian virus 40 enhancer core consensus sequence 5'GTGG$\overset{AAA}{TTT}$G (52), and the thick arrows indicate an inverted repeat. The bold letters indicate the *Hin*dIII recognition sequence, and above them is shown the 5-bp deletion in rR51. b. Northern analysis of deletion mutants. The level of T7rRNA was measured by densitometric quantitation of slot blots as described in Materials and Methods and in the legend to Fig. 3a. rR52 and rR53 contain a 140-bp *Fok*I-*Scr*FI fragment (nucleotides 77 through 217 in panel a) cloned in either orientation into the *Pvu*II site of rR8 (Fig. 1b).

Materials and Methods; Fig. 1c and 5). The *Pst*I and *Pvu*II sites were chosen because they are approximately equidistant from the E-H fragment, which will then be the same distance from the initiation site in all four constructs. If the function of the rDNA enhancer is relatively independent of orientation but strongly dependent on the nature of the flanking sequences, then the relative amounts of T7rRNA produced from rR37 and rR38 should remain similar to those produced from their parental constructs, rR10 and rR14, since the sequences surrounding the enhancer element have remained the same. Indeed, rR37 yields substantially more T7rRNA than does rR38 (Fig. 5), demonstrating that neighboring sequences influence the effectiveness of the rDNA enhancer far more than does orientation.

To examine further the effect of the sequences flanking the rDNA enhancer, different fragments of yeast DNA were inserted at the *Cla*I site of YCprR10 (Fig. 1c) and YCprR14. If pBR322 DNA were inhibiting the enhancer function in rR14, then the insertion of other sequences between vector and enhancer sequences might relieve this inhibition. Three *Cla*I fragments of yeast DNA were used: 0.5 kb of the actin gene coding sequence (10, 35), 2.0 kb of the ribosomal protein S10B gene (*RPS10B*) coding sequence and 3′ flanking DNA and 0.9 kb of *RPS10B* 5′ flanking sequences (24). Coding sequences were used since they should not contain transcriptional control signals. In contrast, the *RPS10B* 5′ flanking sequences are likely to contain RNA polymerase II transcriptional signals. The insertion of any one of the three fragments of yeast DNA relieved the inhibition of the rDNA enhancer in rR14 (rR41, rR42, rR46, rR49, and rR50; Fig. 5), demonstrating that in rR14 the pBR322 sequences adjacent to the E-H fragment are inhibitory. Moreover, the level of transcription generated from the different constructs varies with each fragment, suggesting that the sequence environment of an enhancer element affects its ability to function. A similar conclusion can be drawn from the insertion of different DNA fragments into YCprR10 (rR43, rR44, rR45, rR47, and rR48; Fig. 4), indicating that the sequences that flank either side of the enhancer element can affect its function.

Enhancer activity of the E-H fragment requires sequences at its 5′ and 3′ boundaries. The sequence of the E-H fragment has been determined for 21 independently isolated copies of the rDNA repeating unit (Fig. 6) (6, 45, 49). Whereas the A+T-rich region in the center exhibits substantial sequence polymorphism, both borders are highly conserved and thus likely to be involved in the function of the enhancer. Interestingly, the region near the *Eco*RI site contains an almost perfect run of alternating purines and pyrimidines, potentially able to form Z-DNA (nucleotides 23 through 38) (6, 41).

A computer search of the 190-bp fragment revealed two sets of overlapping inverted repeats. Set A includes two 5-bp inverted repeats that overlap the *Hin*dIII site (Fig. 6a, thick arrows). Because all subcloning of the 190-bp *Eco*RI-*Hin*dIII fragment was done after filling in the *Hin*dIII site (see Materials and Methods), this repeat is intact in all constructs described so far. To determine whether the function of the rDNA enhancer element is dependent upon this sequence, we made a 5-bp deletion at the *Hin*dIII site of rR10 by briefly treating the 5′ ends with mung bean nuclease. The resulting construct, rR51, yields substantially reduced levels of T7 rRNA as shown by Northern analysis (Fig. 6b), suggesting that the inverted repeat has a role in the rDNA enhancer

function. The relative amounts of T7rRNA shown in Fig. 6b were determined by densitometric quantitation of slot blots as described in Materials and Methods.

A second set, B, of 7-bp repeats that overlaps repeat A includes two to the left and one to the right of the *Hin*dIII site (Fig. 6a, thin arrows). Surprisingly, this GTGAAAG sequence is similar to the simian virus 40 enhancer core consensus sequence GTGG$^{AAA}_{TTT}$G (52). However, these repeats are not necessarily critical for the rDNA enhancer activity. For example, the repeat to the right of the *Hin*dIII site is not present in several rDNA constructs that still exhibit enhancer activity (6; E. A. Elion, Ph.D. thesis, Albert Einstein College of Medicine, 1985).

We next asked whether the conserved region near the *Eco*RI site (including the purine-pyrimidine tract) is necessary for enhancer function. If maximal enhancer activity were solely dependent upon the presence of the repeats surrounding the *Hin*dIII site, one might expect a subclone encompassing all of them to function better than the E-H fragment. Therefore, a 140-bp *Fok*I-*Scr*FI fragment which includes nucleotides 77 through 190 of the E-H fragment plus an additional 27 nucleotides downstream of the *Hin*dIII site, was isolated and subcloned into the *Pvu*II site of rR8, generating rR52 and rR53 (Fig. 6a and b). To avoid variations due to differences in flanking sequences the transcription of rR52 and rR53 was compared with that of rR30 and rR31, in which the E-H fragment functions at the same *Pvu*II site. Surprisingly, the *Fok*I-*Scr*FI fragment did not function in either orientation at the *Pvu*II site. Apparently, the rDNA enhancer function requires sequences that lie within the first 77 nucleotides of the E-H fragment. Thus, the repeats alone cannot confer enhancer activity.

DISCUSSION

rRNA major promoter element is an enhancer. We have examined in detail a series of artificial rDNA constructs to analyze the function of the major promoter element of rRNA genes. We conclude that the yeast rRNA major promoter element is an RNA polymerase I enhancer sharing many properties of the well-characterized RNA polymerase II viral enhancer elements. A 190-bp fragment encompassing the promoter element can stimulate transcription on a centromere plasmid when placed in either orientation, upstream or downstream of the rRNA transcription initiation site. In addition, it can also stimulate transcription when placed 5′ or 3′ to the artificial rDNA gene integrated in the genome. Analysis of in vitro run-on transcription products demonstrates that the rDNA enhancer functions by increasing the number of initiating RNA polymerase molecules. Resistance of such transcription to α-amanitin suggests that RNA polymerase I is most likely the enzyme involved.

The RNA polymerase I rDNA enhancer is distinct from the two types of RNA polymerase II enhancer-like elements that have been described for *S. cerevisiae*. Upstream activating sequences can function in either orientation at variable distances 5′ to a TATA box (14, 47, 53). However, they differ from the rDNA enhancer in that they do not function 3′ to a gene (14, 47), nor do they generally function when more than 1 kb from the initiation site (14). Yeast transposable elements stimulate transcription of adjacent RNA polymerase II genes in a manner analogous to retroviruses (8, 37, 42). Genetic selections for up-promoter mutations of several genes have yielded Ty elements transposed into the regulatory regions only at the 5′ ends of yeast genes (7, 42, 43, 54). That no Ty insertions 3′ to any gene have been found suggests that Ty elements may stimulate transcription only from a position 5′ to a gene.

The rDNA enhancer differs from other yeast promoter elements in that extended regions of DNA are necessary for its stimulation of transcription. This is in contrast to upstream activating sequences whose function is often encompassed in short (6- to 30-bp-long) repeats that can function in single or multiple copies (18, 33, 34, 44, 53). Both the rDNA enhancer and Ty enhancer contain sequences homologous to the simian virus 40 enhancer core consensus sequence (8, 42), which might suggest that the two elements interact with the same *trans*-acting factors. However, whereas the Ty element is under mating type control and is repressed in *Mat***a**/*MAT*α diploids (8, 42), the rDNA enhancer is not (data not shown).

Enhancer function is influenced by the adjacent sequences. The stimulation of transcription by the RNA polymerase I enhancer is sensitive to its sequence environment. Flanking pBR322 sequences inhibit the rDNA enhancer when it is positioned in one orientation (rR14, Fig. 5), whereas they have a neutral (or possibly stimulating) effect when the enhancer is positioned in the opposite orientation (rR10, Fig. 5). In addition, we find that the magnitude of enhancer activity is sensitive to its sequence context, since the insertion of several different fragments of yeast DNA at either boundary of the 190-bp fragment led to variations in enhancer activity (Fig. 5). We conclude from these experiments that enhancer activity is not autonomous, but rather dependent upon its sequence environment. Such a finding has been reported for a number of upstream promoter elements including the *HIS4*, *CYC1*, and *GAL1-GAL10* upstream activating sequences in yeast (14, 28, 53) and the simian virus 40 72-bp repeat (31).

Enhancer function is bidirectional. The rDNA enhancer, when placed either upstream or downstream, stimulates transcription of the artificial rDNA gene not only on a plasmid, but also when integrated into a nonribosomal locus in the genome. This finding suggests the enhancer may function bidirectionally in the genome, a particularly interesting idea since the rDNA repeats on chromosome XII are arranged in a tandem head-to-tail array with the enhancer located between two transcription units.

Role of the rDNA enhancer. We conclude that the 190-bp *Eco*RI-*Hin*dIII fragment serves to stimulate transcription of 35S rRNA in vivo. Work by others (48, 49) suggests that the region between nucleotides 158 and 189 contains an RNA polymerase I promoter that is active in vitro (Fig. 6). However, based on our in vivo evidence and the reasons outlined below, it is not clear that this in vitro promoter is physiologically relevant.

Studies by a variety of workers have failed to find substantial transcription of the nontranscribed spacer between the E-H fragment and the beginning of 35S rRNA (23, 27, 48). In vivo transcription of 35S rRNA appears to initiate at the beginning of 35S rRNA (36). The promoter observed in vitro could, however, be an entry site for RNA polymerase I. A comparison of the nucleotide sequences of 17 ribosomal repeats from three yeast strains identified two types, which differ slightly in sequence (49). Type I repeats support efficient in vitro transcription initiation, whereas type II repeats do not. The 190-bp fragment used in our studies is a type II repeat. Therefore, in vitro transcriptional activity does not necessarily correlate with in vivo enhancer activity.

It is premature to propose a detailed model for the role of the rDNA enhancer. Nevertheless, one can speculate that it binds a transcription factor(s) which serves to attract RNA

polymerase I. An extension of this view is that within the genomic rDNA it could serve to catch the RNA polymerase I molecules which have just completed the transcription of the adjacent rRNA gene, i.e., to coordinate transcription termination with reinitiation at the 35S start site. In this connection it is interesting that recent work by J. Klootwijk and R. Planta (personal communication) suggests that the 3′ end of 35S rRNA is a processing intermediate and that termination of transcription actually occurs within the T runs (nucleotides 121 to 151) of the E-H fragment (Fig. 6). Our finding that sequences on both sides of the T runs are important for activity lends some support to the idea that the enhancer serves to trap terminating RNA polymerase molecules.

Are all RNA polymerase I enhancers alike? An RNA polymerase I enhancer has been previously described in the rDNA repeat of *Xenopus* sp. (for review, see reference 39). Like the *S. cerevisiae* rDNA enhancer, the *Xenopus* sp. rDNA enhancer will function in either orientation at a distance of several kilobases 5′ or 3′ to the rRNA transcription start site (25, 39). In addition, its effect can be transmitted through an active rRNA promoter to stimulate transcription of a nonadjacent rDNA gene (25, 39). This characteristic holds true for the *S. cerevisiae* rDNA enhancer since its effect is transmitted across a 5S gene.

However, there are important differences between the enhancers of the two species. The *S. cerevisiae* rDNA enhancer sequence is unique in the rDNA repeat, bearing little or no homology to the downstream promoter region (45). In contrast, the *Xenopus* spp. enhancer consists of variable numbers of 60- to 81-bp repeats that are 90% homologous with a 42-bp domain of the rRNA promoter (39). Therefore, it has been suggested that the *Xenopus* spp. enhancers can act as multiple loading sites either for RNA polymerase I or for transcription factors (39). The role of the *S. cerevisiae* rDNA enhancer is more obscure.

The *Xenopus* sp. rDNA enhancer does not involve the 5S RNA genes, which are unlinked and whose transcription, at least in the well-studied oogenesis system, is not coupled to that of rRNA (9). In *S. cerevisiae*, however, there is some evidence that 5S RNA and rRNA are transcribed in a 1:1 molar ratio (50). Because the enhancer must act across the 5S gene to stimulate 35S rRNA transcription (Fig. 1), it may serve to couple tightly the transcription of 5S and 35S rRNA.

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