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A system to study transcription by yeast RNA polymerase I within the chromosomal context: functional analysis of the ribosomal DNA enhancer and the RBP1/REB1 binding sites

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We have developed a novel system to study transcription by yeast RNA polymerase I (Pol I) of mutated rDNA units within the chromosomal context. For this, complete rDNA units carrying specific oligonucleotide tags in both the 17S and 26S rRNA genes were integrated into the chromosomal rDNA locus. Using this novel system, we analysed the action of the rDNA enhancer in stimulating transcription within the chromosomal context. We found that the enhancer acts as a stimulatory element in both directions, mainly on its two most proximal rRNA operons. Deletion of the sequences between the enhancer and the Pol I promoter in the tagged, integrated unit indicated that this part of the intergenic spacer contains no other transcriptional regulatory elements for Pol I. We also applied the system to study the function of the rDNA binding protein RBP1/REB1. For this purpose, we analysed tagged units in which either one or both of the binding sites for this protein have been inactivated. We found that mutations of both binding sites strongly diminish the transcription of the adjacent operon. The protein is hypothesized to play a crucial role in keeping the chromosomal rDNA units in an optimal spatial configuration by anchoring consecutive enhancers and promoters to the nucle(ol)ar matrix.

Key words: enhancer/REB1/ribosomal DNA/ribosome biogenesis/yeast

Introduction

Regulation of transcription of the rRNA genes may be central to the intricate process of ribosome biosynthesis in response to environmental conditions. In *Saccharomyces cerevisiae* the rRNA genes are organized in a tandem array of ~200 units on chromosome XII (Petes, 1979; Warner, 1989). The genes encoding 17S, 5.8S and 26S rRNA are arranged in a pre-rRNA operon that is transcribed by RNA polymerase I (Pol I) in the nucleolus. Unlike other eukaryotes, the *S.cerevisiae* rDNA unit also contains a 5S rRNA gene, which is located within the spacer regions between the pre-rRNA operons and is transcribed by Pol III (Phillippsen *et al.*, 1978). The *cis*-acting elements involved in yeast Pol I transcription have been extensively studied (reviewed by Raué and Planta, 1991). The boundaries and substructure of the Pol I promoter have been analysed *in vivo* as well as *in vitro* (Musters *et al.*, 1989b; Kulkens *et al.*, 1991). A region from −155 to +27 with respect to the Pol I transcription initiation site is required for accurate and efficient initiation of transcription. In addition a 170−190 bp enhancer element, responsible for a 15- to 30-fold increase in transcription has been identified in the spacer, ~2.2 kb upstream of the initiation site (Elion and Warner, 1984, 1986). The enhancer element also contains the main terminator, T2 (Kempers-Veenstra *et al.*, 1986; Mestel *et al.*, 1989; Van der Sande *et al.*, 1989). The location of the Pol I terminator within the enhancer has led us to propose a model that accounts for efficient recycling of Pol I molecules and/or transcription factors (Kempers-Veenstra *et al.*, 1986). In this model, the so-called 'ribomotor', the terminator/enhancer element, is brought in the vicinity of the Pol I promoter by looping out the Pol I transcription unit. Pol I molecules that have terminated at T2 can immediately be passed on to the promoter by means of the enhancer. To induce the proposed association between promoters and enhancers and to stabilize such a structure a protein factor(s) may be involved. The ribosomal DNA binding protein RBP1/REB1, which binds the rDNA enhancer and also close to the Pol I promoter (Morrow *et al.*, 1989, 1990; Kulkens *et al.*, 1989) seems to be an attractive candidate for this function. In the loop structures that are supposed to be formed this way, the promoter and enhancer of one and the same operon can be juxtaposed or alternatively, promoter and enhancer of adjacent units can be brought together. To distinguish between these different possibilities it is important to know whether the enhancer acts as a stimulatory element on the upstream or downstream unit or whether it works bidirectionally, particularly within the chromosomal context of the tandem array of rDNA units.

Functional analysis of the rDNA enhancer and the RBP1/REB1 binding sites has been performed mainly by using episomal rDNA minigenes or mini rDNA repeats integrated into a non-rDNA locus (Elion and Warner, 1984, 1986; Johnson and Warner, 1989; Kulkens *et al.*, 1989). Obviously these experimental systems cannot account for all specific features of rDNA transcription in its natural locus. Specifically, the high degree of tandem repetition of the rDNA genes and their presence in a specialized area of the nucleus, the nucleolus, containing high concentrations of specific proteins important for rDNA transcription, cannot be mimicked by the systems exploited thusfar. We have seeked a way to circumvent the above-mentioned experimental problems and developed an experimental system that optimally mimics the natural context of Pol I transcription. To this end we used rDNA units carrying oligonucleotide tags in both their 17S and 26S rRNA genes for integration into the rDNA locus. The tags allowed for specific detection of only the transcripts from such a unit, and made it possible to study the effects of rDNA mutations on Pol I transcription

within their natural chromosomal context. Tagged integrated rDNA units were shown to be transcribed with similar efficiency as normal, endogenous rDNA units. Using this novel approach, we studied the effects of mutating the rDNA enhancer and the RBP1/REB1 binding sites on Pol I transcription. The enhancer can stimulate transcription by Pol I in both directions, mainly of the two most proximal units. Mutations of the RBP1/REB1 binding sites that abolish RBP1/REB1 binding decrease Pol I transcription drastically.

Results

A novel system to study the regulation of Pol I transcription

For the study of Pol I transcription under conditions that accurately mimic the chromosomal Pol I transcription, we decided to transform yeast with linear DNA fragments consisting of exactly one complete rDNA unit in which the genes for both 17S and 26S rRNA have been tagged by the insertion of small oligonucleotides. Earlier studies indicated that these insertions behave as neutral mutations with respect to rRNA transcription, ribosome formation and ribosome function (Musters *et al.*, 1989a, 1990). Integration of one or a few of such units in the rDNA locus leads to a situation where the effect of mutations of the rDNA can be studied in the correct chromosomal context. Obviously, the site at which the tagged rDNA is cut determines the site of integration and therefore determines whether the 17S and 26S rRNA tags will be present in two consecutive rDNA units or in one and the same unit. Likewise, it determines the position of the additional mutations to be studied relative to the position of the tags. To allow for some flexibility in generating tagged rDNA units for integration, we constructed a plasmid (pORIS, Oligonucleotide-tagged Ribosomal Integrated DNA of *S.cerevisiae*) that contains somewhat more than a single rDNA repeat in a pUC vector, starting at the *Sph*I site in the 5.8S rRNA gene through the corresponding site in the next rDNA unit and followed by a stretch of rDNA sequence up to *Mlu*I, present in the 26S rRNA gene (Figure 1). In addition, we constructed a pORIS derivative in which the *Hpa*I site in the intergenic spacer was destroyed (pORIS-H; Figure 2B). As a result of these cloning steps, tagged rDNA units for integration into the chromosomal rDNA could be isolated as *Hpa*I (from pORIS-H) or *Mlu*I repeats (from pORIS). When *Mlu*I repeats were used for integration, the tagged 17S and 26S rRNA genes remained part of the same transcription unit (Figure 2A), whereas in the case of a *Hpa*I unit, the tagged genes became part of different Pol I transcription units (Figure 2B). This second type of integration therefore allowed us to simultaneously monitor the transcription of two flanking Pol I transcription units.

Since it is not possible to select directly for yeast transformants that have integrated one or more tagged rDNA units, we set up a procedure for pre-selection of such transformants using co-transformation with a plasmid (YEp13) containing a *LEU2* marker. We transformed *S.cerevisiae* MG34 with both YEp13 and rDNA fragments derived from pORIS (*Mlu*I repeat; Figure 2A) and pORIS-H (*Hpa*I repeat; Figure 2B) respectively. Positive pORIS transformants containing a tagged integrated unit were scored by PCR analysis of DNA of Leu^+ transformants using the 17S and 26S rRNA oligonucleotide tags as primers. When the transformants had one or more tagged units integrated, a PCR product of ~1.3 kb was visible after agarose gel electrophoresis. Using optimal co-transformation conditions (1 μg of YEp13 and 5–10 μg pORIS fragment) the yield of co-transformants was 5–10%.

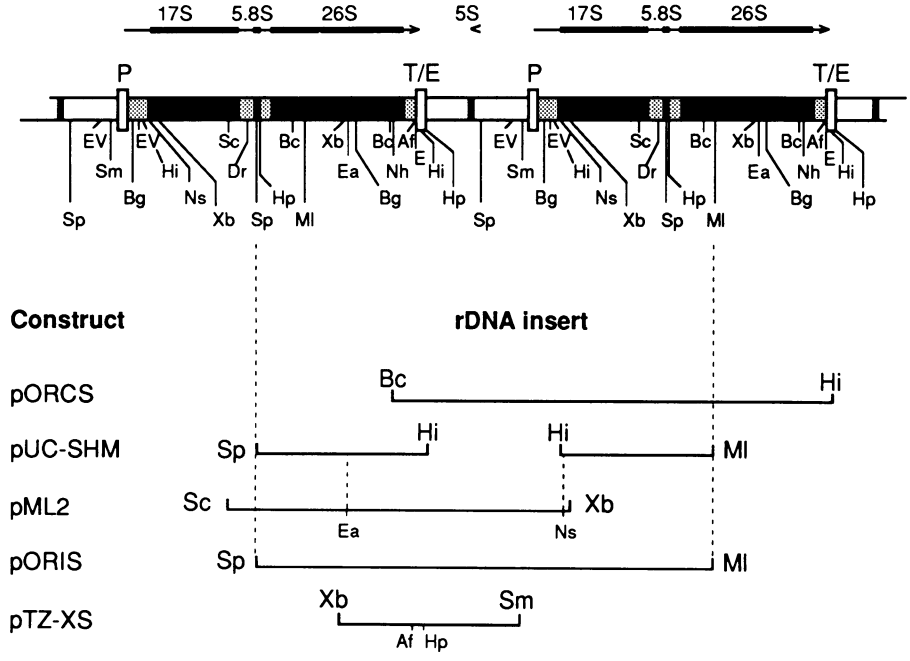

Fig. 1. Construction of pORIS. Two tandemly repeated rDNA units are aligned with the rDNA inserts of various recombinant plasmids. See Materials and methods for details on cloning procedures. Black bars represent the genes for the mature rRNAs (17S, 5.8S, 26S and 5S rRNA), shaded bars represent the transcribed spacer regions and white bars represent the intergenic spacer. Arrows represent the primary Pol I transcript. In addition, the promoter (P) and terminator/enhancer (T/E) element are indicated. Abbreviations for restriction enzyme sites are as follows: Af = *Afl*II, Bc = *Bcl*I, Bg = *Bgl*II, Dr = *Dra*I, Ea = *Eag*I, E = *Eco*RI, EV = *Eco*RV, Hi = *Hin*dIII, Hp = *Hpa*I, MI = *Mlu*I, Nh = *Nhe*I, Ns = *Nsi*I, Sc = *Sac*I, Sm = *Sma*I, Sp = *Sph*I, Xb = *Xba*I.

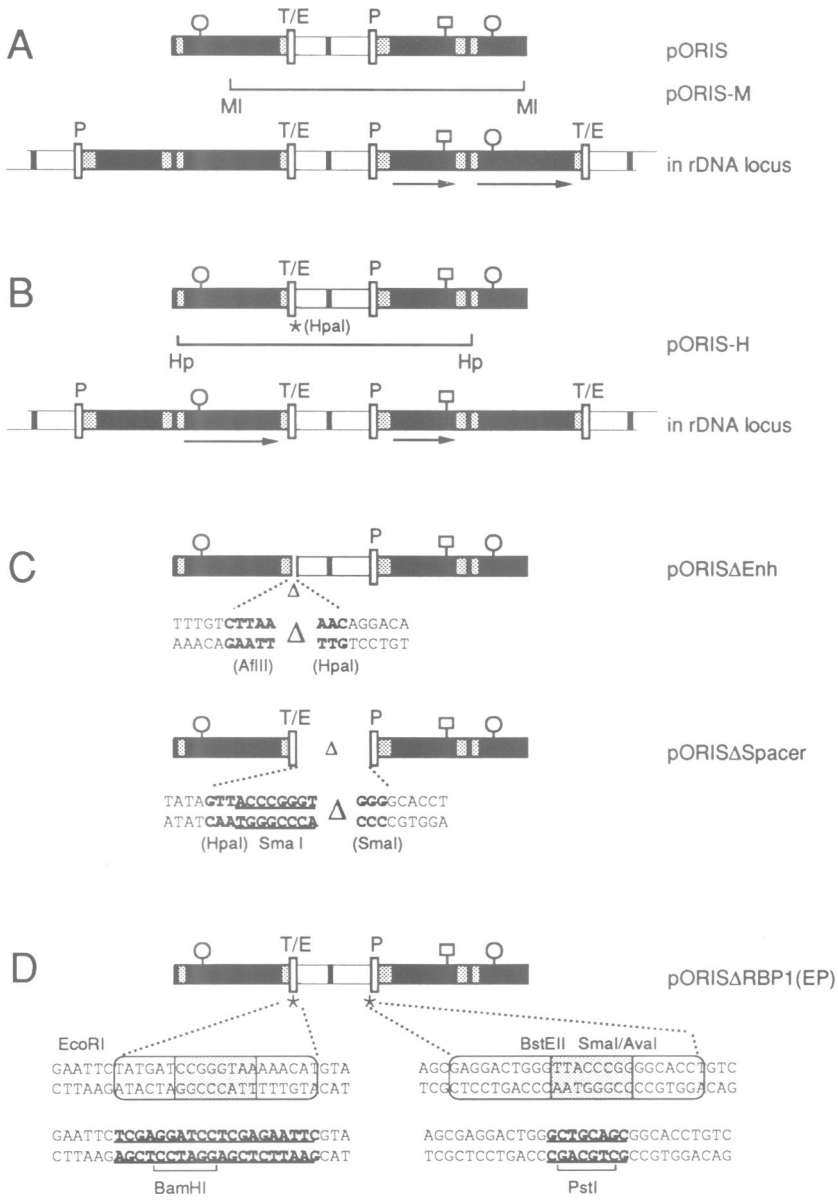

Fig. 2. Construction of pORIS derivatives and integration of pORIS derived rDNA units. (**A**) The wild type pORIS construct and the final situation in the rDNA locus after integration of a *MluI* repeat. (**B**) The construct pORIS-H, which carried a linker insertion (*) in the *HpaI* site within the intergenic spacer and the final situation in the rDNA locus after integration of a *HpaI* repeat. (**C**) The two deletion mutants pORISΔEnh and pORISΔSpacer. The rDNA sequences around the deletions are shown. Destroyed restriction enzyme sites are indicated in bold face and the inserted *SmaI* linker in pORISΔSpacer is underlined. In these constructs the *HpaI* site within the intergenic spacer is destroyed as a result of which *HpaI* as well as *MluI* repeats can be isolated for integration into the rDNA locus. (**D**) The mutant pORISΔRBP1(EP). The two RBP1/REB1 binding sites are shown and the consensus core sequences are indicated by shaded boxes. The binding sites were mutated by linker insertions, indicated in underlined bold face. In the corresponding constructs pORISΔRBP1(E) and pORISΔRBP1(P) only one of the binding sites has been mutated in a similar way. From these constructs. *MluI* repeats can be isolated for integration. In all constructs the position of oligonucleotide tags in 17S or 26S rRNA is indicated by ♀ or ♂, respectively. Arrows represent the tagged rRNAs produced in the situations depicted in Figure 2A or B respectively. See legend to Figure 1 for further explanation of symbols and abbreviations.

To determine the number of copies integrated we then analysed DNA from the co-transformants by Southern hybridization using the 26S rRNA tag and the *TRP1* gene as probes (Figure 3). We also analysed DNA isolated from yeast transformed with an episomal pORCS plasmid, carrying a tagged rDNA unit on a vector with a *TRP1* marker [pORCS(17S* + 26S*), Musters *et al.*, 1989a, 1990]. Using the plasmid copy number of the pORCS transformant as a standard and the signal of the single copy chromosomal *TRP1* gene as an internal control, we established that pORIS transformants having a copy number ranging from 1 (pORIS-H1 and -M1) to 8 (pORIS-H8) were obtained. Since recombination events in the chromosomal rDNA locus may occur occasionally we made sure that each analysis of RNA was accompanied by analysis of DNA isolated from the same culture.

To study the transcription of the tagged integrated units, we analysed the RNA of the various pORIS transformants by Northern blot hybridization using as probes oligonucleotides complementary to either the 26S (Figure 4A) or the 17S rRNA tag (Figure 4B). As an internal control for the amount of RNA loaded, the same blot was reprobed

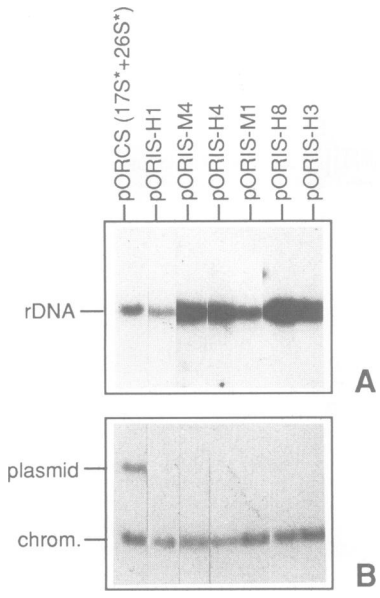

Fig. 3. Southern blot analysis of pORIS transformants. *Bgl*II-digested DNA isolated from various transformants was fractionated on a 1% agarose gel, blotted onto Hybond and hybridized using as a probe an oligonucleotide complementary to the 26S rRNA tag (**A**) or the M13−*TRP1* probe (**B**). In order to determine the number of integrated tagged units in pORIS transformants, DNA of pORCS (17S* + 26S*) was also analysed (see text for explanation). The length of the rDNA band in (A) is 4.5 kb, whereas in (B) the length of the plasmid band is 3.1 kb and that of the chromosomal-derived band is 1.6 kb.

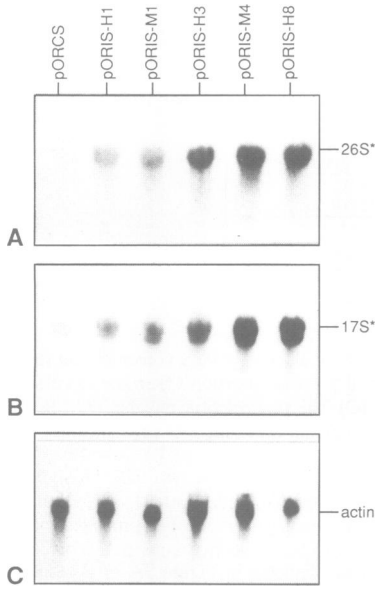

Fig. 4. Northern blot analysis of pORIS transformants. 20 μg of RNA isolated from the various pORIS transformants was fractionated on a 0.8% agarose gel, blotted onto Hybond and hybridized using oligonucleotides complementary to the 26S rRNA tag (**A**), the 17S rRNA tag (**B**) or the M13−actin probe (**C**) as a probe.

with an actin probe (Figure 4C). Clearly all pORIS transformants expressed the tagged 17S and 26S rRNA genes, and furthermore their expression correlated very well with their respective copy numbers. It is noteworthy that the transcription of an integrated tagged unit of pORIS was much more efficient than that of an episomal plasmid pORCS, carrying the tagged unit. As there was no difference in the expression of tagged genes obtained by single copy integration of either a *Hpa*I or a *Mlu*I unit, we infer that the insertion of an 8 bp linker in the *Hpa*I site in the intergenic spacer (necessary for the destruction of the *Hpa*I site) does not affect transcription of flanking Pol I operons. Furthermore, we conclude that there is no difference in the expression of consecutive transcription units. This implies that *Hpa*I units containing an additional mutation in the intergenic spacer between the two tagged genes, after integration, can be used to study specific upstream (monitored via the expression of the tagged 17S rRNA gene) or downstream effects (monitored via the expression of the tagged 26S rRNA gene) of that mutation (see next section).

If our system for studying Pol I transcription really reflects normal chromosomal Pol I transcription, then tagged rDNA units should be expressed with similar efficiency as normal endogenous rDNA units. To check this, we visualized both tagged and untagged RNA species of pORIS-H8 in a single experiment (Figure 5). Primer extension starting from an oligonucleotide complementary to 26S rRNA sequences located just downstream of the site of insertion of the tag resulted in a major 154 nt long extension product derived from the wild type cellular 26S rRNA, as well as an extension product that was 18 nucleotides longer, derived from the tagged units. Comparison of the relative intensities of the two extension products in Figure 5 indicated that in a pORIS transformant which carried eight tagged units, the amount of tagged 26S rRNA was roughly 2−4% of the unmutated 26S rRNA. This number was in line with the 3−4% that would theoretically be produced against a background of 140−200 chromosomal rDNA units, thought to be present in an average yeast cell (Petes, 1979; Warner, 1989). A similar primer extension experiment to quantify the amount of tagged 26S rRNA derived from extrachromosomal rDNA units contained in a pORCS transformant, has demonstrated that when 10 plasmid copies per cell are present, the amount of tagged 26S rRNA is only 0.2% of the total amount of 26S rRNA (Musters *et al.*, 1989a). These results for pORCS and pORIS transformants indicate that tagged rDNA units integrated within the rDNA locus are expressed much more efficiently than extrachromosomal units. In fact they are being transcribed with an efficiency that is virtually indistinguishable from that of the endogeneous rDNA units. Taking these results together we conclude that we have succeeded in the development of a system for studying Pol I transcription that mimics normal chromosomal Pol I transcription.

Studies with tagged integrated rDNA units carrying an enhancer or spacer deletion

As a first application of this novel system, we studied the function of the Pol I enhancer. More specifically, we have tried to answer the question whether the Pol I enhancer can activate one (or more) downstream rDNA units, whether it activates only rDNA units located upstream or whether it can act bidirectionally. For this purpose we constructed a pORIS mutant having its enhancer completely deleted (pORIS-ΔEnh; Figure 2C). From this mutant, we isolated either a *Hpa*I or a *Mlu*I repeat for integration. Following the co-transformation and selection procedure described above, four enhancer mutants were obtained carrying either one (pORISΔEnh-H1, pORISΔEnh-M1), two (pORISΔEnh-

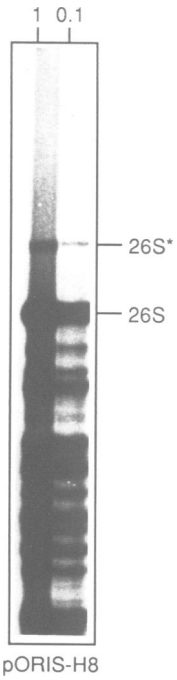

Fig. 5. Quantification of tagged 26S rRNA in pORIS-H8. An oligonucleotide complementary to position +136 to +154, i.e. immediately downstream of the oligonucleotide tag in 26S rRNA, was annealed to RNA isolated from pORIS-H8 and extended by reverse transcriptase in the presence of all four dNTPs. Different amounts of the extension products were separated on a 4% polyacrylamide gel. Bands representing tagged (26S*) and untagged (26S) 26S rRNA are indicated, and their relative intensities were determined by densitometric scanning.

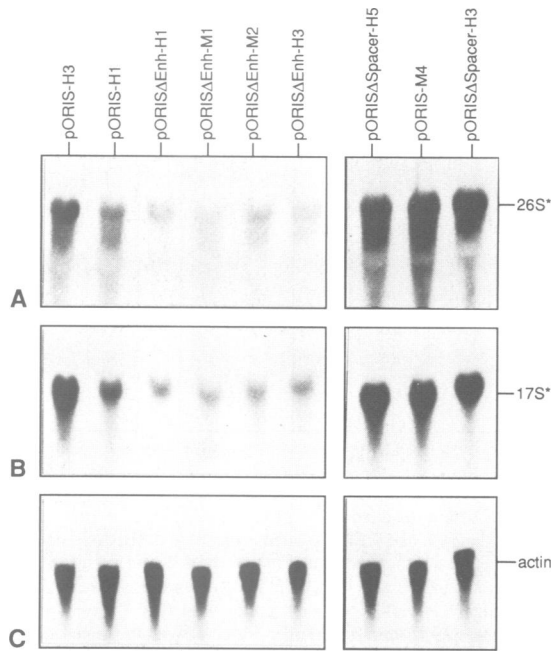

Fig. 6. Northern blot analysis of pORISΔEnh and pORISΔSpacer transformants, using as a probe oligonucleotides complementary to the 26S rRNA tag (**A**), the 17S rRNA tag (**B**) or the M13−actin probe (**C**).

M2) or three (pORISΔEnh-H3) tagged units. We also constructed a pORIS derivative in which almost the entire intergenic spacer was deleted, i.e. the region between the terminator/enhancer element and the promoter (see Figure 2C), and used *Hpa*I units for co-transformation. We obtained two tandem copy transformants carrying three (pORISΔSpacer-H3) or five (pORISΔSpacer-H5) integrated units. We analysed whether multicopy transformants contain either tandemly or dispersely integrated tagged rDNA units by digestion of the DNA with various restriction enzymes and Southern blot hybridization with the 17S or 26S rRNA tag as the probe (e.g. *Nhe*I and *Hin*dIII for ΔEnh mutants and *Mlu*I for ΔSpacer mutants). Thus, it was found that all multicopy transformants carry the integrated units in tandem (results not shown).

Next, RNA of the mutants was analysed by Northern blot hybridization using oligonucleotides complementary to either the 26S (Figure 6A) or the 17S rRNA tag as probes (Figure 6B). Hybridization of the same filter using a probe complementary to actin mRNA was used as an internal standard (Figure 6C). If we compare the level of expression of either the tagged 17S or the tagged 26S rRNA in pORISΔEnh-H1 with that in the control transformant pORIS-H1, we observe a decrease of ~50% in the expression of both tagged rRNA species. This implies that the deletion of a single enhancer affects the transcription of both the preceding as well as the downstream flanking operon. In other words, enhancers can exert their stimulatory action in two directions. In addition, it can be concluded that the extent of enhancement must be similar in either direction, since we do not observe a differential effect on the production of tagged 17S and 26S rRNA upon enhancer deletion.

The total reduction in transcription observed as a result of enhancer deletion may seem rather limited. This is, however, the consequence of the fact that after deletion of a single enhancer an integrated tagged unit is still under the influence of at least one (and maybe more) remaining enhancers (see for example the situation for pORISΔEnh-H1 in Figure 9C). In the simplest model, where one transcription unit is influenced by only one upstream and one downstream enhancer and assuming that the extent of enhancement is the same for both enhancers, deletion of a single enhancer is expected to result in a 50% reduction in expression of both rDNA units flanking the deleted enhancer. Such a reduction in expression of both tagged genes after enhancer deletion can be observed not only for pORISΔEnh-H1, but also for pORISΔEnh-M1 (Figure 6).

Interestingly, the RNA analysis of tandem copy enhancer deletion mutants (pORISΔEnh-H3 and pORISΔEnh-M2) reveals that the amount of tagged 17S and 26S rRNA produced in these transformants was also clearly lower than the amount produced in a single copy control (pORIS-H1) (Figure 6). Moreover, the hybridization signals detected for these two tandem copy enhancer deletion mutants are indistinguishable from those observed for the single copy enhancer deletion mutants (pORISΔEnh-H1 and pORISΔEnh-M1) (Figure 6). Thus, deletion of the enhancer in two or more consecutive rDNA units generates a strong reduction in the expression per integrated tagged unit. To put it another way, tandem integration of additional marked units, which are flanked on both sides by an enhancer deletion, hardly increases the expression of tagged rRNA over the level observed in a single copy enhancer deletion mutant. This strongly suggests that the effect of an enhancer

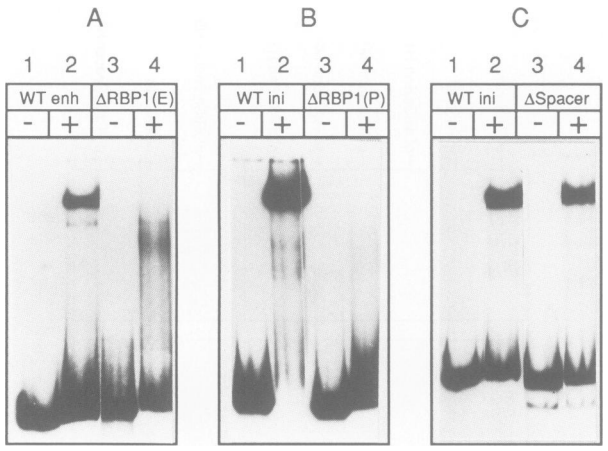

Fig. 7. Gel retardation analysis of RBP1/REB1 binding site mutations. (**A**) 3 fmol of end-labelled *Nhe*I−*Hin*dIII enhancer fragment either wild type (lanes 1 and 2) or isolated from pUCΔRBP1(E) (lanes 3 and 4) was incubated either without protein (lanes 1 and 3) or with 10 μg S100 extract (lanes 2 and 4). (**B**) 3 fmol of end-labelled *Eco*RV−*Eco*RI initiation fragment either wild type (lanes 1 and 2) or isolated from pUCΔRBP1(P) (lanes 3 and 4) was incubated either without protein (lanes 1 and 3) or with 10 μg S100 extract (lanes 2 and 4). (**C**) 3 fmol of end-labelled wild type *Eco*RV−*Eco*RI initiation fragment or *Hin*dIII−*Bgl*II fragment from pORISΔSpacer was incubated either without protein (lanes 1 and 3) or with 5 μg S100 extract (lanes 2 and 4).

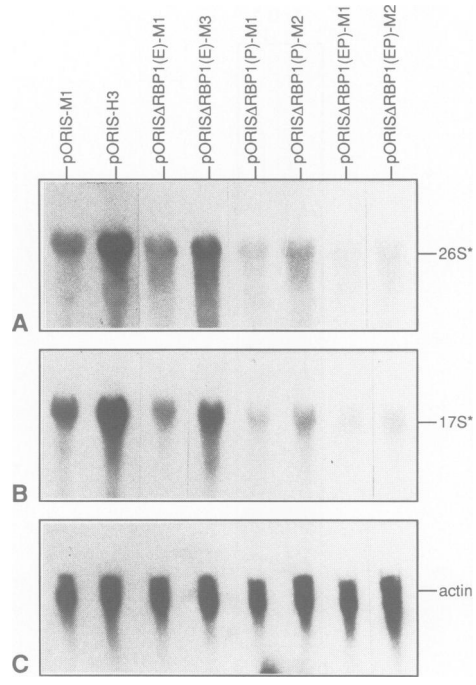

Fig. 8. Northern blot analysis of pORISΔRBP1(E), pORISΔRBP1(P) and pORISΔRBP1(EP) transformants, using a probe oligonucleotides complementary to the 26S rRNA tag (**A**), the 17S rRNA tag (**B**) or the M13−actin probe (**C**).

is sensed almost exclusively by the two most proximal flanking transcription units and hardly (if at all) by rDNA units further down or upstream (see drawings for pORISΔEnh-H1 and pORISΔEnh-H3 in Figure 9C and D, respectively).

RNA analysis of the two spacer deletion mutants clearly shows that the expression of the tagged genes in the spacer mutants was similar to that in control transformants (Figure 6). Thus, deletion of the intergenic spacer does not affect Pol I transcription within the chromosomal context. Furthermore, these experiments again underline that there are no other Pol I regulatory elements within the spacer apart from the terminator/enhancer element and the promoter. As the spacer deletion brought these elements closer together, we also conclude that diminishing the distance between the Pol I enhancer and promoter does not influence the efficiency of Pol I transcription.

Studies with tagged integrated rDNA units carrying mutations of the RBP1/REB1 binding sites

To study the function of RBP1/REB1, derivatives of pORIS were constructed in which either one or both of the RBP1/REB1 binding sites has been destroyed. The binding sites were mutated by replacing (part of) them by a linker, containing a unique restriction enzyme site (see Figure 2D). The effects of the mutations on the *in vitro* binding of RBP1/REB1 were assayed by gel retardation analysis (Figure 7). Incubation of a yeast S100 extract with a ^{32}P-labeled enhancer fragment (Figure 7A, lanes 1−2) or an initiation fragment (Figure 7B, lanes 1−2) shows one major complex as a result of the RBP1/REB1−rDNA interaction. Retardation analysis of the corresponding fragments isolated from the mutated constructs shows that these mutants had completely lost their ability to bind RBP1/REB1 (Figure 7A and B, lanes 3−4). The mutations therefore prevented binding of RBP1/REB1 and allowed the determination of whether one or both binding sites are involved in the regulation of Pol I transcription. In Figure 7C we demonstrate that pORISΔSpacer, although lacking all spacer sequences, could still bind RBP1/REB1. This mutant still contains an almost intact RBP1/REB1 binding site near the promoter, due to a *Sma*I linker that replaces virtually all the deleted sequences relevant for binding RBP1/REB1 (compare with Figure 2C).

pORIS derivatives with mutations of the RBP1/REB1 binding sites were integrated using *Mlu*I units. The transformants obtained carried 1−3 integrated units in tandem. RNA of the mutants was analysed by Northern blot hybridization as described above (Figure 8). All of the mutants produced significantly lower amounts of tagged 17S and 26S rRNA as compared with control pORIS constructs. Mutation of the RBP1/REB1 binding site in the enhancer caused a small decrease in the expression of the tagged rRNAs. In contrast with the tandem copy enhancer deletion mutants, tandem copy mutants of pORISΔRBP1(E) produced more tagged rRNA than the single copy mutant. The level of expression was proportional to the amount of integrated marked units, but always lower than a corresponding multicopy control transformant. These results suggest that RBP1/REB1 is involved in enhancer functioning, but that additional enhancer sequences and DNA-binding proteins play a role as well. Mutation of the RBP1/REB1 binding site near the promoter diminished transcription of the tagged unit to ~50%. In tandem copy mutants of pORISΔRBP1(P) the amount of tagged rRNA correlated with the number of units integrated, but remained reduced compared with control pORIS transformants. When both RBP1/REB1 binding sites were removed the effects were even more drastic and transcription dropped to ~25%. Again, the transcription was dependent on the number of copies integrated in tandem.

Combining these results with those obtained with

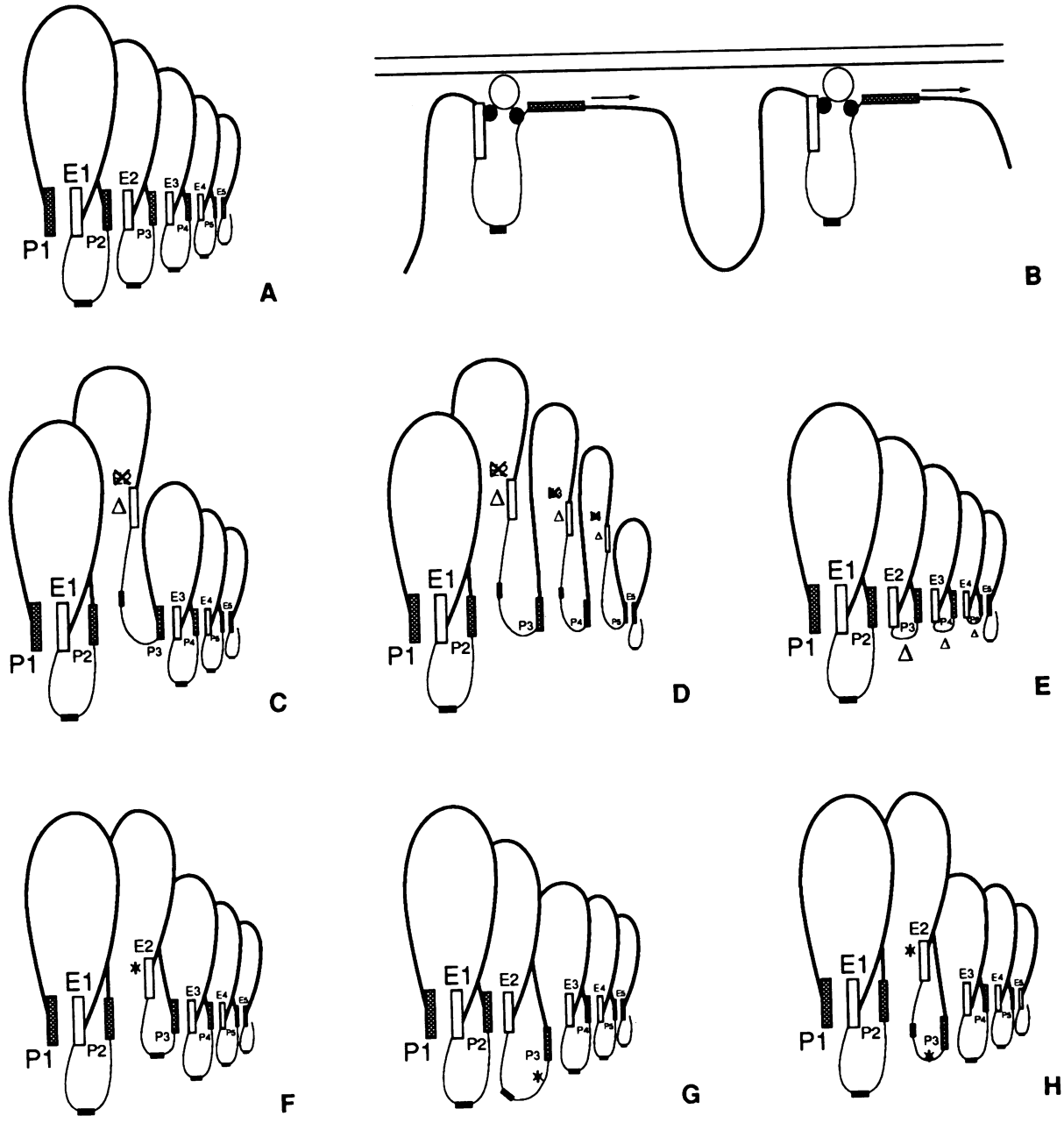

Fig. 9. Model for the regulation of transcription by Pol I. (**A**) Five consecutive rDNA units are indicated. Large loops (thick black lines) represent Pol I operons, whereas the smaller loops (thin lines) represent the intergenic spacer, carrying the 5S rRNA gene (small black box). The Pol I promoter (P) and enhancer (E) are indicated by shaded and open boxes, respectively. (**B**) Matrix attachment model in which the rDNA units are anchored to the nucle(ol)ar matrix via interaction of RBP1/REB1 (black circles) with some unknown (protein) component (white circles). In 9C–H the situation in the rDNA locus is shown after integration of a single-copy enhancer deletion mutant (**C**), a triple copy enhancer deletion mutant (**D**), a triple copy spacer deletion mutant (**E**) and single copy mutants (transcribed from P3) of the RBP1/REB1 binding sites in either the enhancer (**F**), near the promoter (G) or at both the enhancer and promoter (**H**). Deletions are indicated by a Δ and mutations are indicated by a *.

pORISΔEnh and pORISΔSpacer, it can be concluded that RBP1/REB1 is essential for efficient transcription in the chromosomal context. For optimal transcription of an rRNA operon it seems necessary that both RBP1/REB1 binding sites are functionally intact.

Discussion

We have described a novel system to study the regulation of transcription by yeast RNA polymerase I. Tagged units integrated in the rDNA locus were transcribed with an efficiency indistinguishable from that of the endogenous rDNA units. Using the system we showed that the Pol I enhancer, initially identified using artificial minigenes, indeed functions as a stimulatory element when assayed within the rDNA locus. Furthermore, we found that enhancers exerted their function in two directions and that the stimulatory effect in the upstream direction equalled the effect in the downstream direction. We also applied the system to study the function of the rDNA binding protein RBP1/REB1 and showed that this protein is involved in efficient Pol I transcription within the chromosomal context.

On the basis of our results we favour a model in which consecutive promoters and terminator/enhancer elements are

bound to the nucle(ol)ar matrix in a highly ordered, linear fashion (see Figure 9A). RBP1/REB1 is supposed to be involved in this anchoring, either directly (Figure 9A, C–H) or indirectly via some unknown protein (Figure 9B). A similar matrix–attachment model has been proposed for Pol I transcription in HeLa cells by the group of Cook (Jackson and Cook, 1985; Cook, 1989; Dickinson *et al.*, 1990). The DNA loops formed are thought to be organized in such a way as to locate all Pol I transcription units at one side and the 5S rRNA genes (transcribed by Pol III) at the other side. Terminating Pol I molecules can in this model, by some as yet unknown mechanism, be handed over to either the most proximal upstream or downstream promoter. It seems unlikely that just a high local concentration of Pol I molecules is responsible for the enhancing effect, since this would result in additional activation of more distally located promoters. Overall regulation of rRNA transcription is thought to be exerted mainly at the level of the number of promoters and terminator/enhancer elements attached to the nucle(ol)ar matrix, in accordance with the electron microscopical finding that a given rDNA unit is either in a transcriptional on or off mode with little modulation at the level of the number of initiating Pol I molecules (Hamkalo, 1985).

In our model, deletion of an enhancer leads to a situation in which the two operons flanking this deleted enhancer are still being activated by the enhancers that flank these operons on the side opposite to that of the deleted enhancer (Figure 9C). Our conclusion that a given Pol I enhancer mainly acts on the two Pol I operons immediately up- and downstream of that enhancer is based on the following line of reasoning. Both the tagged operon upstream and the operon downstream in a transformant having a single deleted enhancer (pORISΔEnh-H1, see Figure 6) produce ~50% of the amount of tagged RNA produced by a single copy control transformant. Therefore we conclude that up- and downstream enhancing effects are equal. If we assume that the total transcriptional efficiency of a given operon (as estimated by measuring steady state levels of tagged rRNA) will be determined by the combined effects of individual enhancers, it follows that only the first up- and downstream enhancer are involved in enhancement, both contributions being equal and measuring ~50% of the total enhancing effect. A similar conclusion can be reached based upon the data obtained for the triple copy enhancer deletion mutant pORISΔEnh-H3. For this mutant, it has been found that there is very little difference between the amount of tagged rRNA produced compared with the amount produced in a single copy enhancer deletion mutant. The two additional tagged operons in the triple mutant are flanked on both sides by deleted enhancers, so apparently enhancers located further up or downstream do not exert a substantial enhancing effect (compare the situation in Figure 9C and D).

The major difference between our results and the results obtained by Johnson and Warner (1989) is that these authors found an enhancer to act on flanking as well as on more distally located rDNA minigenes, whereas we conclude that enhancing effects on rDNA units not directly flanking the enhancer under study are at best extremely weak. These conflicting results are most probably due to the difference in experimental approach (rDNA minigenes versus intact rDNA repeats containing oligonucleotide tags and integration in the *URA3* locus versus integration into the rDNA locus). Since our test system measures rDNA transcription under conditions that optimally reflect the natural context, we believe that our results are more indicative for the *in vivo* situation.

The way we envisage the role of RBP1/REB1 in the regulation of Pol I transcription of the tandemly organized rDNA units is schematically shown in Figure 9F–H. When RBP1/REB1 binding to the enhancer, E2, is abolished, this enhancer cannot be placed in its optimal position as in the natural context and thus is not able to function efficiently. The non-mutated part of the enhancer may still exert activation, but to a much lower extent due to a less favourable spatial localization (Figure 9F). When the binding site near the promoter is mutated, the correct positioning between enhancer and promoter is similarly disturbed. In this case, however, the interaction of the promoter (P3) with both the upstream (E2) and downstream (E3) enhancer is affected, which may explain the somewhat stronger negative effect on transcription (Figure 9G). When both presumed nucleolar attachment sites are destroyed the level of spatial organization is still further diminished, resulting in an even more strongly diminished transcription enhancement (Figure 9H).

In the *in vivo* situation, up and down regulation of Pol I transcription may very well be regulated by the number of rDNA transcription units organized in the proposed loop structure. This regulation does, however, not necessarily involve RBP1/REB1 itself. In fact it is probable that regulation is exerted via another protein(s) whose state of post-translational modification reflects the metabolic state of the cell and, as a result, can or cannot couple RBP1/REB1 bound rDNA units to the nucleolar matrix. This proposal is instigated by the fact that the RBP1/REB1 protein is supposed to play a much more general role in the yeast cell than solely its involvement in the regulation of rDNA transcription. Binding sites for RBP1/REB1 have, for instance, also been identified in the upstream region of several Pol II transcribed genes, as well as in the centromere *CEN4* and the subtelomeric X and Y regions (Chasman *et al.*, 1990; Wang *et al.*, 1990). RBP1/REB1 appears to be identical to factor Y (Fedor *et al.*, 1988), GRF2 (Chasman *et al.*, 1990) and QBP (Brandl and Struhl, 1990). In some cases the protein stimulates transcription, whereas in others it acts as a repressor (Chasman *et al.*, 1990; Wang *et al.*, 1990). According to Chasman *et al.* (1990) factor Y functions by influencing the chromatin structure and creating a nucleosome-free region surrounding its binding site in the promoter region. The abundant and ubiquitous DNA binding protein RBP1/REB1 is an essential protein for growth of the yeast cell (Ju *et al.*, 1990) and is postulated to have a function in the organization of the DNA in the nucleus and nucleolus. An additional possibility that has been suggested by Johnson and Warner (1991) and is implicated in the results of Mestel *et al.* (1989), is that RBP1/REB1 plays a role in the termination of Pol I transcription. However, we have demonstrated that 3′-end formation can take place at T2 *in vivo* without RBP1/REB1 binding (Kulkens *et al.*, 1989), but we cannot exclude the possibility that RBP1/REB1 plays a role in the efficiency of 3′-end formation at T2, in particular in the chromosomal context.

Elements within the intergenic spacer that are involved in the enhancement of Pol I transcription have also been described for various other eukaryotes, notably *Xenopus* (Reeder, 1984; Labhart and Reeder, 1984, 1985; De Winter and Moss, 1986, 1987; Pikaard and Reeder, 1988; Firek *et al.*, 1989; Pape *et al.*, 1989), *Drosophila* (Grimaldi and Di Nocera, 1988; Grimaldi *et al.*, 1990), mouse (Kuhn and

Grummt, 1987; Tower *et al.*, 1989; Pikaard *et al.*, 1990) and rat (Cassidy *et al.*, 1986, 1987; Dixit *et al.*, 1987, 1989; Garg *et al.*, 1989). These elements include spacer promoters, promoter-proximal terminators and Pol I enhancer elements. Apart from the fact that the yeast rRNA does not contain (genuine) spacer promoters (Mestel *et al.*, 1989; Van der Sande *et al.*, 1989; Riggs and Nomura, 1990), the function of the yeast intergenic spacer may differ in more respects from that described for other organisms. First, experiments performed with pORISΔSpacer described here confirm that the intergenic spacer contains no additional elements involved in stimulation of Pol I transcription. The integration of tagged spacer mutants within the rDNA locus can be envisioned, as shown in Figure 9E. Secondly, the yeast Pol I enhancer differs from the Pol I enhancer elements present in the rDNA of other organisms, in that it does not display a (partial) sequence identity with the gene promoter. Furthermore it does contain the main Pol I termination site, the latter observation being an essential feature in models describing its mode of action. In addition, the recycling models for yeast Pol I predict that in contrast to the Pol I enhancer elements present in the rDNA of other eukaryotes (Reeder, 1984; Labhart and Reeder, 1984, 1986; De Winter and Moss, 1987; Pikaard and Reeder, 1988; Pikaard *et al.*, 1990), the yeast Pol I enhancer will not function in both orientations (i.e. when tested using the pORIS system). This prediction follows from the observation that terminator T2 is not functioning when tested in the opposite orientation (Mestel *et al.*, 1989; Van der Sande *et al.*, 1989). Since the intimate coupling of terminator and enhancer is a key feature in all recycling models, this observation leads to the testable assumption that an oppositely oriented terminator/enhancer will also no longer function as an enhancer.

Unlike other eukaryotes the intergenic spacer in yeast contains the 5S rRNA gene, which is transcribed by Pol III (Phillippsen *et al.*, 1978). Attachment of the rDNA units by RBP1/REB1 and formation of a series of loops (see Figure 9), therefore, may be necessary also to arrange both transcription units in different compartments of the nucleus.

In summary, we conclude that in yeast the sole element involved in stimulation of Pol I transcription is the terminator/enhancer element, which element presumably operates by a mechanism different from that of the Pol I enhancer elements identified in higher eukaryotes, which resemble Pol II enhancers in some respects. Moreover, the rDNA binding protein RBP1/REB1 is probably structurally involved in regulation of Pol I transcription and the interplay of enhancer and promoter and transcription factors within the specific tandem array of rDNA units present in the nucleolus of the cell. The newly developed system for studying Pol I within the chromosomal context offers new possibilities to further unravel the mechanism of regulation of Pol I transcription.

Materials and methods

Strains

Escherichia coli DH1 (F$^-$ *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 λ$^-$) and JM101 [Δ(*lac-pro*AB) *sup*E *thi* [F′.*tra*D36 *pro*AB *lacI*^{q} ZΔM15]] were used for construction and propagation of plasmid and M13 phage DNAs, respectively. *S.cerevisiae* MG34 (*MAT***a** *leu2 trp1 rad2 cir*$^+$) was used for co-transformation of YEp13 and pORIS-fragments.

Construction of pORIS

The *Sma*I site in pUC19 was destroyed by insertion of an 8 bp linker introducing a unique *Mlu*I site. The resulting plasmid was digested with *Sph*I and *Mlu*I and used to accommodate the 3.9 kb *Sph*I−*Hin*dIII fragment and the 3.4 kb *Hin*dIII−*Mlu*I fragment both derived from pORCS (17S* + 26S*) (Musters *et al.*, 1989a, 1990) yielding pUC-SHM (Figure 1). Next, in order to obtain a continuous rDNA sequence, we introduced the 5.3 kb *Eag*I−*Nsi*I fragment from pML2 (Musters *et al.*, 1989a) in pUC-SHM. The resulting plasmid had a direct repeat of >1 kb at both ends, contained part of two Pol I transcription units separated by the intergenic spacer and was termed pORIS (Figure 1).

Construction of enhancer and spacer mutants

All relevant cloning sites are indicated in Figure 1. In order to introduce mutations in the intergenic spacer present in pORIS, we subcloned the 4.4 kb *Xba*I−*Sma*I fragment from pML2 in pTZ18U, yielding pTZ-XS (Figure 1). Prior to this cloning step the *Hin*dIII site in pTZ18U was mutated into a *Xho*I site with the aid of an 8 bp linker, after which the resulting plasmid was first digested with *Xho*I and *Sal*I and then religated. pTZ-XS (Figure 1) was used for construction of various mutations within the intergenic spacer. (i) the *Hpa*I site in pTZ-XS was destroyed by the insertion of an 8 bp *Pst*I linker, yielding pTZ-H. (ii) To delete the enhancer, we removed the 0.3 kb *Afl*II−*Hpa*I fragment from pTZ-XS, yielding pTZ-ΔEnh. For this purpose, we filled in the sticky ends of the *Afl*II site using T4 DNA polymerase (Bethesda Research Laboratories, Rockville, Maryland, USA). (iii) Digestion of pTZ-XS with *Hpa*I and *Sma*I, followed by religation of the blunt ends and including an 8 bp *Sma*I linker, resulted in pTZ-ΔSpacer.

All three mutations were transferred to pORIS via a triple ligation for which a fragment carrying the *Xba*I−*Sma*I insert of the specific pTZ subclone and two fragments from pORIS being the 2.9 kb *Sma*I−*Dra*III fragment and the 5.5 kb *Dra*III−*Xba*I fragment were used. These cloning steps resulted in pORIS-H (Figure 2B), pORIS-ΔEnh and pORIS-ΔSpacer (Figure 2C), from which *Mlu*I units as well as *Hpa*I units can be isolated for integration.

Construction of RBP1/REB1 binding site mutants

The RBP1/REB1 binding site within the enhancer was mutated using a subclone, consisting of the *Eco*RI−*Hin*dIII enhancer fragment in pUC19. The binding site was removed from this subclone by digestion with *Eco*RI and *Acc*I, and ligation with an oligonucleotide that restores the NTS1 sequences up to the RBP1/REB1 binding site. This oligonucleotide destroys the original *Eco*RI site and introduces a *Nhe*I and another *Eco*RI site downstream of this site. Between these new sites the 270 bp *Nhe*I−*Eco*RI rDNA fragment upstream of the enhancer element was cloned to restore all wild type rDNA sequences in the subclone except for the 20 bp RBP1/REB1 binding site as described previously (Kulkens *et al.*, 1989). In the *Eco*RI site of the subclone with the deletion, a 20 bp linker was inserted which restored the length of the deleted sequences and created a unique *Bam*HI restriction enzyme site, yielding pUCΔRBP1(E). The *Bam*HI site can be used to check for the presence of the mutation after integration. The mutation was transferred to pORIS via a triple ligation, using the 440 bp *Nhe*I−*Hin*dIII fragment from pUCΔRBP1(E), the 1.9 kb *Hin*dIII−*Sma*I fragment from pTZ-XS and the 10.2 kb *Sma*I−*Nhe*I fragment from pORIS, yielding pORISΔRBP1(E) (Figure 2D). For mutation of the RBP1/REB1 binding site near the promoter, a subclone (pUC-SH) was constructed consisting of the 1.5 kb *Sph*I−*Hin*dIII initiation fragment, cloned between the *Sph*I and *Hin*dIII sites of a pUC derivative that contains an inverted repeat of polylinkers from pUC18 and pUC9. This vector was chosen because the sites for *Sph*I and *Hin*dIII can be used only when they are >3 bp apart. In the subclone interjacent polylinker sequences were removed by the inserted fragment, so the *Sma*I−*Ava*I site in this fragment could be used to create a deletion in the RBP1/REB1 binding site. Therefore pUC-SH was digested with either *Bst*EII or *Ava*I. The 5′-protruding ends were removed by treatment of 1 μg linearized DNA with 1 U S1 nuclease (Erase-a-Base system, Promega) for 60 min at 16°C in 75 mM potassium acetate, pH 4.6, 0.6 M NaCl, 25 mM $ZnSO_4$, 12.5% glycerol. The reaction was stopped by addition of S1 stop buffer to a final concentration of 30 mM Tris and 5 mM EDTA, followed by extractions with a phenol:chloroform:iso-amylalcohol mixture (49.5:49.5:1) and ethanol precipitation. The blunt-ended DNA fragments were then digested with *Sph*I and the 780 bp *Sph*I−ex*Bst*EII and the 3.4 kb ex*Ava*I−*Sph*I fragments respectively were isolated. Via a triple ligation of these fragments and an 8 bp *Pst*I linker, a mutated subclone (pUCΔRBP1(P)) was constructed. This lacks the 9 bp core of the RBP1/REB1 binding site and contains a unique *Pst*I site instead. The mutation was transferred to pORIS via a triple ligation, using the 510 bp *Eco*RV−*Bgl*II fragment from pUCΔRBP1(P), the 2.2 kb *Nhe*I−*Eco*RV fragment from pTZ-XS and the 9.9 kb *Bgl*II−*Nhe*I fragment from pORIS, obtained by partial digestion with *Bgl*II. The resulting pORIS derivative is called pORISΔRBP1(P) (Figure 2D).

A construct which lacked both RBP1/REB1 binding sites was constructed in a similar way. Using the 510 bp *Eco*RV−*Bgl*II fragment from

pUCΔRBP1(P), the 2.2 kb *Nhe*I–*Eco*RV fragment from pORISΔRBP1(E) and the 9.9 kb *Bgl*II–*Nhe*I fragment from pORIS in a triple ligation resulted in pORISΔRBP1(EP) (Figure 2D). From all these pORIS derivatives *Mlu*I units can be isolated for integration.

Protein–DNA interaction analysis

Preparation of yeast S100 extracts and gel retardation experiments with end-labelled fragments from wild type and mutant constructs were performed as described before (Kulkens *et al.*, 1989). The wild type 440 bp *Nhe*I–*Hin*dIII enhancer fragment was isolated from pSIRT (Musters *et al.*, 1989b). The wild type 430 bp *Eco*RV–*Eco*RI initiation fragment was isolated from pUC-ini (Kulkens *et al.*, 1989). The corresponding mutant fragments were isolated from the subclones pUCΔRBP1(E) and pUCΔRBP1(P), as described above. The 460 bp *Hin*dIII–*Bgl*II fragment was isolated from pORISΔSpacer.

PCR analysis

Total yeast DNA (100 ng) was added to a reaction mixture, containing 5 μl 10 × reaction buffer (15 mM $MgCl_2$, 10 mM Tris, pH 9, 500 mM KCl, 0.1% w/v gelatin, 1% Triton X100), 2 μl dNTP mix (5 mM each), 2.5 μl (~25 pmol) each of two primers (50 ng/μl) and H_2O to 48 μl. Primers were the 26S oligonucleotide tag (non-RNA like strand, 5′-ACTCGAGAGCTTCAGTAC-3′) and the 17S oligonucleotide tag (RNA like strand 5′-CTAGTAGATGCTAGGTACC-3′). After 10 min denaturation at 94°C 2 μl Taq polymerase (0.05 U/μl, HT Biotechnology Ltd) was added and samples were subjected to 32 rounds of temperature cycling (40 s at 94°C for 2 min at 48°C and for 3 min at 72°C) with a final 10 min at 72°C, using a Perkin–Elmer Cetus DNA thermocycler. A portion of each reaction mixture was analysed on agarose gel.

Miscellaneous techniques

Transformation of yeast cells, isolation of DNA and RNA from transformants, blotting techniques and labelling procedures were performed essentially as described previously by Musters *et al.* (1989a, 1990) and Van der Sande *et al.* (1989). Blots probed with oligonucleotides were hybridized at 48°C. Northern blot hybridization using an actin mRNA specific probe and Southern blot hybridization using a *TRP1*-specific probe were performed at 65°C. Construction of the M13–actin and the M13–*TRP1* clones has been described elsewhere by Musters *et al.* (1989a) and Kulkens *et al.* (1989). Where relevant the ratio of the signals obtained in the Northern blot analysis was determined by scanning using an LKB 2222-020 UltroScan XL laser densitometer.

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