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Linker scanning of the yeast RNA polymerase I promoter

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ABSTRACT

To define the RNA polymerase I promoter in the rDNA of *Saccharomyces cerevisiae* more precisely, we have constructed a series of 5'- and 3'-deletion mutants in a novel, plasmid-borne rDNA minigene, that also contains the transcriptional enhancer. Our data show that the Pol I promoter, in this context, extends from position −155 to +27, with 5'-deletions up to −134 and 3'-deletions up to −2 removing essential sequence information. To investigate the internal organization of the yeast Pol I promoter, linker scanning mutants were constructed, that traverse the Pol I promoter region and comprise between 5 and 12 clustered point mutations. Analysis of minigene transcription in yeast cells transformed with these plasmids demonstrates that the Pol I promoter consists of three domains. Mutations in Domain I (from position −28 to +8) and Domain II (−70 to −51) drastically reduce promoter activity, whereas clustered point mutations in Domain III (starts at position −146 and presumably extends to position −76) appear to have less effect. Furthermore, the insertion of 4 nt between Domains I and II diminishes minigene transcription, indicating that the relative positions of these domains is essential.

INTRODUCTION

In eukaryotic cells the tandemly reiterated ribosomal RNA (rRNA) genes are transcribed by a specialized polymerase, RNA polymerase I (Pol I). The sole product of Pol I transcription is a 35−45S precursor rRNA that is processed to yield the mature ~18S, ~28S and 5.8S rRNAs, which together with 5S rRNA (produced by RNA polymerase III) constitute the RNA content of a eukaryotic ribosome. Like in prokaryotes, the production of all components of a eukaryotic ribosome is highly coordinated, and controlled by the cellular demand for protein synthetic capacity in response to *e.g.* the availability of nutrients or the developmental stage of the cells. There is evidence that in yeast this control is primarily exerted at the level of rRNA transcription (1,2). Therefore, the elucidation of how rRNA transcription is regulated is an important step in our understanding of the mechanisms by which the synthesis of ribosomes is influenced by environmental signals.

The development of Pol I *in vitro* transcription systems for a number of organisms has facilitated the identification of *cis*-acting elements (promoters, terminators and enhancers) as well as *trans*-acting factors that are involved in the regulation of Pol I transcription (reviewed in Ref. 3). Though the yeast rRNA genes were among the first eukaryotic rRNA genes to be fully characterized (4−7), it has not been possible as yet to devise a reliable yeast *in vitro* system for Pol I transcription. A system has been reported that permits *in vitro* initiation by Pol I at a specific site in the Non Transcribed Spacer, but it fails to direct Pol I to initiate at the site employed *in vivo* (8−10).

As an alternative approach a system has been developed by several laboratories, that utilizes Pol I minigenes, which, upon transformation to yeast, can be used to study the regulation of Pol I transcription *in vivo* (10–13). Following this approach a number of *cis*-acting elements involved in Pol I transcription in *Saccharomyces cerevisiae* have been identified, *i.e.* a transcriptional enhancer (13,14), a number of 3'-end generating sites (15), and the promoter, which, as we have reported previously (16), is located between nucleotide positions −192 and +15 relative to the point of transcription initiation.

In this paper we describe experiments which reveal that the sequence requirements for initiation of transcription by Pol I are not affected by the presence of a Pol I transcriptional enhancer in *cis*, as well as studies with Linker Scanner Mutants (LSMs), that reveal the internal organization of the yeast Pol I promoter. In close similarity to the structure of rDNA promoters in other eukaryotes (3), the yeast Pol I promoter consists of a core domain that is essential for efficient initiation of transcription, and an upstream domain in which alterations cause less drastic effects. In contrast to the situation in other eukaryotes, the core promoter in *S. cerevisiae* seems to be composed of two elements, that are separated by a region of lesser importance.

MATERIALS AND METHODS

Enzymes, Strains, Plasmids and Oligonucleotides

Restriction enzymes were purchased from Bethesda Research Laboratories, except for *Asu*II and *Sna*BI, obtained from Promega and New England Biolabs, respectively. Polynucleotide kinase, *Escherichia coli* DNA polymerase (Klenow fragment), M-MLV Reverse transcriptase and *Bal*31 exonuclease were from Bethesda Research Laboratories. Zymolyase-100T was used for yeast DNA isolations and obtained from Seikagaku Kyogo, Tokyo, Japan. Helicase, used in the procedure for yeast transformation, was from Industrie Biologique Française, Clichy, France, and Mung Bean nuclease was from Promega. *E. coli* DH1 (F^- *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 λ^-) and JM83 (F^- *ara*Δ(*lac-pro*AB) *rps*L φ80d*lac*ZΔM 15) were used for construction and propagation of all plasmids. *E. coli* JM101 (Δ(*lac-pro*AB) *sup*E *thi*/F' *tra*D36 *pro*AB *lac*I^q ZΔM 15) was used for propagation of M13-actin. *S. cerevisiae* MG34 (*leu2 trp1 rad2 cir*$^+$) was used for expression of SIRT and related minigenes. All yeast rDNA fragments used in this study were taken from pMY60 (17), a plasmid that has been described previously (18). Oligonucleotides were synthesized with an Applied Biosystems 381A DNA synthesizer, except for a 8 bp *Sac*I linker that was obtained from Bethesda Research Laboratories, and a 10 bp *Bam*HI linker that was a gift from J. H. van Boom, Leiden, The Netherlands.

Construction of Pol I Minigenes

pSIRT was constructed in two steps. First, the termination fragment (*Taq*I-*Hpa*I, 921 bp) was inserted between the *Acc*I and *Sma*I sites of pUC18, and the initiation fragment (*Stu*I-*Bgl*II, 2741 bp) was combined with the double stranded reporter oligonucleotide, equipped with *Bgl*II and *Pst*I compatible cohesive ends, between the *Sma*I and *Bam*HI sites of pUC19. The inserts of these two constructs were fused, using the *Pst*I and *Nde*I sites present in both constructs, resulting in pSIRT. This plasmid carries the SIRT minigene (*cf.* Fig. 1), flanked by an inverted repeat that comprises the *Kpn*I, *Sac*I and *Eco*RI sites of the pUC18/19 polylinker. pIRT and pNIRT are derivatives of pSIRT. pIRT contains a 10 bp *Bam*HI linker inserted in the unique *Sma*I site at −208, whereas in pNIRT the 12 bp 'scanning linker' is present at this position (Fig. 5). The *E. coli*-yeast shuttle vector pEMBL Ye30-Δ16

was derived from pEMBL Ye30 (19) by deleting approximately 1.3 kb of non-characterized sequences between the LEU2 gene and the 2μ fragment with *Bal*31 exonuclease (starting from a *Sac*I site in the middle of this region), and inserting a 8 bp *Sac*I linker in the unique *Sma*I site. The minigenes SIRT, IRT, and NIRT were all inserted in the same orientation in the *Sac*I site of pEMBL Ye30-Δ16 (as depicted for SIRT in Fig. 1), resulting in YepSIRT, YepIRT and YepNIRT, respectively .

Construction of Deletions in the Pol I Promoter

In order to be able to employ the strategy for the construction of clustered point mutations as developed by Haltiner *et al.* (20) in yeast vectors, a specific tool was designed: the *Bal*31 cassette (Fig. 2). The first step of its construction involved the insertion of a double stranded oligonucleotide containing sites for *Nco*I and *Nru*I, between the *Bam*HI and *Eco*RI sites of pUC18. Next, the 255 bp *Eco*RI-*Nde*I fragment of this plasmid was used to replace the analogous fragment of pUC9, resulting in the fusion of the pUC9 and pUC18 polylinkers. Finally, a 665 bp *Eco*RI fragment of the rDNA repeat of *S. carlsbergensis* was inserted in the unique *Eco*RI site of this plasmid (Fig. 2). The *Bal*31 cassette can be excised from the resulting plasmid by digestion with either *Hin*dIII, *Pst*I, *Sal*I, *Acc*I, *Hinc*II or *Bam*HI. The latter option was used to insert the *Bal*31 cassette in either the *Bam*HI or *Bgl*II site of pIRT, yielding pBIRT and pIBRT, respectively (Fig. 2). The BIRT and IBRT minigenes were excised with *Sac*I and inserted in the unique *Sac*I site of pEMBL Ye30-Δ16.The resulting YepBIRT and YepIBRT constructs were linearized with *Sma*I, treated with *Bal*31 exonuclease essentially as described previously (18) in order to generate 5′- and 3′-deletions of the Pol I promoter region, digested with *Nru*I to remove remaining 'buffer DNA', and recircularized.

Construction of Linker Scanning Mutants (LSMs)

LSMs[−146;−134], [−121;−108], [−110;−97], [−70;−60], [−33;−26]-12, [−4;+8] and [+27;+37] were constructed by joining appropriate combinations of the approximately 0.9 kb *Sph*I-*Nco*I fragment of 3′-deletion mutants and the approximately 11kb *Sph*I-*Nco*I fragment of 5′-deletion mutants, restoring the YepIRT promoter region except for the substitution of a short stretch of rDNA sequence by the 12 bp scanning linker. LSMs[−102;−91], [−87;−76], [−62;−51], [−49;−38] and [−49;−38]-Δ11 were constructed by combining the approximately 11 kb *Sph*I-*Nco*I fragment of the corresponding 5′-deletion mutant, the *Sph*I-*Sna*BI fragment of IRT, and a set of nested oligonucleotides that restores the rDNA sequences between the *Sna*BI site and the desired position of the scanning linker. LSM[−28;−17] was constructed following essentially the same approach, but starting from the 11 kb *Nco*I-*Sph*I fragment from IRTΔ3′[−28;+128] and the 0.9 kb *Asu*II-*Sph*I fragment from IRT. LSM [+27;+37]* was constructed by joining the 0.9 kb *Sph*I-*Asu*II fragment of IRT to the 11 kb *Nco*I-*Sph*I fragment of LSM[+27;+37] with an appropriate oligonucleotide. LSM[−33;−26]-8 was constructed from LSM[−33;−26]-12 by linearizing the plasmid with *Nco*I, removing the protruding ends with Mung Bean nuclease, and recircularizing the plasmid. All constructions were checked by dideoxy sequencing of double stranded DNA.

Miscellaneous

Transformation of yeast cells, isolation of DNA and RNA from transformants, blotting techniques, labeling of oligonucleotides and hybridizations were performed essentially as described previously (18). Blots probed with the reporter oligonucleotide were hybridized and washed at 52°C; all other hybridization experiments were performed at 65°C. The probe specific for NTS2 was prepared by radiolabeling an excess amount (1μg) of the

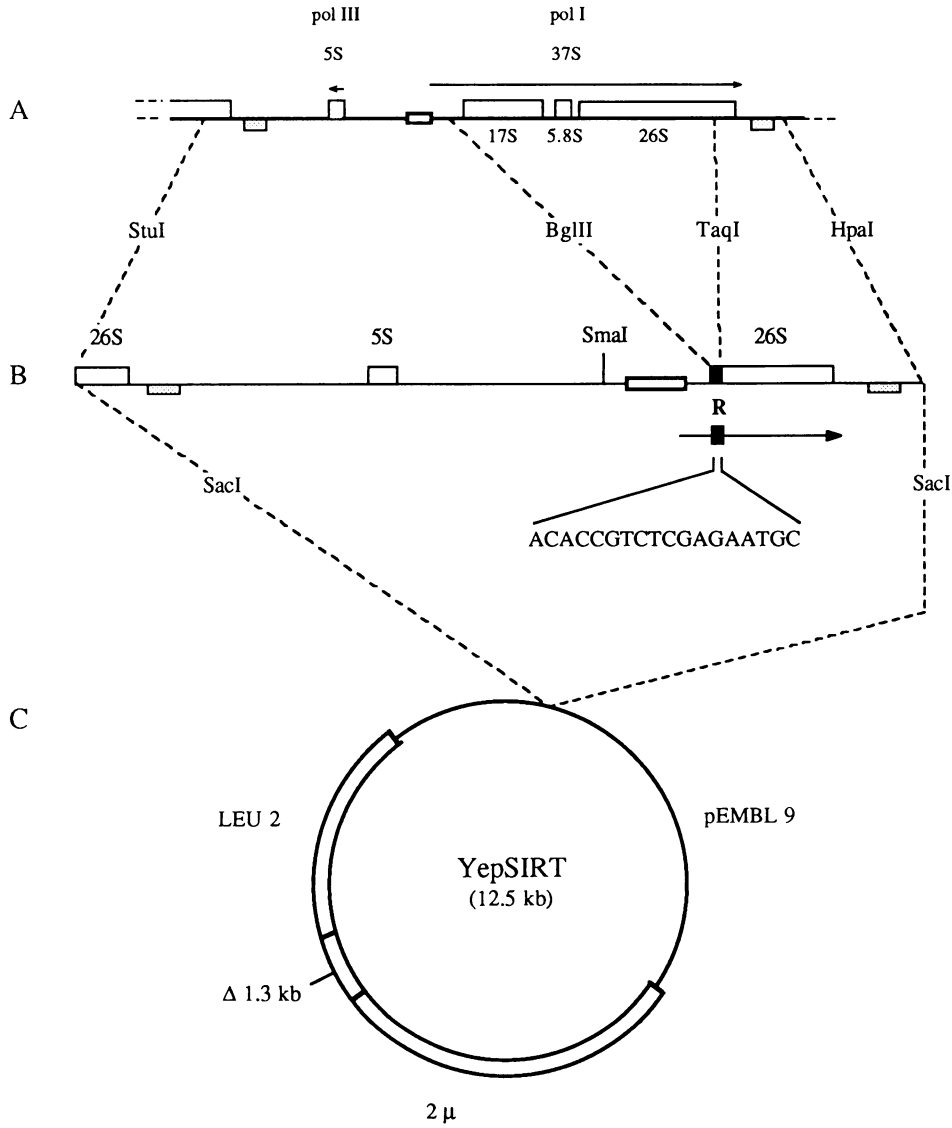

Figure 1. Genetic organization of the rDNA of *S.cerevisiae* (A), the SIRT minigene (B) and YepSIRT (C). Yeast rRNA genes are represented by open bars, the Pol I enhancer by a hatched box, and a fat-lined open box is used to indicate the Pol I promoter. A black box (R) represents the reporter oligonucleotide. Transcripts are indicated by arrows. The site of the *Bal*31 deletion in the vector is indicated (Δ 1.3 kb). See text for details.

610 bp *Sph*I-*Eco*RV fragment of the *S. carlsbergensis* rDNA repeat, using a Prime-a-Gene kit from Promega. The probe specific for actin mRNA was prepared from an M13mp10 construct containing the 1.5 kb. *Bam*HI-*Hin*dIII fragment of the *S. cerevisiae* actin gene (a kind gift of Mr. J.J. van den Heuvel), essentially as described previously (18). Reverse

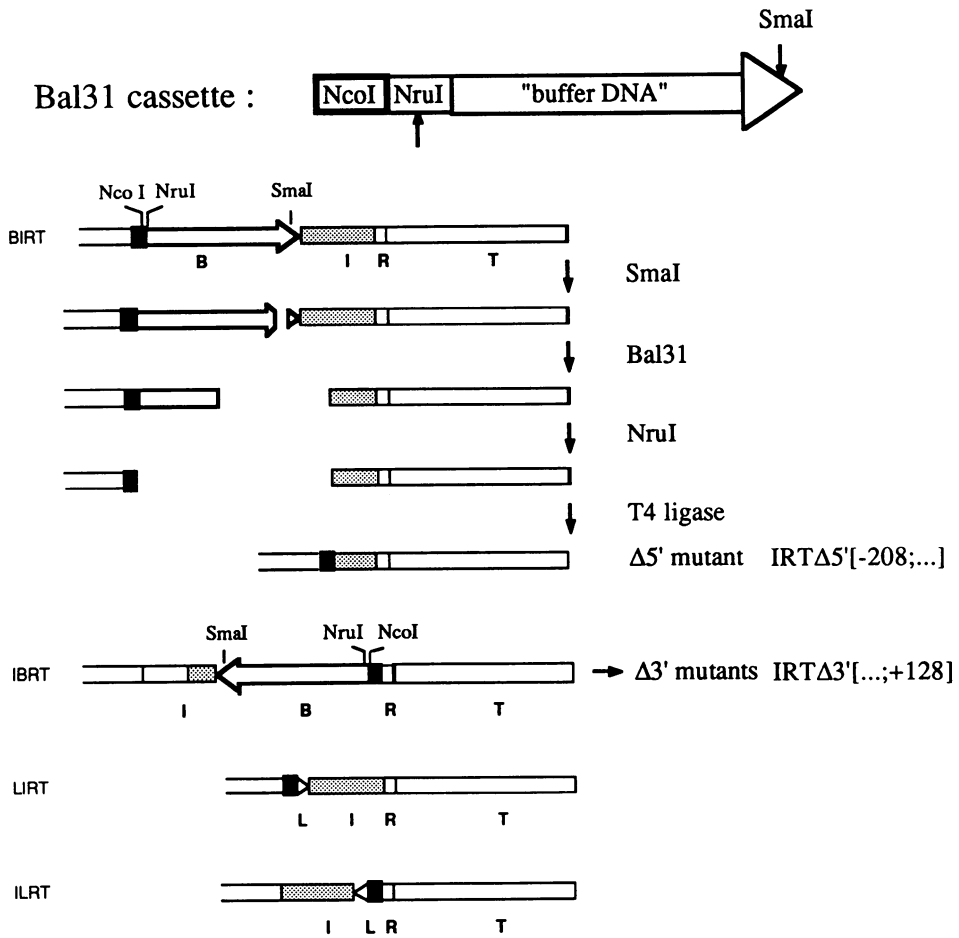

Figure 2. The *Bal*31 cassette and its mode of application.
The *Bal*31 cassette is represented by an open arrow. Deletions are created starting from the *Sma*I site and progressively remove the promoter element (hatched bar) towards one side, and the 'buffer DNA' fragment towards the other side. Following the deletion reaction, the residual part of the 'buffer DNA' fragment is removed by cutting with *Nru*I, resulting in the exposure of the *Nco*I linker (black box) at a blunt end. Intramolecular ligation positions the linker at the end-point of the deletion, recircularizing the plasmid. The sequence of events is illustrated for the generation of a 5'-deletion mutant, starting from the BIRT minigene. A similar procedure was followed to construct a 3'-deletion mutant from IBRT. LIRT and ILRT are control constructs, that result from mock deletions of BIRT and IBRT, respectively. I= initiation fragment, B= *Bal*31 cassette, R=reporter oligonucleotide, T=termination fragment and L=remains of the *Bal*31 cassette.

transcription was performed by annealing 0.2 pmol of the radiolabeled reporter oligonucleotide (specific activity 5.0×10^6 cpm/pmol) to 25 μg of total RNA isolated from transformed yeast cells, in the reaction buffer supplied with the reverse transcriptase. The extension reaction was performed with 200 units of M-MLV reverse transcriptase in the same buffer in the presence of 130 μM actinomycin D and 62.5 μM of each of the four

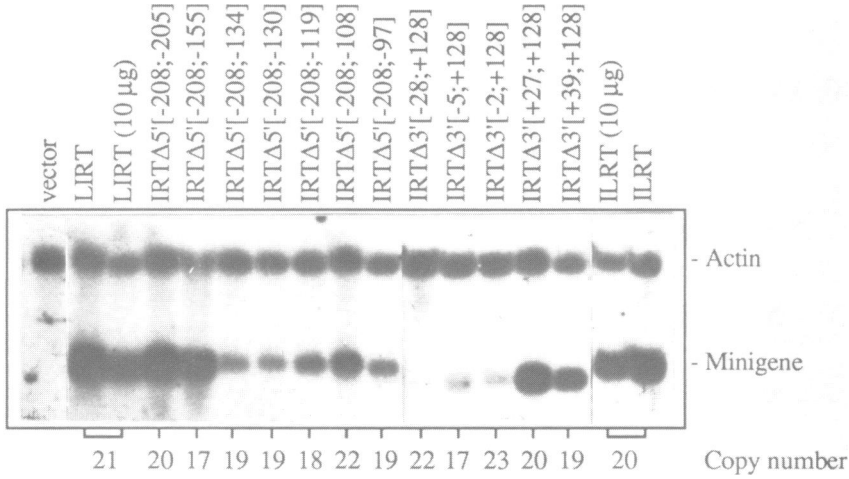

Figure 3. Northern hybridization of deletion mutants.
Unless indicated otherwise, 20 µg of total RNA isolated from yeast cells transformed with the indicated plasmids, was fractionated on a 1.4% agarose gel, blotted to Hybond N and hybridized with the radiolabeled reporter oligonucleotide. The 'vector' transformant was transformed with pEMBL Ye30-Δ16 (see Materials and Methods), and the names of the deletion constructs reflect the first and the last nucleotides that were deleted. The same blot was subsequently analyzed with a probe specific for actin mRNA. The figure is a superposition of two autoradiographs, obtained with the different probes.

dNTPs. Following the reaction, the templates were hydrolyzed by alkali treatment and the reaction products were analyzed by electrophoresis on a denaturing 8% polyacrylamide gel. The intensity of the signals obtained in the experiments depicted in Fig. 6, 7 and 8 was quantitatively determined by scanning laser densitometry of appropriate exposures, using an LKB 2202 Ultroscan.

RESULTS

Construction and Characterization of an RNA Polymerase I Minigene.

In this paper we report on our studies of the internal organization of the yeast Pol I promoter by linker scanning mutagenesis. To allow accurate measurement of the effects of clustered point mutations on the efficiency of transcription initiation by RNA polymerase I, we have constructed a minigene (SIRT, Fig. 1) that gives rise to a relatively high steady-state level of minigene transcripts in the yeast cell. In order to ensure efficient transcription a copy of the rDNA enhancer was included both upstream and downstream of the artificial transcription unit, since it is not yet known in what direction the yeast rDNA enhancer exerts its influence (13,14). We have tried to ensure a high transcript stability by including a relatively large part of the 26S rRNA gene in the minigene and by using a short (19 bp) reporter oligonucleotide, instead of the more commonly used longer heterologous reporter sequences (10−13). In a Northern hybridization of total cellular RNA isolated from cells transformed with the SIRT minigene, a transcript was readily detected using the reporter oligonucleotide as a probe (*e.g.* Fig. 7A, lane SIRT). The migration of the minigene transcript in the gel correlates well with its expected size (681 nt) if the 5′- and 3′-ends are identical with that of 37S pre-rRNA and 26S rRNA, respectively. For the

5'-end this was confirmed by reverse transcriptase mapping, using the reporter oligonucleotide as a primer (data not shown).

We subsequently tested whether the nucleotide requirements for the initiation of transcription by Pol I are affected by the presence of an enhancer in the SIRT minigene. To that end a series of 5'- and 3'-deletion mutants was constructed from a derivative of the SIRT minigene, IRT, which contains a *Bam*HI linker inserted into the *Sma*I site at −208 (outside the previously mapped promoter region [−192;+15], Ref. 16), but is otherwise identical to SIRT. The 5'- and 3'-deletion series were constructed with the aid of a specific tool: the *Bal*31 cassette (Fig. 2). This DNA fragment was inserted into the IRT minigene at the *Bam*HI site for the construction of the 5'-deletion mutants, and at the *Bgl*II site for the construction of the 3'-deletion mutants, yielding the minigene derivatives BIRT and IBRT, respectively (Fig. 2). This approach allows for the one-step construction of unidirectional deletions within the minigene, resulting in the insertion of a single linker at the site of the deletion (see legend to Fig. 2 for details). Following this approach we have constructed a small library of 5'- and 3'-deletion mutants and about 100 clones of each deletion type were characterized by dideoxy sequencing. The extent of transcription of a selection of minigenes with progressive 5'- or 3'-deletions in the Pol I promoter was analyzed by Northern hybridization with the reporter oligonucleotide (Fig. 3). As a control, minigenes with a mock deletion (i.e. removal of the 'buffer DNA' by cutting with *Sma*I and *Nru*I, omitting treatment with *Bal*31) were constructed from BIRT and IBRT, yielding LIRT and ILRT, respectively. As can be seen from Fig. 3, the minigene transcription of both LIRT and ILRT is not affected by the presence of the 18 bp remains of the *Bal*31 cassette in these minigenes. In the IRT minigene, the 5'-boundary of the gene promoter is located between nucleotide positions −155 and −134, since in IRTΔ5'[−208;−134] the level of the minigene transcript is reduced by at least a factor 5 as compared to IRTΔ5'[−208;−155] (Fig. 3). Comparison of the hybridization signals obtained with the 3'-deletion mutants (Fig. 3) leads to the conclusion that the 3'-boundary of the gene promoter in the IRT minigene is located upstream of nucleotide position +27, and, most likely, downstream of nucleotide position −2. We cannot rule out, however, the formal possibility that the lowered transcript levels in IRTΔ3'[−2;+128] and IRTΔ3'[−4;+128] result from destabilization of the transcripts, due to the removal of a stabilizing element.

To further investigate the residual transcriptional activity of minigenes carrying partially deleted Pol I promoters, the 5'-ends of their transcripts were analyzed by reverse transcription (Fig. 4). Comparison of the lanes marked IRTΔ5'[−208;−154] and IRTΔ5'[−208;−134] confirms that the 5'-boundary of the Pol I promoter is located between −154 and −134, since transcripts initiated at +1 are almost completely absent when the deletion extends beyond nucleotide position −134. Additionally, all lanes show bands lower in the gel (especially prominent around position +80). Since no signals corresponding to transcripts initiated at this site were detected in the Northern experiment (Fig. 3), these bands must be derived from premature termination of the reverse transcriptase, presumably due to the secondary structure of the template. Also, all lanes show transcripts that have initiated between nucleotide positions −10 and −45. These minor starts have also been observed in experiments using other minigenes (11,13), and it is not clear whether they are produced by Pol I or by a different polymerase. Our working hypothesis is that their presence is caused by the fact that not all components of the Pol I transcriptional machinery are available to all plasmids at all times, due to the location of (part of) the artificial templates outside of the nucleolus. It is of particular importance to note that these 'upstream starts'

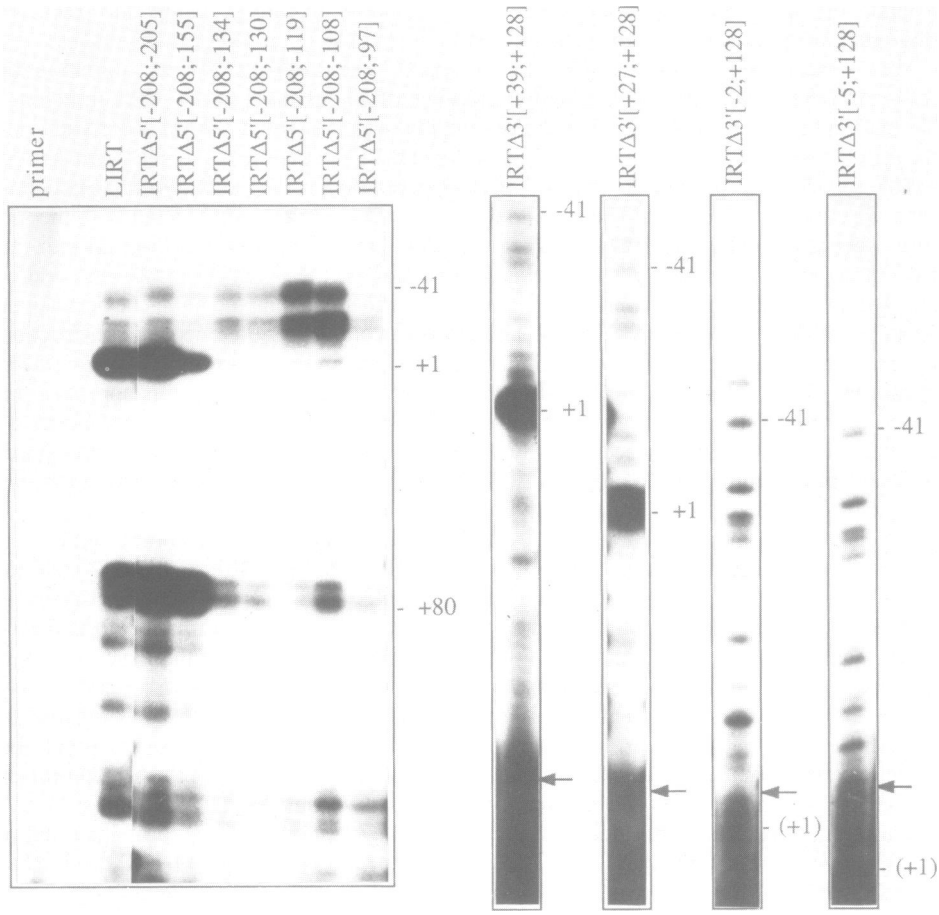

Figure 4. 5′-End mapping of the minigene transcripts from deletion mutants.
The radiolabeled reporter oligonucleotide was annealed to total RNA isolated from the indicated transformants, or to *Bacillus licheniformis* rRNA (primer lane), and extended by reverse transcriptase. The extension products were analyzed on denaturing 8% polyacrylamide gels (separate experiments for 5′- and 3′-deletion series). For each lane, the expected positions for transcripts initiated at +1 and −41 are indicated. For IRTΔ3′[−2;+128] and IRTΔ3′[−5;+128] brackets around +1 indicate that this position has been deleted in these constructs. The arrow marks the 3′-end of the reporter oligonucleotide.

are generated to the same extent by all the control constructs that have been used: SIRT, NIRT (Fig. 8), LIRT and ILRT (Fig. 4), and that they represent only a minor fraction of the total of minigene transcripts.

Residual transcription of minigenes with partially deleted Pol I promoters, as detected in Fig. 3, can largely be attributed to these minor transcripts (Fig. 4) Though all the minigenes give rise to the production of these transcripts, their relative abundance is higher in IRTΔ5′[−208;−119] and IRTΔ5′[−208;−108] transformants, indicating that the level of these transcripts may vary with different mutations.

SacI
SnaBI
-200
-180
-160
-140
-120
-100
SIRT
NIRT
LSM [-146;-134]
LSM [-121;-108]
LSM [-110;-97]
LSM [-102;-91]
LSM [-87;-76]
LSM [-70;-60]
LSM [-62;-51]
LSM [-49;-38]
LSM [-49;-38]Δ11
LSM [-33;-26]-8
LSM [-33;-26]-12
LSM [-28;-17]
LSM [-4;+8]
LSM [+27;+37]
LSM [+27;+37]*

-80
-60
-40
-20
+1
+20
AsuII
+40
SIRT
MIRT
LSM [-146;-134]
LSM [-121;-108]
LSM [-110;-97]
LSM [-102;-91]
LSM [-87;-76]
LSM [-70;-60]
LSM [-62;-51]
LSM [-49;-38]
LSM [-49;-38]a11
LSM [-33;-26]-8
LSM [-33;-26]-12
LSM [-28;-17]
LSM [-4;+8]
LSM [+27;+37]
LSM [+27;+37]*

Given the combined results of the Northern hybridization assay and the reverse transcription experiment, we feel confident that the Pol I promoter is located within the region [−155, +27], with 5′-deletions up to position −134 and 3′-deletions up to position −2 removing essential sequence information. This result for the IRT minigene is in full agreement with the results obtained previously with other minigenes (16,21), and clearly demonstrates that the presence of the enhancer element in *cis* does not alter the sequence requirements for Pol I promoter activity.

Construction and Characterization of Linker Scanning Mutants

From the 5′- and 3′-deletion mutants 12 Linker Scanning Mutants (LSMs) were constructed, each carrying the complete Pol I promoter region, a small part of which was replaced with a 12 bp linker. These linker substitutions introduce a number of clustered point mutations within the Pol I promoter at a different site in each plasmid (Fig. 5). For 5 LSMs the substitution caused an additional mutation, in the form of an insertion or deletion of 1 or 2 bp as the deletions in the corresponding 5′- and 3′-deletion mutants did not exactly 'match'. LSM[−33;−26]-8 is exceptional in that the length of the scanning linker in this plasmid is 8 rather than 12 nucleotides. It was constructed from LSM[−33;−26]-12 in order to restore the original length of the promoter region. LSM[+27;+37] was constructed as a control downstream of the Pol I promoter region, whereas as a control upstream of this region the NIRT minigene was constructed, which contains the scanning linker inserted into the *Sma*I site at position −208. Since the yeast vector used for the propagation of these minigenes contains a 2μ-derived origin of replication, we anticipated a considerable degree of variation in the copy number of the LSM plasmids. Therefore, the plasmid copy number was determined for each LSM transformant by Southern hybridization, using the 2.7 kb HindIII fragment of the rDNA repeat as an internal standard (Fig. 6). Assuming that the rDNA repeat is present at 140 copies per cell (22), the plasmid copy numbers range from about 14 for LSM[−33;−26]-12 to about 24 copies per cell for LSM[+27+37] (Fig. 6), which is well within the expected range of variation for a 2μ-derivative in a *cir*$^+$ host.

Northern hybridization of the LSM minigene transcripts with the reporter oligonucleotide (Fig.7A) reveals that mutations in different parts of the Pol I promoter affect promoter activity to different extents. This indicates that the Pol I promoter is not composed of one continuous element, but that it contains multiple elements that affect promoter activity to different degrees.

The experiments described in the previous section demonstrated that the hybridization signal in the Northern assay is caused by two classes of transcripts: those that are correctly initiated at +1, and transcripts that are initiated upstream of +1 (Fig. 4). In order to correct the hybridization signals in the Northern analysis of the LSM series (Fig. 7A) for aberrantly initiated transcripts, reverse transcription of the minigene transcripts was performed using the reporter oligonucleotide as a primer (Fig. 8). From this experiment it is evident that the results obtained with the SIRT and NIRT minigenes are essentially the same, with

Figure 5. Positions of the linker substitutions in the Pol I promoter region.
For each construct the point mutations resulting from the linker substitution are represented in reversed contrast. Deletions are indicated by a hyphen, and insertions are accommodated so as to maintain sequence alignment. The site of Pol I transcription initiation is indicated by an arrow, as are the sites for restriction enzymes used in some constructions (see Materials and Methods). The bracketed number at the end of each sequence indicates the number of base pairs that has been inserted or deleted in each LSM, relative to the wild-type sequence of the promoter region between −155 and +27. Additional insertions are boxed.

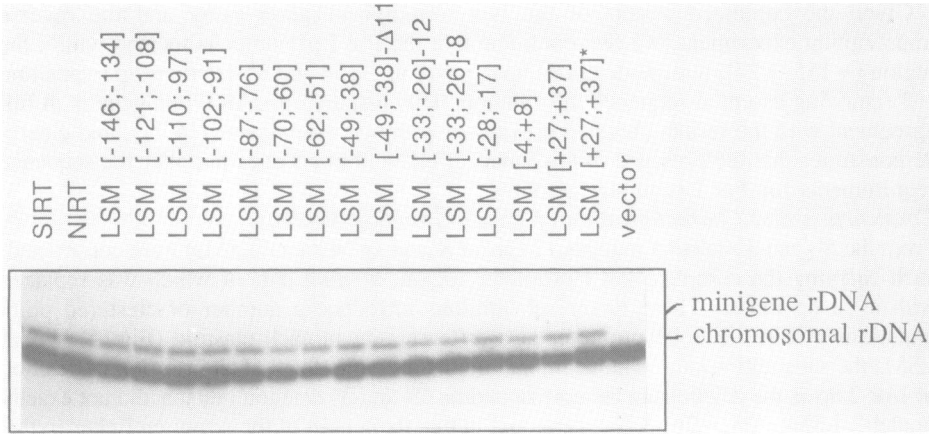

Figure 6. Copy number analysis of the LSM transformants.
*Hin*dIII digests of total DNA isolated from yeast cells transformed with the indicated plasmids (vector=pEMBL Ye30-Δ16), were fractionated on a 0.8% agarose gel, blotted onto Hybond N and hybridized with a probe specific for the NTS2 of the rDNA repeat. Minigene rDNA: the 3.1 kb *Hin*dIII fragment derived from the plasmids.Chromosomal rDNA: the 2.7 kb *Hin*dIII fragment derived from the chromosomal rDNA, comprising the NTS2 from the rDNA repeat.

respect to both the specificity and the intensity of the signals. In either case the aberrantly initiated transcripts contribute only a few percent to total transcription. For the LSMs this percentage appears to be higher, which is mainly due to the fact that the initiation of transcription at +1 is greatly reduced in some of these transformants (*e.g.* LSM[−70;−60],

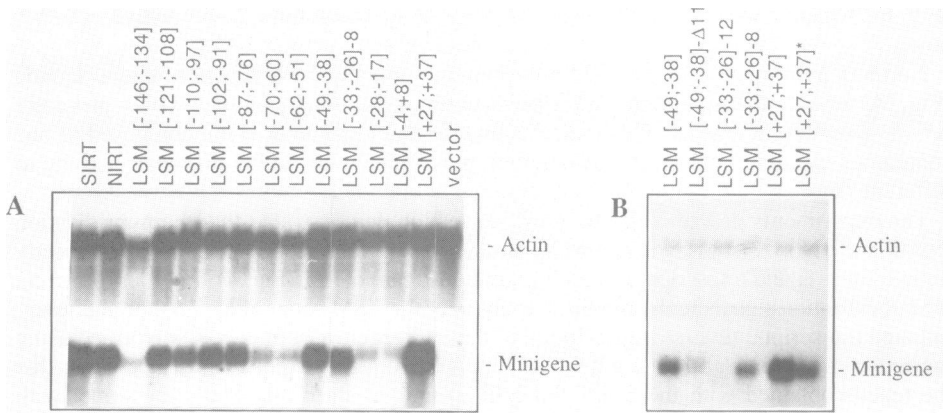

Figure 7. Northern analysis of minigene transcripts from LSMs.
20 μg of total RNA isolated from yeast cells transformed with the indicated plasmids (vector=pEMBL Ye 30-Δ16), was fractionated on a 1.4% agarose gel, blotted onto Hybond N and hybridized with the radiolabeled reporter oligonucleotide ('minigene'). Following autoradiography and removal of the probe, the same filter was hybridized with a probe specific for the actin mRNA ('actin'). The figure (both panels A and B) is a composite of these autoradiographs. **A**: The LSM series. An overexposure was chosen for presentation in order to visualize all the transcripts in their original relative abundancy. **B**: a shorter exposure of three special couples of LSM transformants (see text for details).

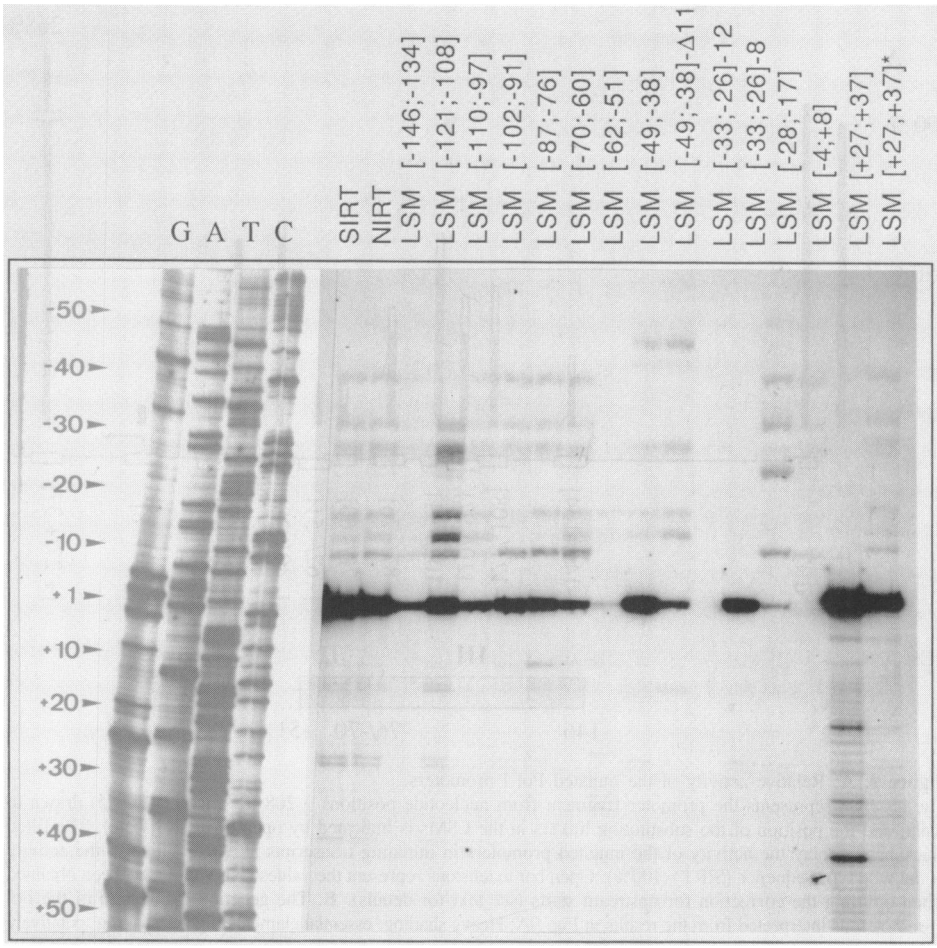

Figure 8. 5′-End mapping of minigene transcripts derived from LSMs.
The radiolabeled reporter oligonucleotide was annealed to total RNA isolated from yeast cells transformed with the indicated plasmids, and extended by reverse transcriptase. The extension products were analyzed on a denaturing 8% polyacrylamide gel. G, A, T, C: dideoxysequencing of YepSIRT double stranded DNA, using the reporter oligonucleotide as a primer. Numbering starts at the site of transcription initiation.

Fig. 8), whereas the total amount of 'upstream starts' is the same for most LSMs. LSM[−121;−108] displays an exceptionally high amount of upstream initiated transcripts. It is noteworthy that the 5′-deletion mutants in which the deletion endpoint maps in this same region of the promoter (IRTΔ5′[−208;−119] and IRTΔ5′[−208;−108]) display a similar pattern (Fig. 4).

The Northern hybridization signals obtained for the LSM transformants were corrected for aberrantly initiated transcripts, variations in plasmid copy numbers and differences in the amounts of RNA applied on the gel. The obtained values were normalized by setting the activity of the Pol I promoter in SIRT to 100% and the resulting relative promoter

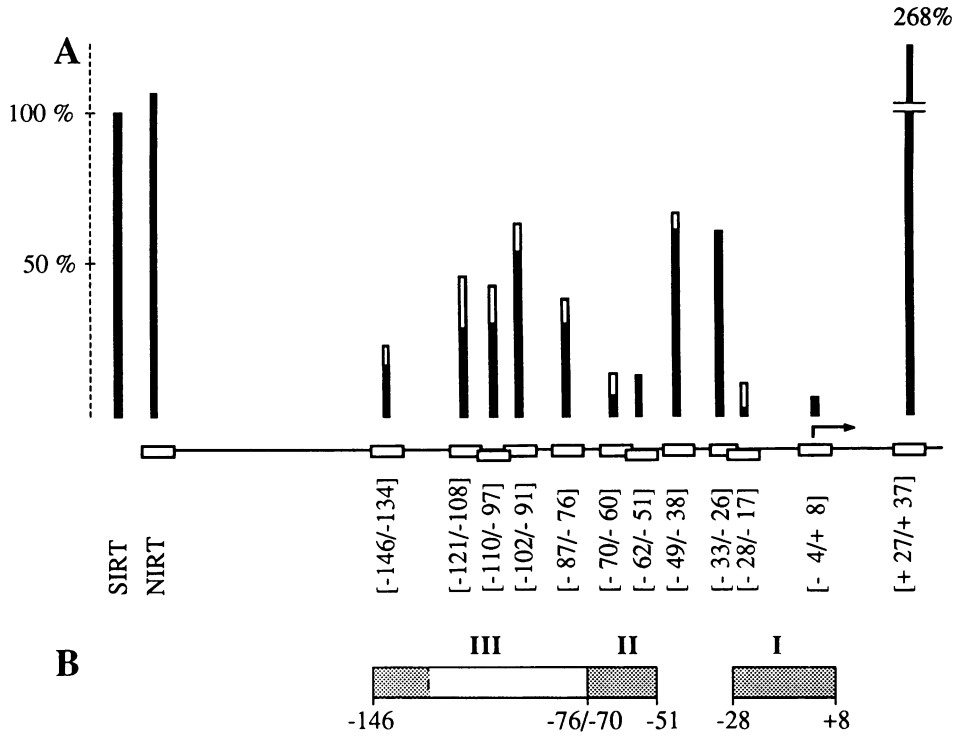

Figure 9. A: Relative activity of the mutated Pol I promoters.
The abscissa represents the promoter fragment from nucleotide positions −208 to +40 (*cf.* Fig. 5) drawn to scale, and the position of the substituting linkers in the LSMs is indicated by open boxes (to scale). The filled black bars display the activity of the mutated promoters in initiating transcripts at +1, relative to the activity of the wild type sequence (SIRT = 100%). Open bar extensions represent the values that would have been obtained when omitting the correction for upstream starts (see text for details). **B**: The genetic organization of the Pol I promoter, as interpreted from the results in Fig. 9A. Heavy shading: essential elements, lighter shading: putatively less important region (see text for details)

activities for the LSM series are graphically depicted in Fig. 9A. The results for the SIRT and NIRT minigenes are essentially the same, indicating that the insertion of a linker in the SmaI site at position −208 is a neutral mutation with respect to the initiation of transcription by Pol I. Also, it turns out that correction of the data for the presence of the aberrantly initiated transcripts, does not have a significant effect on the overall results of the experiment. The histogram in Fig. 9A is not appreciably altered by applying this correction (compare open and filled bars), which means that the obtained result is valid even when the 'upstream transcripts' are considered as genuine Pol I transcripts.

From these data it is apparent that the yeast Pol I promoter contains two domains that are essential for efficient initiation of transcription : domain I from position −28 to +8 and domain II from −70 to −51 (Fig. 9B). Due to the limitations of the linker substitution technique, the actual boundaries of the promoter domains may be located a few nucleotides up- or downstream of the given values. In addition, a third domain can be discerned, in

which the presence of point mutations displays only an intermediate effect on transcription initiation. This third domain is located between position −146 and −76. Though the LSM experiment suggests that it serves only accessory functions with respect to the initiation of transcription by Pol I, 5′-deletions in this element severely affected this process (Figures 3 and 4). This might indicate that an essential element is located at the 5′-end of domain III (Fig. 9B). The linker substitution of part of domain III in LSM[−102;−91] reduces promoter activity much less than substitution of the neighbouring regions. We cannot yet distinguish whether domain III actually consists of two closely positioned functional domains, or whether it represents only one functional region, in which the mutations introduced in LSM[−102;−91] simply affect activity to a lesser extent. We favor the latter possibility, since the position of the scanning linker in LSM[−110;−97] partially overlaps that in LSM[−102;−91], and the results obtained with LSMs[−121;−108], [−110;−97] and [−87;−76] are almost identical.

The data obtained for LSM[+27;+37] suggest that the Pol I promoter in this mutant is far more efficient than the wild-type promoter, which is rather puzzling since the point mutations introduced by the linker substitution in this mutant are located outside the Pol I promoter, as it was defined by deletion analysis (15, this paper). The linker substitution in this LSM creates an inverted repeat within the transcribed region (Fig. 5), which could give rise to the formation of a small stem-loop structure near the 5′-end of the minigene transcripts. This putative secondary structure might increase the stability of the minigene transcripts. In order to test this hypothesis the three left-most nucleotides of the scanning linker in LSM[+27;+37] were interchanged, so as to disrupt the inverted repeat, yielding LSM[+27;+37]* (Fig. 5). The promoter activity in this LSM was only 60%, as compared to over 200% for LSM[+27;+37]. Thus the apparent increase in the activity of the Pol I promoter in LSM[+27;+37] is due to stabilization of the minigene transcripts, caused by the presence of a stem-loop structure near the 5′-end of these transcripts. Further support for this explanation is derived from the reverse transcription assay (Fig. 8), where a strong stop at +38 in the lane LSM[+27;+37] is present, which is absent in the LSM[+27;+37]* lane.

In order to study the effect of changes in the relative positions of the domains of the promoter, two additional LSMs were constructed. LSM[−49;−38]Δ11 is identical to LSM[−49;−38] except that 11 nucleotides (position −78 to −68) have been deleted (Fig. 5). This shortens the distance between domains II and III by a little over one helical turn of the DNA, but leaves the spatial orientation of the two domains intact. The deletion results in a relative promoter activity for the LSM[−49;−38] transformant of less than 10%, as compared to about 60% obtained with LSM[−49;−38] (Figs. 6, 7B, 8 and 9). It cannot be excluded, however, that the malfunctioning of the promoter is caused by the fact that the deletion comprises part of domain II, rather than by a difference in the spacing between domains II and III. If this is true, the 5′-boundary of domain II must be located upstream of position −68.

LSM[−33;−26]-12 is identical to LSM[−33;−26]-8, but for the length of the scanning linker, which is 12 instead of 8 nt, resulting in the insertion of 4 nt between domains I and II. In this mutant the spatial orientation of these two promoter domains is affected, as well as the distance between them. The relative promoter activity in this mutant turned out to be only about 10%, as compared to about 60% for LSM[−33;−26]-8 (Figs. 6, 7B, 8 and 9). The reduction in promoter activity probably reflects the disruption of interactions between (a) *trans*-acting factor(s) that bind to these two promoter domains.

DISCUSSION

By analyzing mutated Pol I promoters in suitable *in vitro* systems (23–26), as well as *in vivo* (27–29), much progress has been made in the last few years, in unraveling the internal organization of eukaryotic rDNA promoters. Some of the apparently conflicting results that were initially obtained for different organisms, as well as by different groups studying the same organism (24,30), were later shown to be largely attributable to differences in assay conditions. Recently a general model for the structure of the eukaryotic rDNA promoter has been proposed (3), in which a core promoter domain (−40/−10) is involved in the binding of a species-specific transcription factor (factor D, according to the nomenclature of Muramatsu (31)), and an upstream domain of the promoter (about −150/−110, detected only when using stringent conditions) makes a further contribution to the acquisition of transcription factors. RNA polymerase I, activated by either a modification of Pol I itself, or by association with factor C, is positioned at the site of transcription initiation by protein-protein contacts with the transcription factors.

The structure of the yeast Pol I promoter, as revealed by our experiments, shares some characteristics with the general model, but also shows some remarkable differences. The yeast rDNA promoter contains two regions that are essential for efficient transcription initiation by Pol I, domain I from position −28 to +8, and domain II from position −70 to −51. Because of the severe effect of point mutations in both domain I and II on the Pol I promoter activity we propose that these two elements together constitute the yeast equivalent of a core promoter domain. In agreement with the general model, it includes the point of transcription initiation (+1), and it also comprises a large part of the [−9/+14] element, that was previously inferred from phylogenetic studies to be of importance to Pol I transcription in yeasts (32). However, our results suggest that the yeast core promoter extends further into the upstream region, as compared with the general model (−70 vs −40) and that it is interrupted by a region of lesser importance (−26 to −9). Though the presence of point mutations in this region was not completely neutral with respect to the efficiency of transcription initiation by Pol I, the effect of alterations in the flanking regions was much more pronounced. This may mean that the yeast core promoter interacts with multiple transcription factors, or that it contacts a single factor at two separate DNA regions. Interestingly, a similar observation has been reported from a linker-scanning analysis of the *Xenopus laevis* Pol I promoter (28).

In agreement with the general model for Pol I promoters, the yeast rDNA promoter contains a 5′-terminal domain that influences the activity of the promoter to a lesser degree than the core promoter domain. We suggest that domain III is functionally equivalent to the upstream domain found at the 5′-end of Pol I promoters in other eukaryotes.Compared with other organisms the yeast element seems to be somewhat larger at its 3′-end (−140/−76 vs −150/−110). The *X. laevis* Pol I promoter, however, may have a similar organization, since in this organism the region from nucleotide positions −100 to −70 has been shown to interact with proteins (33), and conflicting results have been reported for the effect of clustered point mutations in this region by different groups (28,29). On the other hand, the results we have obtained with LSM[−121/−108] allow for ambiguity in positioning the 3′-boundary of domain III, which may very well be located upstream of nucleotide −121, thus resembling the situation in other eukaryotes.

In order to investigate whether the deviations from the general model represent an essentially different organization of the Pol I promoter in yeast, or whether these are just

minor variations on the general theme of eukaryotic rDNA promoter structure, we are currently trying to identify the yeast Pol I transcription factors.

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