Intragenic Promoter Adaptation and Facilitated RNA Polymerase III Recycling in the Transcription of SCRI, the 7SL RNA Gene of Saccharomyces cerevisiae*

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Giorgio Dieci‡, Silvia Giuliodori, Manuela Catellani, Riccardo Percudani, and Simone Ottonello§
From the Dipartimento di Biochimica e Biologia Molecolare, Università di Parma, I-43100 Parma, Italy

The SCRI gene, coding for the 7SL RNA of the signal recognition particle, is the last known class III gene of Saccharomyces cerevisiae that remains to be characterized with respect to its mode of transcription and promoter organization. We show here that SCRI represents a unique case of a non-tRNA class III gene in which intragenic promoter elements (the TFIIIC-binding A- and B-blocks), corresponding to the D and T/C arms of mature tRNAs, have been adapted to a structurally different small RNA without losing their transcriptional function. In fact, despite the presence of an upstream canonical TATA box, SCRI transcription strictly depends on the presence of functional, albeit quite unusual, A- and B-blocks and requires all the basal components of the RNA polymerase III transcription apparatus, including TFIIIC. Accordingly, TFIIIC was found to protect from DNase I digestion an 80-bp region comprising the A- and B-blocks. B-block inactivation completely compromised TFIIIC binding and transcription capacity in vitro and in vivo. An inactivating mutation in the A-block selectively affected TFIIIC binding to this promoter element but resulted in a much more dramatic impairment of in vivo than in vitro transcription. Transcriptional competition and nucleosome disruption experiments showed that this stronger in vivo defect is due to a reduced ability of A-block-mutated SCRI to compete with other genes for TFIIIC binding and to counteract the assembly of repressive chromatin structures through TFIIIC recruitment. A kinetic analysis further revealed that facilitated RNA polymerase III recycling, far from being restricted to typical small sized class III templates, also takes place on the 522-bp-long SCRI gene, the longest known class III transcriptional unit.

The most represented RNA polymerase III (Pol III)1-transcribed genes, those coding for the tRNAs and the 5 S rRNA, have a highly conserved intragenic promoter comprising the binding sites for the general transcription factor TFIIIC (A- and B-blocks) and for the 5 S-specific factor TFIIIA (C-block). This conservation probably reflects the dual function of the above elements as both nucleation sites for transcription complex assembly and key determinants of tRNA and 5 S rRNA structure. Within the same genes, in fact, an extremely high sequence variability is displayed by the structurally unconstrained, vicinal upstream region. This region provides the binding surface for the initiation factor TFIIIB (1) and can modulate the strength of the intragenic promoter (see Refs. 2–4 and references therein). TFIIIB, which in yeast is minimally composed of the TATA-box-binding protein, the TFIIIB-related factor BRF (or TFIIIB70), and the Pol III-specific factor B* (or TFIIIB90), is generally assembled on tRNA genes in a TFIIIC-dependent manner (5). One extreme case of 5′-flanking sequence effect, however, has recently been documented for some tRNA genes of Saccharomyces cerevisiae, which, due to the presence of a canonical TATA box in their 5′-flanking region, are capable of autonomous TFIIIB binding and TFIIIC-independent in vitro transcription (6). Another indication of the constraints imposed on intragenic promoter elements by their overlapping structural and functional roles is the remarkable variability of promoter organization displayed by the minority of class III genes not coding for tRNAs and 5 S rRNAs. One group of such genes, well exemplified by the metazoan U6 snRNA and the human 7SK RNA genes, entirely relies for transcription on upstream promoter elements similar to those of RNA polymerase II-transcribed genes. Another group, which includes the Xenopus selenocysteine tRNA gene and the EBER2 gene of the Epstein-Barr virus, is characterized by mixed promoters composed of both intragenic and extragenic elements (reviewed in Ref. 5). The highly flexible organization of these genes is best illustrated by the 7SL RNA genes, coding for the conserved RNA component of the signal recognition particle, which in eukaryotes have undergone a remarkable variation in their mode of transcription. In humans, 7SL RNA gene transcription requires both an extended upstream region (7), including a binding site for the RNA polymerase II activator ATF (8), and an unusual intragenic promoter element that stimulates transcription through a structural motif at the 5′ end of the nascent transcript (9–11). At variance with the human genes, plant 7SL RNA gene transcription only requires an upstream promoter composed of a TATA box and an upstream stimulatory element identical to that of all plant U-snRNA gene promoters (12). Yet another promoter organization is found in the 7SL genes of protozoans of the family Trypanosomatidae, whose transcription depends on the A- and B-blocks of a divergently oriented, companion tRNA gene positioned 100 bp upstream of the 7SL transcription start site (13, 14). As a final example of promoter divergence, the 7SL genes of the yeasts Schizosaccharomyces pombe and S. cerevisiae both contain intragenic sequences resembling the A- and B-blocks (15, 16), but an upstream TATA box has been shown to play an essential transcriptional role in the fission yeast 7SL RNA gene.

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‡ To whom correspondence may be addressed. Tel.: 39-0521-905646; Fax: 39-0521-905151; E-mail: gdieci@unipr.it.
§ To whom correspondence may be addressed. Tel.: 39-0521-905646; Fax: 39-0521-905151; E-mail: sottonell0@unipr.it.
1 The abbreviations used are: Pol III, RNA polymerase III; nt, nucleotide(s); WT, wild type; snRNA, small nuclear RNA.
(17). Despite the critical evolutionary position of S. cerevisiae as one of the most primitive lower eukaryotes and the fact that its RNA polymerase III transcription system is by far the best characterized biochemically, the 7SL RNA gene of this organism, SCR1, is still uncharacterized. This single copy gene was identified more than a decade ago because of the extremely high abundance of its RNA product (15) but was never subjected to transcriptional analysis, and only very recently was it shown to be transcribed by RNA polymerase III (18). In particular, the contribution of the putative A- and B-blocks and the factor requirement for SCR1 transcription are unknown. Another interesting, yet unanswered question is how the very abundant SCR1 product, which accounts for ~0.2% of total yeast RNA (15), can be efficiently synthesized from a single copy gene. By taking advantage of a highly purified and well-characterized RNA polymerase III in vitro transcription system and of a viable, slow growth S. cerevisiae strain lacking SCR1 (19), we have carried out an extensive in vitro and in vivo analysis of SCR1 promoter architecture, initiation complex assembly, and transcription elongation and reinitiation properties.

**MATERIALS AND METHODS**

**Sequence Analysis of SCR1**—198 types of A-block sequences and 60 types of B-block sequences were derived from the alignment of 931 eukaryotic tRNA gene sequences.2 These unique sequences were used to construct updated weight matrices for Pol3Scan (available on the World Wide Web at irisi.bio.unipr.it/pol3scan.html), a program based on weight matrix analysis of tRNA gene promoters (20). 246 bp of 5'-flanking and 224 bp of 3'-flanking sequences, was verified using wild type S. cerevisiae SCR1 template and transcription components in the presence of 0.5 mM ATP and GTP and 2.5 μM [α-32P]UTP (Amersham Biosciences; 800 Ci/mmol). The output of a single round of transcription was then evaluated by adding CTP (0.5 μM), together with excess unlabeled UTP (2 μM), with or without 100 μM heparin, and allowing transcription elongation to proceed for 1 min. RNA size markers were generated by T7 RNA polymerase (Amersham Biosciences) in vitro transcription (23) of linearized pBlueScript-KS constructs bearing inserts of different sizes in the following site: the S. cerevisiae ITAT/LR1 tRNA gene and flanking regions (302 bp (6)) and the sequences coding for yeast ribosomal proteins L13 (600 bp (23)) and S24 (408 bp).3

**DNase I Footprinting and Gel Retardation Assays**—For the DNase I footprinting experiment in Fig. 4A, a 992-bp SCR1 fragment, 5'-end labeled on the sense strand, was generated by PCR using 5'-labeled SCR1fw and unlabeled SCR1rev as primers and pBlueScript-SCR1 as a template. The fragments utilized for the footprinting experiments of Fig. 4B (256 bp) were 5'-end-labeled on the antisense strand by PCR using a 5'-labeled oligonucleotide primer hybridizing between positions +224 and +200 (5'-GGCGGGACACCTTCTAGAGGCGAC), the 5'-32 oligonucleotide as a forward primer, and the SCR1 5'-32, Adown, and C4T template and transcription components in the presence of 50 mM ATP and GTP and 2.5 μM [α-32P]UTP (Amersham Biosciences; 800 Ci/mmol). The output of a single round of transcription was then evaluated by adding CTP (0.5 μm), together with excess unlabeled UTP (2 μM), with or without 100 μM heparin, and allowing transcription elongation to proceed for 1 min. RNA size markers were generated by T7 RNA polymerase (Amersham Biosciences) in vitro transcription (23) of linearized pBlueScript-KS constructs bearing inserts of different sizes in the following site: the S. cerevisiae ITAT/LR1 tRNA gene and flanking regions (302 bp (6)) and the sequences coding for yeast ribosomal proteins L13 (600 bp (23)) and S24 (408 bp).3

**Transcription of the Yeast 7SL RNA Gene**

Footprinting mixtures were phenol-extracted, ethanol-precipitated in 70% ethanol, and resuspended in 10 mM Tris-HCl (pH 8.0), 10% glycerol, 90 mM (NH4)2SO4, 1 mg/ml ultrapure heparin, and allowing transcript elongation to proceed for 2 min. RNA size markers were generated by T7 RNA polymerase (Amersham Biosciences) in vitro transcription (23) of linearized pBlueScript-KS constructs bearing inserts of different sizes in the following site: the S. cerevisiae ITAT/LR1 tRNA gene and flanking regions (302 bp (6)) and the sequences coding for yeast ribosomal proteins L13 (600 bp (23)) and S24 (408 bp).3

**In Vitro RNA Analyses**—The yeast strain YRA130 (a kind gift of Peter Wach, University of California, San Francisco), in which the entire SCR1 gene, except for the first 14 nucleotides, has been deleted and replaced with the HIS3 gene (19), was utilized for in vitro complementation and expression assays. This strain was transformed with the different Yep352-SCR1 constructs by the lithium acetate procedure (26), and the resulting transformants were selected for uracil auxotrophy on SD plates supplemented with tryptophan, lysine, and adenine. The transformants were prepared as a crude lysate to a previous structure (27). Primer extension reactions were carried out as described (6), using 5 μg of total yeast RNA and a 5'-labeled oligonucleotide primer (5'-GCCTTGCGAAAAGGGGCTGACAACTC) complementary to the coding region of SCR1 between positions +90 and +115. Complementation

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2 R. Percudani, unpublished results.

3 L. Bottarelli and G. Dieci, unpublished results.
Transcription of the Yeast 7SL RNA Gene

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Fig. 1. SCR1 control elements identified by weight matrix analysis. A, frequency matrices for tRNA gene promoter regions. Each column corresponds to an individual position along the A-block or B-block sequence. Intragenic promoter boundaries for a consensus tRNA are +7/+25 (A-block) and +52/+62 (B-block) and are referred to a mature tRNA sequence. The top line (Total) indicates the total number of unique tRNA sequences in which each position is represented. Columns where this number is lower than the sample number (198 for the A-block and 60 for the B-block) indicate a position that in some cases is deleted. The SCR1 putative promoter elements identified with Pol3scan (score = -37.95) are compared with the 75% consensus of the frequency matrix. The consensus is written following IUPAC notations: R = A/G; Y = T/C; B = G/C/T; V = G/C/A; D = G/A/T; N = G/C/A/T. B, secondary structure model of the 5’-end of the S. cerevisiae 7SL RNA (adapted from Ref. 46). The sequences corresponding to the A- and B-blocks are shown in boldface type. Mutations in the putative promoter elements analyzed in this study are indicated in boxes above the wild type sequence.

The YRA130 slow growth phenotype (19) was qualitatively evaluated by visual inspection of selective (SD) or nonselective (YPD) plates, on which cultures of freshly transformed clones were spotted.

Strains UKY403 and MHY308 (a kind gift of Michael Grunstein (UCLA)) were employed to analyze the effects of nucleosome disruption on SCR1 transcription. Strain UKY403, in which the two histone H4 genes have been disrupted, survives with a unique, centromeric plasmid-borne histone H4 gene under the control of the GAL1 promoter (28). MHY308 is isogenic to UKY403, except that its sole histone H4 gene is under the control of its own wild type promoter (29). Both strains were transformed with YEp352 constructs carrying WT and mutant (5’-Δ32 and Adown) SCR1 minigene variants, in which 120 bp at the 3’ terminus had been deleted by PCR using the SCR1 mini oligonucleotide (5’-AAAAAATTGGTGTATCCCGGCGCCTCC) as a reverse primer, either SCR1f or 5’-Δ32 as forward primers, and WT or Adown SCR1 as PCR templates. The SCR1 mini oligonucleotide introduces an artificial terminator sequence at position +400 of the SCR1 sequence, so that transcription of the various minigene templates yields ~400-nt-long transcripts that are easily distinguishable from the endogenous 522-nt-long SCR1 RNA. The glucose shift experiment was carried out as described previously (30). For RNA gel blot analysis, RNA samples (5 μg) were electrophoresed on 6% polyacrylamide, 7 M urea gels and transferred to Hybond-N membranes (Amersham Biosciences), which were then probed with the same 5’-labeled oligonucleotide utilized for primer extension analysis. Hybridization was carried out overnight at 28 °C in 5x SSC, 5x Denhardt’s solution, 0.1 mg/ml denatured salmon sperm DNA, 0.5% (w/v) SDS, followed by three short washings in 2x SSC, 0.1% SDS. Hybridization products were visualized by autoradiography and quantified by phosphorimaging.

Results

Predicted Control Elements of the SCR1 Gene—Pol3scan, a program based on weight matrix analysis of tRNA gene promoters (20, 31), was used to locate A- and B-blocks in the SCR1 sequence. No such element was identified with the default cutoff score (~34.14) usually employed for the identification of tRNA gene promoters. With a more permissive cutoff (~38), however, putative A- and B-blocks, with a spacing almost perfectly matching that of mature tRNAs, were identified at positions +9 and +51, respectively (Fig. 1A). A search of the Signal Recognition Particle Database (32), conducted with the same parameters, revealed the presence of A- and B-blocks above the ~38 cutoff threshold only in the case of fungal 7SL RNA genes (Yarrowia lipolytica and S. pombe). In tRNA genes, these two promoter elements code for highly conserved structural modules of the tRNA (the D and TΨC arms, respectively); their sequence conservation is thus influenced by factors not necessarily related to promoter strength. As shown in Fig. 1B, the A- and B-blocks of SCR1 are embedded in a very distinct structural context, so that sequence variations may be expected because of the different structural constraints. Indeed, the putative promoter elements of SCR1 display distinguishing features as compared with the consensus of the tDNA A- and B-blocks (Fig. 1A). The most prominent of them is the substitution of the canonical B-block starting sequence GTTT (in which the invariant T at the fourth position corresponds to the precursor of the essential pseudouridine residue of the tRNA TΨC arm) with GAA, a sequence that never occurs in tRNA gene promoters but is present in RPR1, another noncanonical yeast class III gene coding for the RNA subunit of RNase P (33). Another sequence feature never occurring in tRNA genes, but found in SCR1, is TC at positions +16 and +17, corresponding to positions +14 and +15 of the consensus tRNA gene A-block (Fig. 1A; numbering starts from the first position of the mature tRNA), which are sites of important tertiary interactions in tRNA structure (34). Other, more evident features of SCR1 are a TATA element upstream of the transcription start site (position −31) and, as already noted (15), a typical T-rich terminator element at position +518.

In Vitro Transcription of SCR1—The coding region of SCR1, plus 246 bp of 5’-flanking and 224 bp of 3’-flanking sequence, was PCR-amplified from S. cerevisiae genomic DNA and inserted into the pBlueScript KS vector. The resulting construct was then assayed in a Pol III-specific in vitro transcription system containing balanced amounts of recombinant TATA-
Fig. 2. **SC1 RNA synthesis in a reconstituted *in vitro* transcription system.** A, the size of *in vitro* synthesized SC1 (lane 4) was measured by comparison with marker RNAs of the indicated sizes in nucleotides (lanes 1–3, M). The arrowhead on the right indicates the migration position of the SC1 transcript. B, the RNA products of a 2 times scaled up *in vitro* transcription reaction programmed with the SC1 template (lane 1, in vitro), or 5 μg of *in vivo* synthesized total RNA (lane 2, *in vivo*) were subjected to primer extension analysis as described under “Materials and Methods.” The migration position of the fully extended SC1-specific primer is indicated on the left (scRNA). Shown in lanes 3–6 are the results of dideoxy chain termination sequencing reactions conducted with the same 5'-labeled oligonucleotide utilized for primer extension. The sequence of the nontranscribed DNA strand around the start site (+1) is indicated on the right. C, SC1 in *in vitro* transcription was carried out either in the presence of the entire set of Pol III components (lane 1, ALL) or in partially reconstituted systems lacking the individual transcription components indicated above each lane.

box-binding protein and BRF proteins, partially purified B' and TFIIIC fractions, and highly purified RNA polymerase III (6, 24). Transcription products were run on a polyacrylamide/urea gel (Fig. 2A, lane 4) in parallel with standard RNAs of known size produced by T7 RNA polymerase (Fig. 2A, lanes 1–3). A single transcript, with a size very close to that of the natural scRNA (519 nt for strain ATCC 25657 (15) and 522 nt for strain 5288C; see “Materials and Methods”), was synthesized in the *in vitro* reconstituted Pol III system. A comparison between the *in vitro* and the *in vivo* synthesized scRNA is presented in Fig. 2B, which shows the results of a primer extension analysis that was carried out to map the SC1 transcription start site. Both *in vitro* (lane 1) and *in vivo* (lane 2) synthesized transcripts initiated at the same A residue corresponding to the first nucleotide of the scRNA (15). Thus, the *in vitro* reconstituted Pol III system supports the efficient and faithful transcription of the SC1 gene. The transcription factor requirements for scRNA synthesis are reported in Fig. 2C, which shows that both TFIIIC and the three components of yeast TFIIIB are essential for SC1 transcription. Very low levels of TFIIIC-independent transcription were observed in some experiments. In accord with the presence of a TATA element at position −31 (6), this background, TFIIIC-independent transcription was abolished by TATA-box inactivation (data not shown). When the natural B' fraction was replaced by recombinant yeast TFIIIB90 (35), SC1 transcription was reduced by about 7-fold and was not significantly stimulated by the addition of the TFIIIE fraction (data not shown (36)).

**Organization of the SC1 Promoter—**Mutations were next introduced into the different putative control elements previously identified by sequence analysis, and the resulting mutants were assayed for template activity both *in vitro* and *in vivo*. For the *in vivo* analysis, mutagenized SC1 derivatives were inserted into the multicopy plasmid YEp352 (22) and transformed into the scr1::HIS null mutant strain YRA130 (19). This strain displays a slow growth phenotype that could be reversed upon introduction of the wild type SC1 gene carried by YEp352 (not shown). This enabled us to monitor the phenotypic effects of the introduced mutations. Fig. 3A summarizes the transcription activities and functional complementation phenotypes of the different mutants, whereas Fig. 3B and C, shows representative results of *in vitro* (Fig. 3B) and *in vivo* (Fig. 3C) transcription analysis. In the experiment of Fig. 3C, the steady state levels of the 7SL RNA were measured by primer extension in the transformed null mutant. This allowed us to reveal simultaneously the *in vivo* transcriptional output of SC1 mutants as well as possible defects in start site selection. The results of these experiments showed that the B-block is the only cis-acting element absolutely required for SC1 transcription. In fact, a C56G point mutation within such an element abolished transcription *in vitro* (Fig. 3B, lane 4), reduced the *in vivo* steady state amount of the 7SL RNA to undetectable levels (Fig. 3C, lane 4), and completely destroyed complementation capacity (Fig. 3A). By comparison, a double point mutation in the A-block (CC in place of GG at the uni-
versely conserved +19 and +20 positions; Adown mutant) had a much less severe effect on in vitro transcription (Fig. 3B, compare lane 5 with lane 2), even in combination with a TATA box-inactivating mutation (lane 6). The same A-block mutation, however, reduced in vitro 75S RNA levels by about 20-fold as compared with wild type SCR1 (Fig. 3C, lanes 5 and 6) and resulted in a partial loss of slow growth complementation (Fig. 3A). In tRNAs, the A-block region is an important structural determinant, and reduced in vitro levels of SCR1 Adown transcripts might thus in principle derive from a decreased stability of the RNA product, rather than from a transcription defect. This possibility was tested by RNase A digestion experiments, which revealed an identical nuclease sensitivity (with respect to enzyme amount and time course of degradation) of in vitro synthesized, wild type and Adown SCR1 transcripts (data not shown). A less dramatic in vitro/in vivo discrepancy was observed with a mutant (5′Δ-32) carrying a deletion of the SCR1 5′-flanking region from −245 to −33. This mutation resulted in a slightly increased transcription efficiency in vitro (Fig. 3B, lane 2), while it caused a 4-fold decrease of in vivo SCR1 transcription (Fig. 3C, lane 2), a relatively small effect that was not reproduced in independent experiments carried out in a different yeast strain with a 3′-truncated version of the 5′-Δ-32 mutant (see below). Similarly, inactivation of the TATA box in the 5′-Δ-32 context did not produce any significant effect (Fig. 3, B and C, lanes 3), and also without effect on in vitro transcription was the deletion of the entire SCR1 coding sequence downstream of the B-block (Fig. 3B, lane 7). In the latter case, the introduction of an artificial T (7) terminator at position +90 led to the synthesis of a correspondingly shortened (−90-nt-long) transcript. The apparently reduced accumulation of this transcript is not due to a transcriptional defect but simply reflects the decreased incorporation of radiolabeled U residues during in vitro transcription. In fact, normalization for the number of incorporated U residues gave an estimate of in vitro transcription efficiency identical to that of the WT SCR1 gene (Fig. 3A). Also, at variance with the yeast U6 snRNA gene, in which TATA box and A-block mutations have been shown to result in an altered initiation site selection (37–39), transcription correctly initiated at the same A residue, both in vivo and in vitro, in all of the tested SCR1 mutants (Fig. 3C, lanes 3, 5, and 6; data not shown).

**Binding of TFIIIC to the SCR1 Gene**—As revealed by the above results, the A- and B-blocks located within the first 70 bp of the SCR1 coding region are essential promoter elements. To verify whether such elements behave like the internal promotors of tRNA genes, binding of TFIIIC to SCR1 was analyzed by DNase I footprinting. As shown in Fig. 4A, TFIIIC protected the entire intragenic region from position +5 to +84 of the sense strand (5 bp upstream of the A-block to 23 bp downstream of the B-block). Both the extent of the observed protection and the presence of an intensified cleavage site at the 3′ border of the protected region closely resemble the protection patterns previously reported for yeast tRNA genes (40) and the tRNA-like leader of the PRP1 gene (33). The TFIIIC-binding properties of the Adown and Bdown SCR1 templates were then examined to find out whether the TFIIIC binding ability of such mutants correlates with their in vitro and/or in vivo transcription activity. As shown in Fig. 4B, on the 5′-Δ-32 template, bearing wild type A- and B-blocks and utilized as a control for these experiments (lanes 5–7), TFIIIC protected a region comprised between positions +9 (the 5′ border of the A-block, also corresponding to a site of intensified cleavage), and +77 (16 bp downstream of the B-block) of the antisense strand. In contrast, TFIIIC binding was completely abolished in the case of the Bdown template (lanes 11–13), in which the C56G mutation per se determined a local alteration of the DNase I sensitivity pattern (compare lane 11 with lanes 5 and 8). A somewhat intermediate situation was observed in the case of the Adown template (lanes 8–10). Here, the interaction of TFIIIC with the B-block was barely affected, whereas protection was significantly decreased from position +9 (with the loss of the TFIIIC-induced hypersensitive site) to +38 (12 bp downstream of the A-block). Thus, the A-block mutation interferes with the correct positioning of the upstream portion of TFIIIC. As further revealed by the gel retardation experiment reported in Fig. 5A, an immediate consequence of this suboptimal promoter occupancy is a reduced affinity for TFIIIC. In fact, the Adown template was much less effective than the control 5′-Δ-32 template in TFIIIC binding (compare lanes 4–7 with lanes 11–14), and a 9-fold reduction in TFIIIC-Adown SCR1 DNA complex formation, as compared with WT SCR1 (compare lanes 4 and 11), was observed in the presence of 100 ng of partially purified TFIIIC, the standard amount used for in vitro transcription experiments.

The decreased in vivo RNA output of the Adown mutant thus appears to correlate with a defective TFIIIC binding, while the in vitro/in vivo transcription discrepancy observed with the same mutant (Fig. 3) may be explained by the lack, in the purified in vitro system, of both competitor templates and interfering chromatin structure effects. This hypothesis was first tested by transcription competition experiments, reported in Fig. 5B, in which wild type SCR1 (lanes 1–7) or the Adown mutant (lanes 8–14) were transcribed in vitro in the presence of increasing concentrations of a competitor tRNAPro gene. Transcription of the Adown mutant was much more sensitive to tDNAPro than WT SCR1 transcription. For example, in the presence of an equimolar amount of competitor DNA, WT SCR1 transcription was reduced by 2-fold (compare lanes 1 and 4) as compared with the 20-fold inhibition observed under the same conditions with the Adown mutant (compare lanes 8 and 11), a 10-fold effect approaching the previously measured difference between in vitro (uncompeted) and in vivo transcription (Fig. 3). This marked competition sensitivity specifically pertains to the Adown mutant, because no such effect was observed in similar competition experiments conducted with the upstream SCR1 deletion mutant 5′Δ-32 (data not shown).

We then asked whether chromatin-mediated transcriptional interference could also contribute to the much more dramatic defect displayed by the Adown SCR1 mutant under in vivo conditions. A yeast strain (UKY403) in which the two genes coding for histone H4 have been disrupted and which survives with a single histone H4 gene under the control of the GAL1 promoter was used to test this hypothesis (28). Shifting the UKY403 strain to a glucose-supplemented medium blocks histone H4 gene expression, thus causing a global disruption of chromatin structure and consequent growth arrest. For the experiment of Fig. 6, this strain and the control strain MHY308 (isogenic to UKY403 except that its sole histone H4 gene is under the control of its own WT promoter and is thus glucose-insensitive (29)) were transformed with WT or Adown SCR1 constructs carrying 3′-shortened derivatives of both genes, so as to distinguish between plasmid-derived and endogenous, full-length SCR1 transcripts. Transformants were grown to an A600 value of 0.5 in selective galactose medium and then shifted to glucose-containing medium for an additional 6 h, and total RNA from both cultures was subjected to RNA gel blot analysis. The levels of shortened RNAs transcribed from episomal SCR1 minigenes were in general much lower than those of endogenous SCR1 transcripts, most likely because of a decreased in vivo stability of the truncated RNAs. In either strain, Adown SCR1 transcripts were undetectable during growth on galac-
FIG. 3. Transcriptional and complementation phenotypes of SCRI mutants. A, schematic representation of the different SCRI mutants. Putative intragenic (filled boxes) or extragenic (empty boxes) control elements and the transcription termination site (Tn) are indicated; crossed boxes indicate mutagenized elements (see "Materials and Methods" for details). In vitro transcription levels, reported in the first column, are given as percentages of wild type SCRI transcription; values are the average of at least five independent measurements that differed by no more than 15% of the mean. Normalized transcription values for the 3'Δ+90 mutant (asterisk; see also lane 7 in B) were calculated by taking into account that 5 times fewer U residues are incorporated into the early truncated 3'Δ+90 transcript as compared with WT scR1. In vivo expression levels, reported in the middle column, were determined by primer extension analysis of total RNA extracted from the YRA130 strain transformed with...
Transcription of the Yeast 7SL RNA Gene

Transcription Elongation and Reinitiation on SCR1—The typical products of 7SL RNA genes (~300 nt) are unusually long with respect to the ~100-nt-long RNAs commonly encoded by class III genes (e.g. tRNAs and the 5 S rRNA). This is even more so for the S. cerevisiae 7SL RNA gene, whose 522-nt transcript is the longest known RNA synthesized by Pol III. The Pol III system has fairly unique transcription reinitiation properties, being able to complete an entire transcription cycle in vitro in time intervals as short as 20 s and to carry out multiple rounds of transcription on the same gene without polymerase dissociation (41, 42). These peculiar recycling properties may in principle be a direct consequence of the small size of class III transcriptional units that, by keeping the transcription complexes in close proximity to the promoter, may augment its probability of reinitiating on the same gene (43). We thus set out to analyze, by single round transcription experiments, the kinetics of reinitiation on the SCR1 gene, a 5 times longer elongation track as compared, for example, with a typical tRNA gene. Classical single round transcription analysis (1) relies on the formation, upon NTP omission, of stalled ternary complexes (composed of template DNA, transcriptional proteins, and nascent RNA) that are resistant to heparin concentrations completely inhibiting reinitiation. The nucleotide sequence of SCR1 is such that a stalled ternary complex incorporating a 4-nt-long RNA can be generated at best by the omission of UTP. On the basis of previous studies, showing slippage of transcripts shorter than 5 nt (44), we expected that an initiated complex containing a nascent RNA shorter than 5 nt might not be sufficiently stable to resist heparin treatment. To solve this problem, we introduced a C to T substitution at position +4 of SCR1. In the absence of CTP, this mutant template (SCR1-C4T), whose in vitro transcription efficiency was identical to that of WT SCR1 (not shown), yielded stalled ternary complexes bearing a 12-nt RNA and supposedly standing heparin concentrations (~100 µg/ml) that completely inhibit reinitiation (1). The latter assumption was verified with the pulse-chase experiment reported in Fig. 7A, in which the 12-mer was synthesized in the presence of ATP, GTP, and [α-32P]UTP at a high specific radioactivity, followed by the resumption of transcription through the addition of CTP and an 800-fold molar excess of unlabeled UTP, either alone (lane 1) or with heparin (100 µg/ml) (lane 2). Under these conditions, because of isotopic dilution, only full-length transcripts synthesized during the first transcription cycle will incorporate enough radioactivity so to contribute significantly to the observed signals. Accordingly, the transcription signal in lane 1 is a measure of the total number of unperturbed elongation-competent ternary complexes, while the signal in lane 2 corresponds to the fraction of such complexes that have resisted heparin perturbation. In the experiment shown and in two additional independent experiments, the intensities of these two signals were found to be nearly identical (± 8%), thus proving the almost complete heparin resistance of stalled SCR1 elongation complexes. Similar results were obtained when ternary complexes were challenged with heparin for up to 2 min prior to NTP addition and resumption of transcription elongation (data not shown). Having established that heparin does not affect the stability of stalled elongation complexes formed on the SCR1-C4T template, we conducted the transcription reinitiation analysis reported in Fig. 7B. To this end, stalled elongation complexes were first formed on SCR1-C4T in the absence of CTP, and then CTP was added, either alone (lane 1) or in combination with varying concentrations of heparin (lanes 2–7), and transcription was allowed to proceed for 5 min. A heparin concentration of 25 µg/ml was found to be sufficient to block reinitiation, whereas elongation from initiated complexes was unaffected even by a 24-fold higher heparin concentration. In the absence of heparin, about eight transcription cycles took place on the SCR1-C4T gene in 5 min, corresponding to a cycle duration time of ~40 s. The experiment reported in Fig. 7C was next carried out to evaluate the duration time of a single elongation step on SCR1. Stalled elongation complexes were assembled as in the experiment reported in Fig. 7A, and then elongation was resumed (and reinitiation was blocked) by the addition of CTP and heparin (100 µg/ml). Aliquots of this reaction mixture were sampled and stopped at times ranging from 5 to 60 s. As apparent from the data in Fig. 7C, no more than 20–30 s was required to complete elongation. Since the transcribed region of SCR1 is 522 bp long, an elongation rate of ~20 nt/s can be inferred from these data. Such a value is in good agreement with previous measurements of yeast Pol III elongation rate on a tRNA gene (45). As implied by these results, Pol III termination and reinitiation on SCR1 take altogether no more than 20 s. Since the recruitment of free Pol III by preinitiation complexes is a relatively slow process requiring a few minutes (41), it can be concluded that facilitated reinitiation does indeed take place on the SCR1 gene.

Discussion

Despite its unusual length and promoter architecture, SCR1, the gene coding for the 7SL RNA of S. cerevisiae, is transcribed by RNA polymerase III through the same intragenic control elements (A- and B-blocks), transcriptional components, and basic mechanisms operating in the case of classical tRNA genes. If compared with the predominantly extragene promoter organization of the 7SL genes from other eukaryotes, this finding further attests to the remarkable plasticity in promoter organization of class III genes other than the tDNAs.

Plasmids carrying the different SCR1 variants. The reported values, expressed as percentages of the RNA levels obtained with WT SCR1, were normalized using the tRNA65(UAU) as an internal standard (see C). Complementation of the slow growth phenotype of the YRA130 strain by the different mutants and by WT SCR1 is reported in the third column. –, no complementation; +, intermediate complementation; ++, full complementation; ND, not determined. B, in vitro transcription of wild type (lane 1) and mutant (lanes 2–7) SCR1 genes. The migration positions of full-length (scRNA) and 3’-truncated (Δ+90) SCR1 transcripts are indicated on the right. C, in vivo transcriptional output of wild type (lane 1) or mutant (lanes 2–6) SCR1 genes determined by primer extension. The migration position of the fully extended SCR1-specific product is indicated on the left (scRNA). Shown on the right are the results of dyeoxy chain termination sequencing reactions primed with the same radiolabeled oligonucleotide utilized for primer extension (lanes G, T, A, and C). The sequence of the nontranscribed strand around the start site (+I) is indicated on the right. tRNA65(UAU) extension products, obtained from the same RNA samples and utilized as internal controls, are shown at the bottom.
and the 5 S rDNAs (16). This probably reflects the different exploitation for transcriptional purposes of intragenic control elements, whose origin as determinants of tRNA structure largely predates their utilization as TFIIIC binding sites. Following the separation of eukaryotic lineages, the adaptation to or elimination of the constraints imposed by such a dual role...
has produced at least three different results. At one extreme, there is the full coadaptation of structural and transcriptional roles as observed in present day tRNA and 5 S rRNA genes. At the other extreme, there is the evolution of structurally unconstrained upstream control elements that tend to confer a complete TFIIIC independence to higher eukaryotic class III genes such as those coding for the human 7SK and U6 RNAs (5). The third, somewhat intermediate situation relies on the maintenance of tRNA gene-like control elements and, concomitantly, of TFIIIC function. This is the predominant case in S. cerevisiae, where TFIIIC function as a transcription complex assembly factor has been preserved for all of the known class III genes. In yeast, the use of TFIIIC-binding blocks has been reconciled with new and variable RNA structural features either by modeling intragenic A- and B-blocks on the structure-function requirements of the new RNA or by dislocating one or both of these elements extragenically. The latter is the case of the yeast RPRI and SNR6 genes, whose transcription depends,
respectively, on an upstream tRNA gene-like leader (33) and on a downstream extragenic B-block (37, 39), whereas a most clear and revealing example of structural-functional adaptation is provided by the SCR1 gene characterized in this work. An evident signature of such an adaptation is the replacement of two consecutive thymines at the third and fourth position of the tDNA B-block consensus with two adenine residues (see Fig. 1). Such thymine residues are highly conserved in tRNA genes. In particular, the thymine at the fourth position is absolutely invariant, most probably because it is the precursor of the essential pseudouridine residue of the tRNA 5′ arm. The adenosines that in the B-block of SCR1 replace these two conserved thymine residues probably favor RNA folding and/or function (see Ref. 46 and Fig. 1B) without being detrimental to TFIIC binding (47).

Important peculiarities of SCR1 promoter organization emerge from our analysis. The first is the stronger A-block requirement in vivo as compared with what is observed under in vitro conditions. Such a discrepancy does not result from an increased instability of the A-block-altered 7SL RNA. In fact, the double CC → GG substitution at positions +19 and +20 of the A-block minimally alters the predicted secondary structure of the resulting 7SL RNA (46), and, more importantly, wild type and A-block-mutated SCR1 transcripts displayed identical sensitivities to nuclease digestion and prolonged incubation with yeast crude nuclear extracts (data not shown). An explanation for the observed discrepancy between in vitro and in vivo transcription was provided by comparative DNA binding and transcription competition assays as well as by in vivo nucleosome disruption experiments, carried out with different SCR1 templates. As revealed by the results of gel retardation and footprinting analyses, the Adown mutation considerably reduces the affinity of SCR1 for TFIIC and specifically impairs the interaction of the A-block with the upstream portion of TFIIC. This weakened binding does not result in a proportionally reduced in vitro transcription efficiency for at least three possible reasons. The first of them rests upon the peculiar assembly properties of TFIIB, which once recruited onto the 5′-flanking region of class III genes through interaction with TFIIC remains tightly bound to template DNA for multiple rounds of transcription. Thus, a defective interaction between TFIIC and the A-block can result in only a moderate transcriptional impairment, provided that such interaction is stable enough to allow the formation of long lived (kinetically trapped (48)) TFIIB-DNA complexes. Accordingly, a mutation in BRF1, the gene coding for the TFIIC-interacting component of yeast TFIIB, was previously selected as an extragenic suppressor of an A-block-inactivating mutation (49), thus implying that a defective TFIIC-DNA interaction may still direct TFIIB assembly as long as a TFIIC-anchoring site is maintained on the B-block. A second reason for the more dramatic effect of the Adown mutation in vivo is the existence in the nucleus of many potentially competing class III templates. In fact, when in vitro transcription was carried out in the presence of increasing amounts of a com-
petior tDNA, transcription of the Adown mutant was much more severely reduced than that of the wild type SCR1 gene. Finally, as revealed by in vivo nucleosome depletion experiments, TFIIC, when suboptimally bound to the Adown SCR1 mutant, displayed a dramatically reduced capacity to counteract repressive chromatin assembly. A similar observation has been reported previously for mutant variants of SNR6, the yeast gene coding for the U6 snRNA (30). In this case, the TFIIC recruiting ability of the Adown mutant was totally compromised, and the very low transcription of the mutant SCR1 gene upon nucleosome loss was attributed to TATA box-mediated, TFIIC-independent transcription. The case of compromised, and the very low transcription of the mutant TFIIIC recruiting ability of the Adown mutant was totally reactivated repressive chromatin assembly. A similar observation accordingly, TFIIIC appears to be required for human 7SL gene transcription of total RNA (15). Since the RNA/DNA ratio in a rapidly growing yeast cell (100-min duplication time) is calculated that not less than 5000 7SL RNA molecules/cell must be synthesized. The estimated in vivo transcriptional output of the single copy SCR1 gene is thus on the order of 50 RNA molecules/min!

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Transcription of the Yeast 7SL RNA Gene

Intragenic Promoter Adaptation and Facilitated RNA Polymerase III Recycling in the Transcription of *SCRI*, the 7SL RNA Gene of *Saccharomyces cerevisiae*

Giorgio Dieci, Silvia Giuliodori, Manuela Catellani, Riccardo Percudani and Simone Ottonello

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