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Intragenic Promoter Adaptation and Facilitated RNA Polymerase III Recycling in the Transcription of *SCR1*, the 7SL RNA Gene of *Saccharomyces cerevisiae**

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The *SCR1* 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 *SCR1* 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 TΨC 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, *SCR1* 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 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 *SCR1* 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 *SCR1* 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 TFIIB-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 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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[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, as 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 irisbioc.bio.unipr.it/pol3scan.html), a program based on weight matrix analysis of tRNA gene promoters (20). Pol3scan, with properly modified cut-off parameters, was then used to locate A- and B-block-like elements in *SCR1*.

Amplification and Cloning of SCR1—The *S. cerevisiae SCR1* gene was PCR-amplified from yeast genomic DNA (strain S288C) using the high fidelity Deep Vent DNA polymerase (New England Biolabs) and the following oligonucleotide primers: SCR1fw (5′-TGATCAACTTAGCCAGGACATCC) and SCR1rev (5′-GTTCTAAGTATTCTCATTTTATCC). Amplification conditions and insertion into the pBlueScript KS (+) vector (Stratagene) were as described (6). The identity of the 992-bp amplified fragment, containing the *SCR1* coding sequence (522 bp) plus 246 bp of 5′-flanking and 224 bp of 3′-flanking sequences, was verified by dideoxy chain termination sequencing. The sequence of the amplified *SCR1* fragment, which exactly matches the one retrieved from the Munich Information Center for Protein Sequences (MIPS) Web site (mips.gsf.de/proj/yeast/CYGD), presents some differences in the coding region with respect to the originally published *SCR1* sequence (15). These are three insertions (G at position +49, G at position +98, and C and A at positions +362 and +363, respectively) and one deletion (a missing G between positions +403 and +404); numbering refers to the MIPS (and our) sequence of the *SCR1* coding region.

In Vitro Mutagenesis of Putative SCR1 Promoter Elements—The 5′Δ-32, TATAdown, Adown, and C4T *SCR1* mutants were obtained by PCR using wild type *SCR1* in pBlueScript-KS (pBlueScript-*SCR1*) as template and the SCR1rev oligonucleotide (see above) together with the following mutagenic 5′ oligonucleotides (mutated positions are underlined) as primers: 5′Δ-32, 5′-GTATAAAATCGAAAGTTTATTCCAATTG; TATAdown, 5′-GTGTAAAATCGAAAGTTTATTCCAATTG; Adown, 5′-GTATAAAATCGAAAGTTTATTCCAATTGTGCTAGGCTGTAATGGCTTTCTCCTGGGATGGGATACG; C4T, 5′-GTATAAAATCGAAAGTTTATTCCAATTGTGCTAGGTTGTAATGG.

The (A/TATA)down mutant was derived from *SCR1* Adown by mutagenic PCR using TATAdown and SCR1rev primers. The Bdown *SCR1* mutant was constructed by recombinant PCR (21). Two overlapping PCR primary products were generated using the 5′Δ-32 oligonucleotide in combination with Bdown-rev (5′-CGCGAGGAAGGATTTCTTCCTGGCC) and the SCR1rev oligonucleotide in combination with the Bdown-fw primer (5′-GGCCAGGAAGAAATCCTTCCTCGCG). Mutated positions in Bdown-rev and Bdown-fw are underlined. After gel purification, primary amplification products were mixed and used as templates in a subsequent amplification reaction, employing SCR1fw and SCR1rev as "outside" primers, which yielded the desired full-length secondary product. The 3′Δ+90 mutant was obtained by PCR using WT *SCR1* as template, the 5′Δ-32 oligonucleotide as a forward primer, and the 3′Δ+90 oligonucleotide (5′-AAAAAAACGTGCAATCCGTGTCTAGCCGCG) as a reverse primer, which allowed us to introduce an artificial Pol III terminator. All of the mutated *SCR1* fragments were inserted into the pGEM-T Easy vector (Promega) and sequence-verified. For *in vivo* analyses, *SCR1* variants were subcloned as *Bam*HI-*Hin*dIII (WT *SCR1*) or *Sph*I-*Sac*I (all of the mutants) fragments into the YEp352 vector (22) cut with the same enzymes. All restriction and modification enzymes were from Amersham Biosciences, Inc.

In Vitro Transcription Assays—Multiple round and single round *in vitro* transcription of *SCR1* using recombinant or purified Pol III transcription components was carried out as described (6) except for the use of SUPERase-In (Ambion) as an RNase inhibitor. In the single round transcription experiments of Fig. 7, *B* and *C*, UTP was present at a concentration of 100 μM. The heparin resistance of the 12-mer RNA-containing ternary complex assembled on *SCR1*-C4T (Fig. 7*A*) was evaluated as follows. Ternary complexes were assembled by incubating *SCR1*-C4T template and transcription components in the presence of 0.5 mM ATP and GTP and 2.5 μM [α-^{32}P]UTP (Amersham Biosciences; 800 Ci/mmol). The output of a single round of transcription was then evaluated by adding CTP (0.5 mM), together with excess unlabeled UTP (2 mM), with or without 100 μM heparin, and allowing transcript 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 *Sma*I site: the *S. cerevisiae* I(TAT)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. 4*A*, 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. 4*B* (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′-GCCGGGACACTTCAGAACGGAC), the 5′Δ-32 oligonucleotide as a forward primer, and the *SCR1* 5′Δ-32, Adown, and Bdown mutants as PCR templates. Radiolabeled fragments were purified by agarose gel electrophoresis followed by elution with the QIAquick Gel Extraction Kit (Qiagen); the specific radioactivity of purified fragments (250 ng each) was about 1500 cpm/fmol. DNase I digestion mixtures (20 μl) contained 16 fmol of the *SCR1* fragment, 10 ng/μl pBlueScript-KS, 20 mM Hepes/KOH (pH 8.0), 170 mM KCl, 5% (v/v) glycerol, 0.1 mg/ml ultrapure bovine serum albumin (Ambion), 0.5 mM dithiothreitol, and 50–100 ng of affinity-purified TFIIIC (24). Briefly, TFIIIC-DNA complexes, formed upon incubation for 15 min at 20 °C, were treated for exactly 1 min with 0.35 ng of pancreatic deoxyribonuclease I (Amersham Biosciences; E2215Y type), followed by the addition of 22 μl of blocking solution (20 mM EDTA, 1% (w/v) SDS, 0.2 M NaCl). Footprinting mixtures were phenol-extracted, ethanol-precipitated in the presence of 30 μg of carrier RNA (Sigma; R 6625 type), and fractionated on 6% polyacrylamide, 7 M urea sequencing gels, which were then dried and phosphorimaged with a Personal Imager FX (Bio-Rad). DNA fragments for gel retardation assays were radiolabeled by PCR, using 5′-labeled amplification primers as described above for the preparation of DNA fragments for footprinting analysis. DNA binding reactions were conducted in a final volume of 15 μl and contained 25 mM Tris-HCl (pH 8.0), 10% glycerol, 90 mM $(NH_4)_2SO_4$, 1 mg/ml ultrapure bovine serum albumin, 15 μg/ml supercoiled plasmid DNA (pBlueScript-KS), 4 fmol of radiolabeled DNA fragment (~8,000 cpm), and varying amounts of TFIIIC purified up to the DEAE-Sephadex A-25 step (24). Native gel electrophoresis and subsequent analysis were carried out as described (25).

In Vivo RNA Analyses—The yeast strain YRA130 (a kind gift of Peter Walter, 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 vivo* 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. Total RNA was prepared according to a previously described procedure (27). Primer extension reactions were carried out as described (6), using 5 μg of total yeast RNA and a 5′-labeled oligonucleotide primer (5′-CCCTTGCCAAAGGGCGTGCAATCCG) complementary to the coding region of *SCR1* between positions +90 and +115. Complementation of

[2] R. Percudani, unpublished results.

[3] L. Bottarelli and G. Dieci, unpublished results.

A

	A-block																					B-block										
Total	198	198	198	198	198	198	198	198	198	198	63	198	198	198	114	24	198	198	198	198	198	60	60	60	60	60	60	60	60	60	60	60
%G	45	0	57	93	1	27	12	2	79	5	2	99	99	2	4	4	4	52	33	60	4	55	97	0	0	2	77	0	15	2	2	7
%A	31	3	38	1	0	13	7	98	19	4	6	0	0	10	3	0	96	26	28	39	1	23	2	15	0	0	18	98	42	17	0	8
%T	20	97	3	1	39	29	41	1	1	77	68	0	0	79	73	75	0	21	11	0	28	10	2	83	100	2	3	2	33	53	5	28
%C	4	0	3	5	60	31	40	0	1	15	24	1	1	9	20	21	0	2	29	1	68	12	0	2	0	97	2	0	10	28	93	57
tRNA Consensus	+7	R	T	R	G	Y	B	Y	A	G	T	-	G	G	T	Y	-	A	R	V	R	Y	+25	+52	R	G	T	T	C	G	A	D	Y	C	Y	+62
SCR1	+9	A	T	G	G	C	T	T	T	C	T	-	G	G	T	-	-	G	G	G	A	T	+26	+51	G	G	A	A	C	A	A	A	T	C	C	+61

B

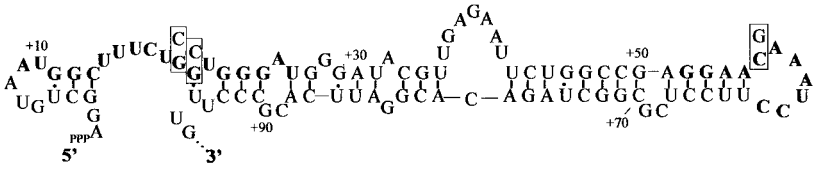

FIG. 1. **SCR1 control elements identified by weight matrix analysis.** *A*, frequency matrices for tRNA gene promoter regions. Each *column* corresponds to an individual position along the A-block or B-block sequence. Intragenic promoter boundaries for a consensus tRNA are +7/+25 (A-block) and +52/+62 (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 = G/A; 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′Δ-32 and Adown) *SCR1* minigene variants, in which 120 bp at the 3′ terminus had been deleted by PCR using the SCR1_mini oligonucleotide (5′-AAAAAAAATGTGCTATCCCGGCCGCCTCC) as a reverse primer, either SCR1fw or 5′Δ-32 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 5× SSC, 5× Denhardt's solution, 0.1 mg/ml denatured salmon sperm DNA, 0.5% (w/v) SDS, followed by three short washings in 2× 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. 1*A*). 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. 1*B*, 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. 1*A*). The most prominent of them is the substitution of the canonical B-block starting sequence GGTT (in which the invariant T at the fourth position corresponds to the precursor of the essential pseudouridine residue of the tRNA TΨC arm) with GGAA, 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. 1*A*; 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-

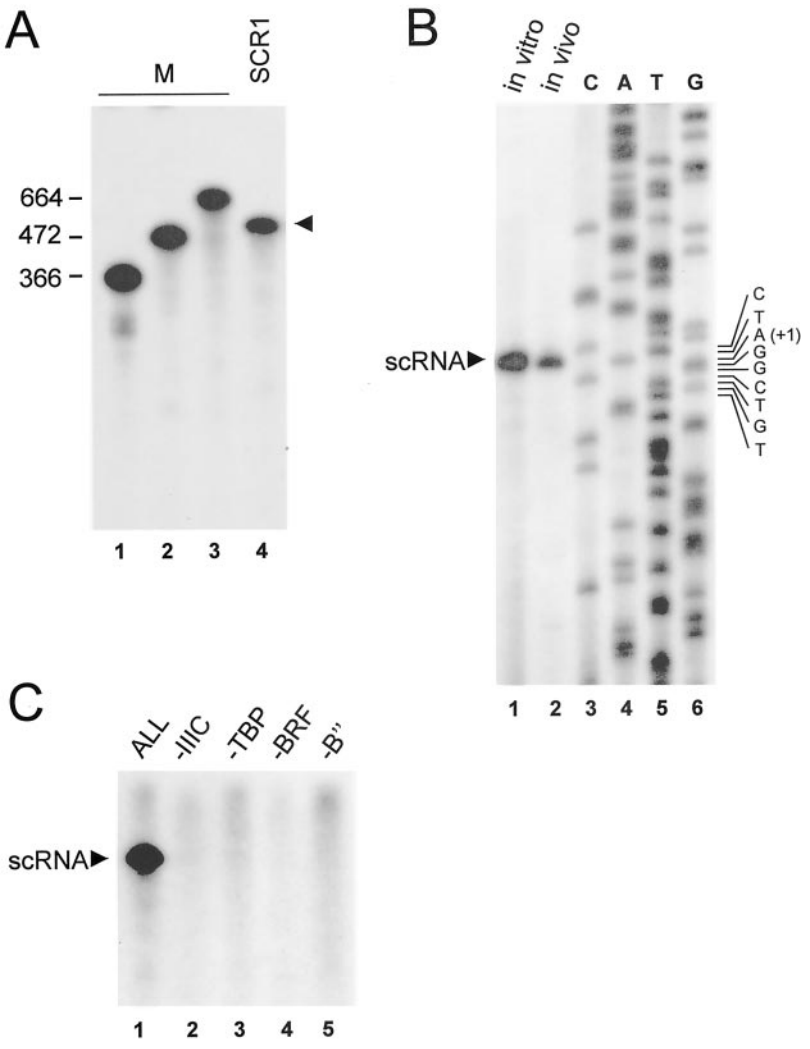

FIG. 2. **SCR1 RNA synthesis in a reconstituted *in vitro* transcription system.** *A*, the size of *in vitro* synthesized *SCR1* (*lane 4*) was measured by comparison with marker RNAs of the indicated sizes in nucleotides (*lanes 1–3*, *M*). The *arrowhead* on the *right* indicates the migration position of the *SCR1* transcript. *B*, the RNA products of a 2 times scaled up *in vitro* transcription reaction programmed with the *SCR1* 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 *SCR1*-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*, *SCR1 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. 2*A*, *lane 4*) in parallel with standard RNAs of known size produced by T7 RNA polymerase (Fig. 2*A*, *lanes 1–3*). A single transcript, with a size very close to that of the natural scR1 RNA (519 nt for strain ATCC 25657 (15) and 522 nt for strain S288C; 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 scR1 RNA is presented in Fig. 2*B*, which shows the results of a primer extension analysis that was carried out to map the *SCR1* 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 scR1 RNA (15). Thus, the *in vitro* reconstituted Pol III system supports the efficient and faithful transcription of the *SCR1* gene. The transcription factor requirements for scR1 RNA synthesis are reported in Fig. 2*C*, which shows that both TFIIIC and the three components of yeast TFIIIB are essential for *SCR1* 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), *SCR1* 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 SCR1 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 *SCR1* 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 *SCR1* gene carried by YEp352 (not shown). This enabled us to monitor the phenotypic effects of the introduced mutations. Fig. 3*A* summarizes the transcription activities and functional complementation phenotypes of the different mutants, whereas Fig. 3, *B* and *C*, shows representative results of *in vitro* (Fig. 3*B*) and *in vivo* (Fig. 3*C*) transcription analysis. In the experiment of Fig. 3*C*, 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 *SCR1* 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 *SCR1* transcription. In fact, a C56G point mutation within such an element abolished transcription *in vitro* (Fig. 3*B*, *lane 4*), reduced the *in vivo* steady state amount of the 7SL RNA to undetectable levels (Fig. 3*C*, *lane 4*), and completely destroyed complementation capacity (Fig. 3*A*). By comparison, a double point mutation in the A-block (CC in place of GG at the uni-

versally conserved +19 and +20 positions; Adown mutant) had a much less severe effect on *in vitro* transcription (Fig. 3*B*, 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 vivo* 7SL RNA levels by about 20-fold as compared with wild type *SCR1* (Fig. 3*C*, *lanes 5* and *6*) and resulted in a partial loss of slow growth complementation (Fig. 3*A*). In tRNAs, the A-block region is an important structural determinant, and reduced *in vivo* 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. 3*B*, *lane 2*), while it caused a 4-fold decrease of *in vivo SCR1* transcription (Fig. 3*C*, *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. 3*B*, *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. 3*A*). 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. 3*C*, *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 promoters of tRNA genes, binding of TFIIIC to *SCR1* was analyzed by DNase I footprinting. As shown in Fig. 4*A*, 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 *RPR1* 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. 4*B*, 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. 5*A*, 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. 5*B*, 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 $tRNA^{Pro}$ gene. Transcription of the Adown mutant was much more sensitive to $tDNA^{Pro}$ competition 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 A_{600} 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-

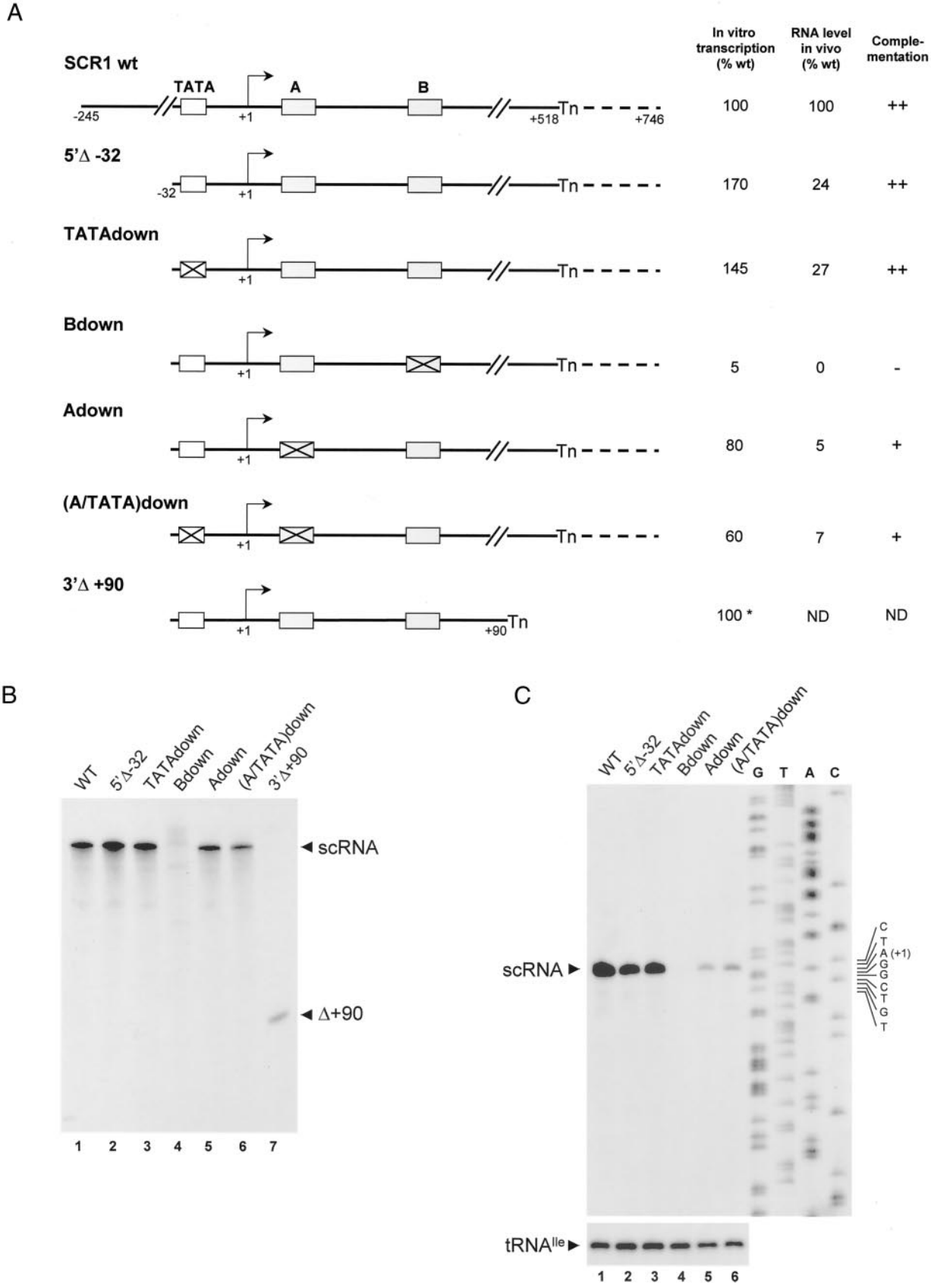

FIG. 3. **Transcriptional and complementation phenotypes of *SCR1* mutants.** *A*, schematic representation of the different *SCR1* mutants. Putative intragenic (*filled boxes*) or extragenic (*empty boxes*) control elements and the transcription termination site (*Tn*) are indicated; *crossed boxes* 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 *SCR1* 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

tose (*lanes 2* and *6*) and were still undetectable in the control strain after a shift to the glucose medium (*lane 4*). Interestingly, however, Adown transcript levels rose to up to 40% of those of WT *SCR1* upon glucose shift of the UKY403 strain (compare *lanes 7* and *8*), in which chromatin had been disrupted because of histone H4 depletion. This finding indicates that suboptimal TFIIIC binding to A-block-mutated *SCR1* results in a reduced ability to counteract the assembly of repressive chromatin structures. Once again, this effect specifically pertains to the Adown mutant. In fact, regardless of the particular yeast strain or growth conditions, shortened *SCR1* RNAs transcribed from either 5′Δ-32 or TATAdown mutant minigenes accumulated at exactly the same level as WT *SCR1* minigene transcripts (data not shown).

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 transcribing Pol III 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. 7*A*, in which the 12-mer was synthesized in the presence of ATP, GTP, and [α-^{32}P]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. 7*B*. 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. 7*C* 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. 7*A*, 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. 7*C*, 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 extragenic 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 tRNA^{Ile}(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 dideoxy 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 (*+1*) is indicated on the *right*. tRNA^{Ile}(UAU) extension products, obtained from the same RNA samples and utilized as internal controls, are shown at the *bottom*.

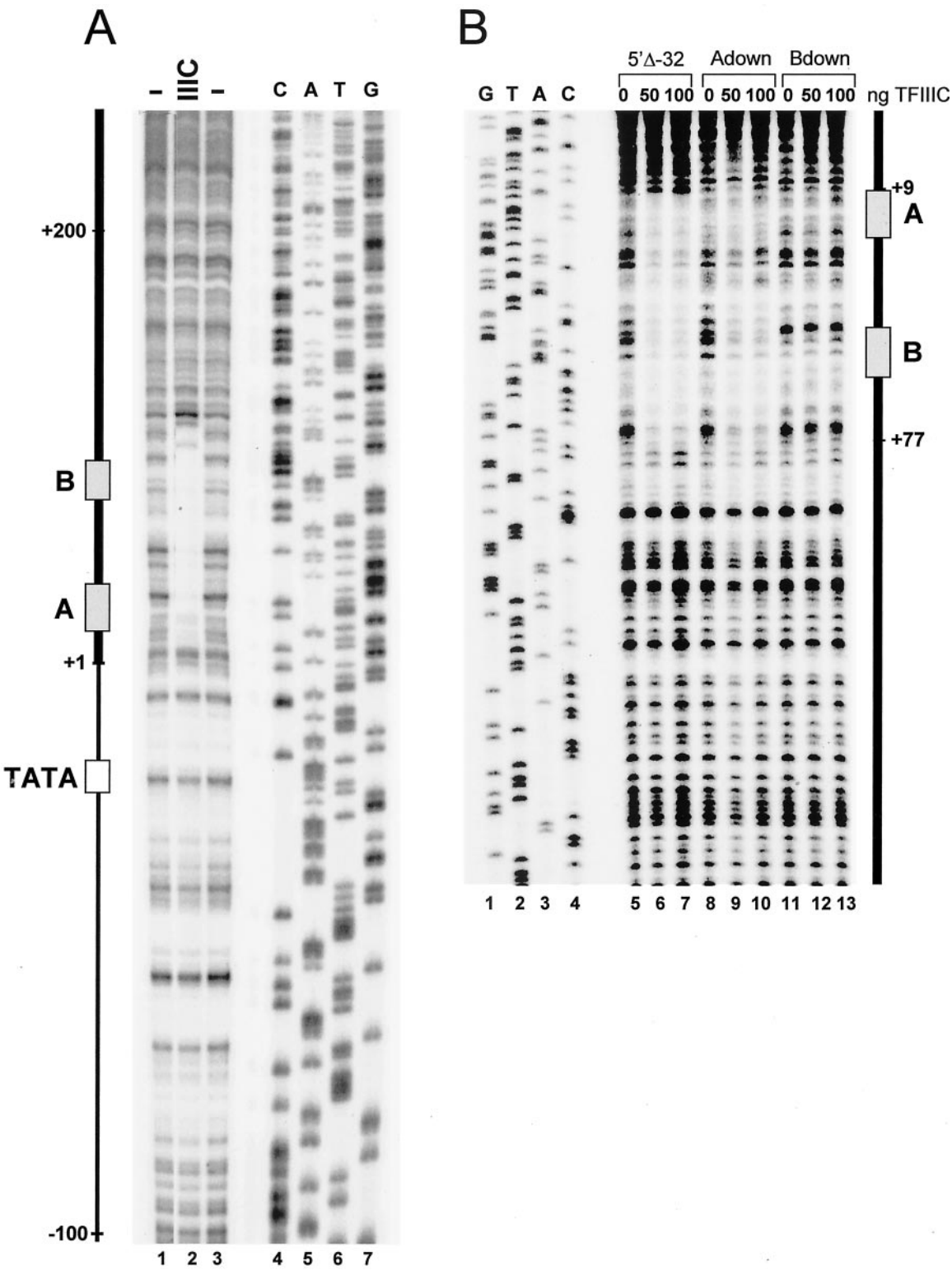

FIG. 4. **Binding of TFIIIC to *SCR1*.** *A*, TFIIIC binding to WT *SCR1*. A WT *SCR1*-containing fragment (16 fmol), radiolabeled on the sense strand, was incubated in the presence (*lane 2*) or in the absence (*lanes 1* and *3*) of affinity-purified TFIIIC (75 ng), digested with DNase I, and processed as described under "Materials and Methods." Shown in *lanes 4–7* are the results of dideoxy chain termination sequencing reactions conducted with the same radiolabeled oligonucleotide utilized to amplify the *SCR1* fragment. Sequence element references on the coding (*thick solid bar*, +1 and above) and the upstream nontranscribed (*thin solid bar*, −100 to +1) regions of *SCR1* are indicated on the *left*. *B*, TFIIIC binding to *SCR1* mutants. DNA fragments radiolabeled on the antisense strand and containing the 5′Δ-32 (*lanes 5–7*), Adown (*lanes 8–10*), or Bdown (*lanes 11–13*) *SCR1* derivatives were incubated with the indicated amounts of TFIIIC before DNase I digestion. Reference sequencing reactions were run in *lanes 1–4*. Indicated on the *right* are the positions of the A- and B-blocks and the borders (+9/+77) of TFIIIC protection.

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

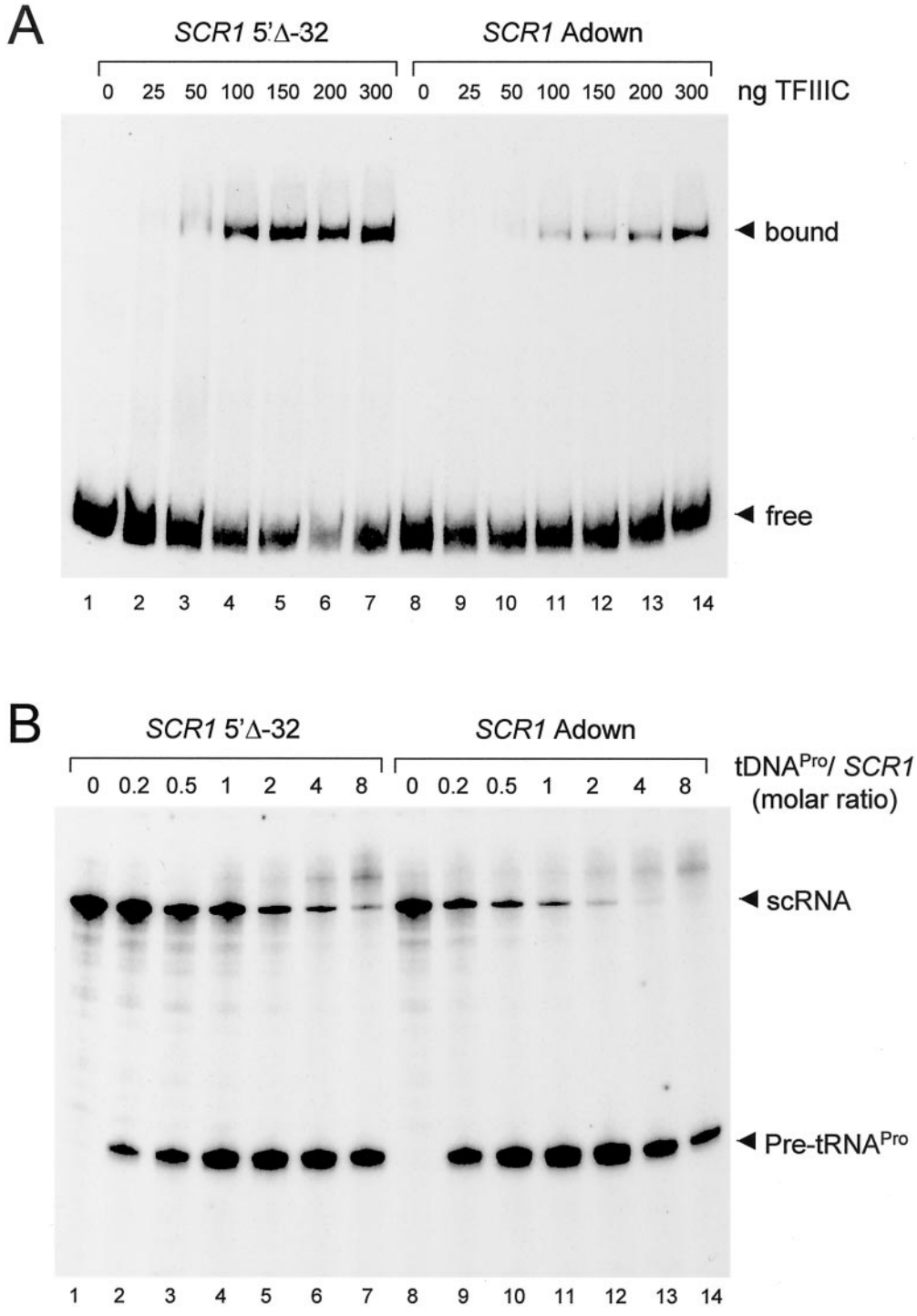

FIG. 5. **TFIIIC binding and competition ability of A-block-mutated *SCR1*.** *A*, the indicated amounts of TFIIIC were incubated with radiolabeled fragments (4 fmol) derived from either 5′Δ-32 (*lanes 1–7*) or Adown (*lanes 8–14*) *SCR1* derivatives, followed by electrophoretic fractionation of TFIIIC-bound and free DNA molecules on a native polyacrylamide gel. *B*, *in vitro* transcription reactions were conducted in reaction mixtures containing 20 fmol of either WT (*lanes 1–7*) or Adown (*lanes 8–14*) *SCR1* and increasing concentrations of a $tDNA^{Pro}$(TGG) competitor (6) at the molar ratios indicated *above* each *lane*. The migration positions of *SCR1* (*scRNA*) and $tDNA^{Pro}$ (*Pre-$tRNA^{Pro}$*) transcripts are indicated on the *right*.

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 *RPR1* and *SNR6* genes, whose transcription depends,

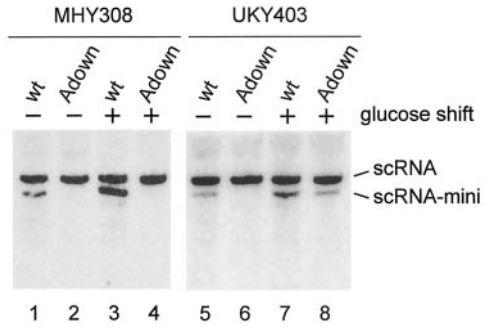

FIG. 6. **Effect of nucleosome disruption on *in vivo* transcription of WT and A-block-mutated *SCR1*.** Total RNA (5 μg) extracted before (*lanes 1*, *2*, *5*, and *6*) or after (*lanes 3*, *4*, *7*, and *8*) glucose shift of the MHY308 (*lanes 1–4*) or UKY403 (*lanes 5–8*) strains transformed with 3′-shortened variants of either WT *SCR1* (*lanes 1*, *3*, *5*, and *7*) or the Adown mutant (*lanes 2*, *4*, *6*, and *8*) was gel-fractionated and probed with a radiolabeled *SCR1* antisense oligonucleotide. The migration positions of endogenous, full-length *SCR1* transcripts (*scRNA*) and of plasmid-derived shortened transcripts (*scRNA-mini*) are indicated on the *right*.

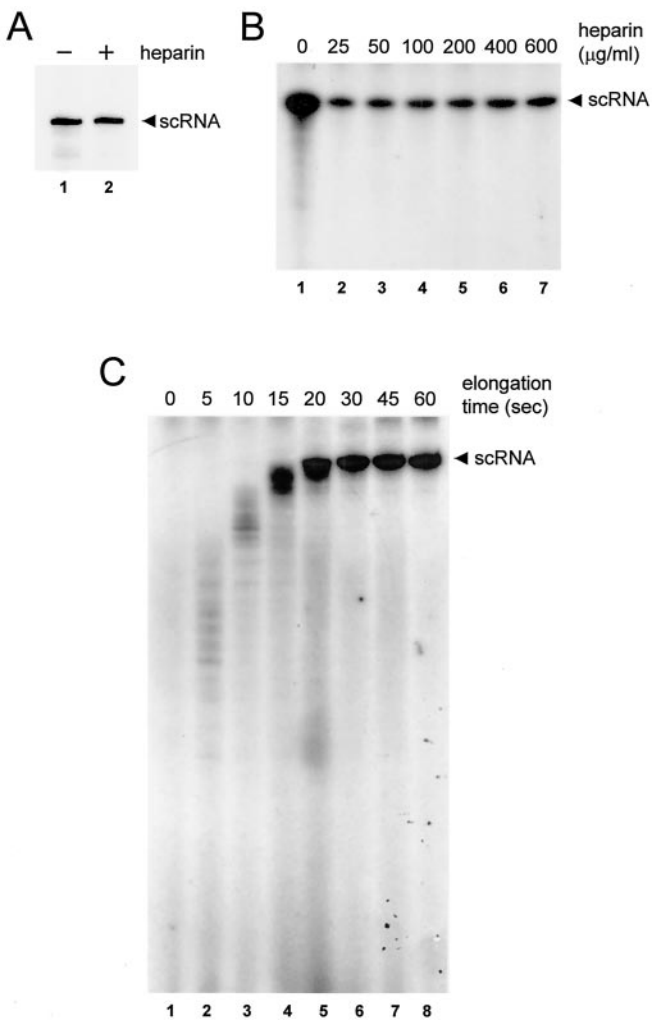

FIG. 7. **Transcription elongation and reinitiation on *SCR1*.** *A*, heparin stability of ternary complexes assembled on *SCR1*-C4T. Ternary complexes carrying the transcript encoded by the first 12 bp of *SCR1*-C4T (^{32}P-labeled at its four U residues) were formed by incubation with an NTP mixture lacking CTP and then fully elongated by the addition of CTP in the presence of an 800-fold molar excess of unlabeled UTP with (*lane 2*) or without (*lane 1*) heparin (100 μg/ml). *B*, single round transcription analysis of reinitiation. Stalled, 12-mer RNA-containing ternary complexes were first formed on the *SCR1*-C4T template, and elongation was then resumed by the addition of CTP together with increasing concentrations of heparin as indicated above each lane; multiple rounds of transcription were allowed to proceed for 5 min in the heparin-lacking reaction mixture loaded in *lane 1*. *C*, time course of transcription elongation. Elongation by stalled ternary complexes (formed as in *B*) was resumed by the addition of CTP, together with heparin (100 μg/ml) to abolish reinitiation. At the times indicated *above* each *lane*, aliquots of the reaction mixture were transferred to tubes chilled in dry ice to stop the reaction. A reaction mixture aliquot sampled before the addition of CTP was loaded in *lane 1*. In all *panels*, the migration position of the full-length *SCR1* transcript (*scRNA*) is indicated on the *right*.

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 TΨC arm. The adenines 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. 1*B*) without being detrimental to TFIIIC 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 *in vivo* 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 TFIIIC and specifically impairs the interaction of the A-block with the upstream portion of TFIIIC. 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 TFIIIB, which once recruited onto the 5′-flanking region of class III genes through interaction with TFIIIC remains tightly bound to template DNA for multiple rounds of transcription. Thus, a defective interaction between TFIIIC 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)) TFIIIB-DNA complexes. Accordingly, a mutation in *BRF1*, the gene coding for the TFIIIC-interacting component of yeast TFIIIB, was previously selected as an extragenic suppressor of an A-block-inactivating mutation (49), thus implying that a defective TFIIIC-DNA interaction may still direct TFIIIB assembly as long as a TFIIIC-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-

petitor 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, TFIIIC, 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 TFIIIC recruiting ability of the Adown mutant was totally compromised, and the very low transcription of the mutant *SNR6* gene upon nucleosome loss was attributed to TATA box-mediated, TFIIIC-independent transcription. The case of *SCR1* is different, because despite the loss of A-block-mediated TFIIIC contacts in the Adown mutant (Fig. 4*B*), TFIIIC is still able to recruit TFIIIB on this template, albeit less efficiently. The *SCR1* A-block can thus be viewed as a dual function promoter element through which TFIIIC exerts both TFIIIB recruitment and chromatin antirepression effects.

Two putative A-blocks have also been recognized between positions +5 and +22 of the human 7SL RNA gene (11), and accordingly, TFIIIC appears to be required for human 7SL gene transcription (50). In this case, however, the first 46 bp of the transcribed region (including the potential A-blocks) have been shown to activate transcription through a new mechanism involving a structural motif at the 5′ end of the nascent transcript (11). A similar structural motif is not evident in the yeast 7SL RNA (46), and the A-block mutation we introduced, although severely affecting transcription *in vivo* and competition ability *in vitro*, is not predicted to disturb the secondary structure of the 7SL RNA (46) (Fig. 1*B*). Moreover, footprinting analyses showed that the A-block region of *SCR1* is specifically contacted by TFIIIC and that such contacts are lost in the Adown mutant. The core function of the *SCR1* A-block thus appears to be the promotion of an optimal TFIIIC-DNA interaction.

Another interesting feature revealed by the present analysis is the ability of the Pol III transcription machinery to support multiple cycles of facilitated reinitiation on the unusually long *SCR1* gene (522 bp). Facilitated recycling was first described in yeast as a mechanism allowing a Pol III molecule to repeatedly transcribe the same tRNA gene without dissociating from it (41), and it has been proposed to play important roles also in human and plant Pol III systems (2, 51, 52). The extremely short length of tRNA genes (100 bp on average) and the high protein occupancy of the transcribed region (an estimated 1.5 MDa for a fully assembled Pol III machinery) probably result in a compact, higher order nucleoprotein complex in which the transcribing Pol III always remains in close proximity of the transcription initiation site. Such a structural compactness may satisfy a minimal requirement for repeated Pol III reattachment to the same transcriptional unit (43). The fact that facilitated recycling also takes place on a much longer transcriptional unit leads us now to exclude the possibility that this process is functionally restricted to small sized genes. Rather, it favors the idea that fast recycling (hyperprocessivity) is a general property of the Pol III system, resulting from its ability to bypass, at each cycle, slow dissociation-reassociation steps with a wide tolerance for the distance between initiation and termination sites. Fast recycling on *SCR1* is likely to be essential for generating the high levels of 7SL RNA required for cell growth. In *S. cerevisiae*, the 7SL RNA is one of the most abundant cytoplasmic RNA species, accounting for about 0.2% of total RNA (15). Since the RNA/DNA ratio in a rapidly growing yeast cell (100-min duplication time) is ~50:1 (53), it can be 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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