Eukaryotic Cell

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Eukaryotic Cell **2012, 11(8):1067. DOI: 10.1128/EC.00141-12.**
Published Ahead of Print 15 June 2012.

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The *Schizosaccharomyces pombe inv1*$^+$ Regulatory Region Is Unusually Large and Contains Redundant *cis*-Acting Elements That Function in a SAGA- and Swi/Snf-Dependent Fashion

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The *Schizosaccharomyces pombe inv1*$^+$ gene encodes invertase, the enzyme required for hydrolysis of sucrose and raffinose. Transcription of *inv1*$^+$ is regulated by glucose levels, with transcription tightly repressed in high glucose and strongly induced in low glucose. To understand this regulation, we have analyzed the *inv1*$^+$ *cis*-regulatory region and the requirement for the *trans*-acting coactivators SAGA and Swi/Snf. Surprisingly, deletion of the entire 1-kilobase intergenic region between the *inv1*$^+$ TATA element and the upstream open reading frame SPCC191.10 does not significantly alter regulation of *inv1*$^+$ transcription. However, a longer deletion that extends through SPCC191.10 abolishes *inv1*$^+$ induction in low glucose. Additional analysis demonstrates that there are multiple, redundant regulatory regions spread over 1.5 kb 5′ of *inv1*$^+$, including within SPCC191.10, that can confer glucose-mediated transcriptional regulation to *inv1*$^+$. Furthermore, SPCC191.10 can regulate *inv1*$^+$ transcription in an orientation-independent fashion and from a distance as great as 3 kb. With respect to *trans*-acting factors, both SAGA and Swi/Snf are recruited to SPCC191.10 and to other locations in the large *inv1*$^+$ regulatory region in a glucose-dependent fashion, and both are required for *inv1*$^+$ derepression. Taken together, these results demonstrate that *inv1*$^+$ regulation in *S. pombe* occurs via the use of multiple regulatory elements and that activation can occur over a great distance, even from elements within other open reading frames.

The regulation of eukaryotic transcription initiation is a complex process that requires the coordinated action of many different regulatory factors (23, 32). In metazoans, regulatory regions are usually large, are sometimes several kilobases in length, and contain many *cis*-acting regulatory regions (4). In contrast, in the well-studied yeast *Saccharomyces cerevisiae*, which has a much more compact genome, regulatory regions are typically smaller, about 150 to 400 base pairs, with an average intergenic size of 455 base pairs (11, 22, 29), although there are some genes with larger regulatory regions (for example, see reference 31).

In the fission yeast *Schizosaccharomyces pombe*, transcriptional regulation has been studied far less than it has in *S. cerevisiae*, with transcription regulatory regions being deciphered in only a small number of cases (for example, see references 14 and 36). *S. pombe* intergenic regions, which average 829 base pairs, are generally larger than those in *S. cerevisiae* (22, 39), suggesting that regulatory regions may also be larger.

In this work, we have studied regulation of the glucose-repressed *S. pombe inv1*$^+$ gene, encoding invertase, required for *S. pombe* to grow on sucrose or raffinose as a carbon source. We chose *inv1*$^+$, as many important insights into transcriptional regulation have come from previous studies of glucose repression in *S. pombe* (for example, see references 14, 16, and 17) and of the *inv1*$^+$ orthologue in *S. cerevisiae*, *SUC2* (for example, see references 6 and 18). Previous studies have shown that *inv1*$^+$ transcription is repressed in high glucose and induced in low glucose, yet its regulation is distinct from that of another glucose-regulated gene, *fbp1*$^+$ (19, 35). Additional work has shown that repression of *inv1*$^+$ in high glucose requires the factors Scr1 (35) and Ssn6/Tup (9). Furthermore, *inv1*$^+$ is regulated by histone acetylation, as a histone deacetylase, Clr6, is required for the full repression of *inv1*$^+$, acetylation of H3K14, H4K12, H4K16, and H4K5 in the intergenic region of *inv1*$^+$ is elevated in a *clr6-1* mutant, and *inv1*$^+$ mRNA levels are decreased in the absence of the histone acetyltransferase Elp1 (28, 38).

In our work, we have analyzed both the *cis*-acting elements and *trans*-acting factors required for *inv1*$^+$ induction in low glucose. Our results demonstrate that the *inv1*$^+$ regulatory region is large, over 1.5 kb in length, and that it includes several redundant regulatory regions. The most distal region, which contains the upstream open reading frame (ORF) SPCC191.10, can confer glucose-mediated transcription to *inv1*$^+$ in the absence of other elements and can confer glucose-mediated transcription when placed 5′ of a gene not normally regulated by glucose levels. Furthermore, this region can function in an inverted orientation and at distances up to 3 kb. Finally, we show that *inv1*$^+$ induction requires both the Swi/Snf and SAGA coactivators and that these complexes are physically associated across the *inv1*$^+$ regulatory region. Taken together, these results reveal a large degree of complexity in a glucose-regulated gene and suggest that *cis*-acting regulatory elements are not confined to intergenic sequences in *S. pombe*.

MATERIALS AND METHODS

***S. pombe* strains, genetic methods, media, and oligonucleotides.** The *S. pombe* strains used in this study are listed in Table 1. General genetic methods were carried out as described previously (10). Standard rich medium, yeast extract supplement (YES) with 3% glucose (10), was used

Received 7 May 2012 Accepted 7 June 2012
Published ahead of print 15 June 2012
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doi:10.1128/EC.00141-12

TABLE 1 *S. pombe* strains used in this study

Name	Genotype
FWP449	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1*
FWP450	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-utrΔ1*
FWP451	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-utrΔ2*
FWP452	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-utrΔ3*
FWP453	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-utrΔ4*
FWP454	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-utrΔ5*
FWP455	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-utrΔ6*
FWP456	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-tata*
FWP457	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-prΔ1*
FWP458	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-prΔ5*
FWP459	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-prΔ6*
FWP460	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-prΔ7*
FWP461	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-prΔ2*
FWP462	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-prΔ3*
FWP463	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-prΔ4*
FWP464	*h*$^+$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-his5*
FWP465	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl1*
FWP466	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl2*
FWP467	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl3*
FWP468	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl4*
FWP469	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl9*
FWP470	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl10*
FWP471	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl11*
FWP472	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl12*
FWP473	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl13*
FWP474	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl5*
FWP475	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl6*
FWP476	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl7*
FWP477	*h*$^-$ *ura4-D18 leu1-32 ade6-m216 his5-rpl6*
FWP478	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-rpl1*
FWP479	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-rpl2*
FWP480	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-rpl3*
FWP481	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-flp1*
FWP482	*h*$^-$ *ura4-D18 leu1-32 ade6-m210 his5Δ1 inv1-flp2*
FWP165	*h*$^+$ *ura4-D18 leu1-32 ade6-m216*
FWP229	*h*$^+$ *ura4-D18 leu1-32 ade6-m216 snf22Δ::ura4*$^+$
FWP422	*h*$^+$ *ura4-D18 leu1-32 ade6-m216 spt7Δ::ura4*$^+$
FWP308	*h*$^+$ *ura4-D18 ade6-m216 gcn5Δ::ura4*$^+$
FWP314	*h*$^+$ *ura4-D18 ade6-m216 spt8Δ::ura4*$^+$
FWP316	*h*$^+$ *ura4-D18 ade6-m216 leu1-32 ubp8Δ::ura4*$^+$
FWP317	*h*^{90}
FWP331	*h*^{90} *spt7-13xmyc::NatMX*
FWP491	*h*^{90} *spt7-13xmyc::NatMX snf22Δ::ura4*$^+$ *ura4-D18*
FWP492	*h*^{90} *spt7-13xmyc::NatMX snf22Δ::ura4*$^+$ *ura4-D18*
FWP165	*h*$^+$ *ura4-D18 leu1-32 ade6-m216*
FWP219	*h*$^+$ *ura4-D18 leu1-32 ade6-M216 snf22*$^+$*-TAP*
FWP495	*h*$^+$ *ura4-D18 leu1-32 ade6-M216 snf22*$^+$*-TAP spt7Δ::ura4*$^+$

unless otherwise noted. The low-glucose medium used was YES with 0.1% glucose and 3% glycerol, and the high-glucose medium was YES with 8% glucose. To grow cultures for *inv1*$^+$ analysis, 50-ml *S. pombe* cultures were grown to 1×10^7 to 2×10^7 cells/ml in YES with 3% glucose at 32°C, washed once in sterile water, and then divided into low-glucose medium (derepressing condition) or high-glucose medium (repressing condition) and grown for another 60 min. All constructs at *inv1*$^+$ and at *his5*$^+$ were made using a previously described method for two-step gene replacement (33). Briefly, a PCR fragment that contains the *ura4*$^+$ gene (2) flanked by 75 to 80 base pairs of homology to the site of integration was first integrated at the appropriate genomic location by homologous recombination. Then, the strain with the *ura4*$^+$ cassette was transformed with a PCR fragment that contained the final construct and whose ends direct homologous recombination at the correct location. Transformants were selected for resistance to 5-fluoroorotic acid. Some PCR constructs were generated by two-step fusion PCR (15), and all strains were confirmed by PCR. Oligonucleotides used for strain constructions, rapid amplification of cDNA ends (RACE), probes in Northern blot analyses, and chromatin immunoprecipitation are listed at http://genepath.med.harvard.edu/~winston/supplemental.htm.

Measurements of RNA levels. Total RNA was prepared by the hot phenol method (24) from 25 ml of *S. pombe* cells grown to 1×10^7 to 2×10^7 cells/ml. For Northern blots, 15 μg of total RNA was denatured with formamide and subjected to electrophoresis in a 1% agarose gel containing 1% formaldehyde and blotted onto nylon membranes (GeneScreen; Perkin Elmer). After hybridization, the blot was exposed on Biomax MR film (Kodak). *cam1*$^+$ was used as a loading control. Probes for Northern blot analyses (*inv1*$^+$, *his5*$^+$, and *cam1*$^+$) were made by PCR amplification, followed by random hexamer priming in the presence of [α-^{32}P]dATP, as previously described (1). All Northern blot analyses were done at least three times. Quantitations were performed using ImageJ software (http://rsbweb.nih.gov/ij/) analysis of scanned films. The values for representative Northern blot analyses shown in the figures were calculated by the value of the experimental signal (*inv1*$^+$ or *his5*$^+$) divided by the value of the loading control (*cam1*$^+$) and then normalized to a value of 1.0 for the wild-type derepressed sample. Measurement of RNA levels by real-time PCR was done as previously described (12).

Mapping the 5′ and 3′ ends of the *inv1*$^+$ mRNA. The mapping of the 5′ and 3′ ends of the *inv1*$^+$ mRNA was done using RACE. Total RNA was prepared as described above and was treated with DNase using a Turbo DNA-free DNase kit. cDNA synthesis and PCR were performed using a SMART-RACE kit (Clontech) as described previously (8). Of 13 clones that were completely sequenced and that mapped to the *inv1*$^+$ gene, 11 had a 5′ end beginning at −872 bp from the *inv1*$^+$ ATG, 1 clone had an insert beginning at −1297 bp, and one clone had an insert beginning at −116 bp. Inserts from 12 other clones mapped to other genes, including *gas2*$^+$, *sam1*$^+$, and *rpl1101*$^+$, showing that nonspecific PCR amplification products were present in the reaction mixture. For the 3′ end determination, three clones were sequenced: two clones had inserts ending at +48 bp from the *inv1*$^+$ stop codon followed by a poly(A) tail, and one had an insert ending at +43 bp followed by a poly(A) tail.

ChIPs and Western blot analysis. Chromatin immunoprecipitation (ChIP) experiments were done as previously described (12). ChIP of Spt7 was performed using an Spt7-myc fusion (13), and ChIP of Snf22 was performed using a Snf22-TAP fusion (27). For Western blot analyses, protein extracts were prepared as follows. First, cultures were grown to approximately 1×10^7 cells/ml, pelleted, and frozen at −70°C. Next, the pellets were thawed on ice and to each tube was added 200 μl of 20% trichloroacetic acid (TCA), 200 μl of TCA buffer (20 mM Tris-HCl, pH 8.0, 50 mM ammonium acetate, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride [PMSF]), and acid-washed glass beads up to the meniscus. Next, the cells were broken open by bead beating to achieve 100% cell lysis, and the cell extract was removed by centrifugation into a fresh tube. This tube was put on ice for 20 min and then spun at 14,000 rpm for 20 min at 4°C. The supernatant was then removed carefully to remove all TCA, and this step was repeated. Finally, the pellet was resuspended in 400 μl of TCA-Laemmli solution (1 ml contains 480 μl SDS-glycerol stock solution, 400 μl Tris-EDTA stock solution, 50 μl β-mercaptoethanol, and 20 μl 100 mM PMSF; the SDS-glycerol stock solution is 7.3% [wt/vol] SDS, 29.1% glycerol, 83.3 mM Tris base, and bromphenol blue; the Tris-EDTA stock solution was 0.2 M Tris base and 20 mM EDTA). Resuspension of the pellet was accomplished by carefully pipetting up and down until the pellet was completely in solution. Finally, the samples were boiled for 10 min and then centrifuged at 14,000 rpm for 10 min at 4°C. Equal loading was controlled by staining the transfer membranes with Ponceau red (Sigma). Antibodies for ChIP and Western blot analyses were an anti-Myc antibody (A14; Santa Cruz Biotechnology) or the peroxidase-antiperoxidase (PAP) antibody (Sigma).

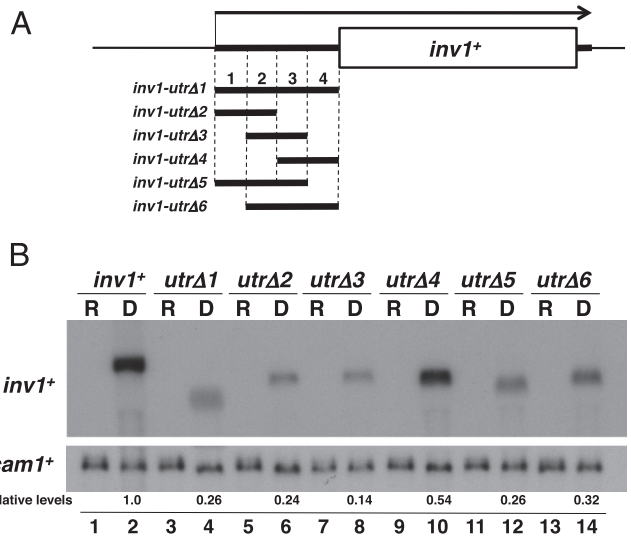

FIG 1 Analysis of the *inv1*$^+$ 5′ UTR. (A) Diagrammed are six deletions that were constructed to remove all or part of the *inv1*$^+$ 5′ UTR. The black bars indicate the regions that were deleted. Each was integrated into the *S. pombe* genome, replacing the wild-type *inv1*$^+$ 5′ UTR. (B) Northern analysis of the 5′ UTR deletions. Strains were grown under either repressing (R) or derepressing (D) conditions, and Northern blot analyses were done as described in Materials and Methods. *cam1*$^+$ serves as a loading control. The strains used were FWP449 to FWP455. Quantitation of the Northern blot shown in the figure was performed as described in Materials and Methods. Values are presented for the derepressed (D) lanes only, and the values are normalized to 1.0 for the wild-type strain (lane 2).

***S. pombe* deletion set screen.** The *S. pombe* deletion set (21) was purchased from Bioneer. Our version of the haploid library contains 3,214 haploid strains, each of which has one nonessential ORF replaced by the *KanMx* cassette. The number of nonessential genes in *S. pombe* is estimated to be 3,576 (21), making the library approximately 90% complete. To screen for mutant phenotypes, the library was spotted onto YES-3% glucose and YES-3% raffinose plus 1 μg/ml antimycin A. The first 31 plates were manually scored for growth both 2 and 5 days after incubation and were manually scored on a scale of from 0 to 5. Strains with a score difference of more than 2 between the YES and YES-raffinose on either of the days were scored Raf$^-$. The screen was performed two times with the entire set, and potential hits were retested a third time. Strains scored Raf$^-$ were tested by several additional tests, including Northern blot analysis of *inv1*$^+$ mRNA levels in the original deletion set strain and reconstruction of each candidate deletion, followed by Northern or real-time analysis of *inv1*$^+$ RNA levels.

RESULTS

The long *inv1*$^+$ 5′ untranslated region (UTR) is required for normal levels of *inv1*$^+$ mRNA. We initiated our studies by mapping the 5′ and 3′ ends of the *inv1*$^+$ mRNA by RACE (see Materials and Methods). Our results show that *inv1*$^+$ has a long 5′ UTR of 872 bases and a short 3′ UTR of 48 bases, in approximate agreement with other studies (30, 37). These sizes are unusual, as the median sizes of *S. pombe* 5′ and 3′ UTRs are 152 and 169 nucleotides (37). Although long 5′ UTRs are not common in *S. pombe*, unstable mRNAs tend to have longer 5′ UTRs (37), suggesting that the *inv1* 5′ UTR may have a role in stability.

To test whether the *inv1*$^+$ 5′ UTR controls *inv1*$^+$ mRNA levels, we constructed a set of 5′ UTR deletions and tested them by Northern analysis. First, a deletion of the entire 5′ UTR caused a greatly reduced level of *inv1*$^+$ mRNA (Fig. 1, lanes 3 and 4). Analysis of smaller 5′ UTR deletions suggested that sequences in the 5′ half of the UTR play the most important role in controlling *inv1*$^+$ mRNA levels, as deletion of the 3′ half of the UTR has a smaller effect on *inv1*$^+$ mRNA levels (Fig. 1, lanes 9 and 10). The results from the rest of the deletions are most consistent with the idea that sequences in segment 2 are the most strongly required for a full level of *inv1*$^+$ mRNA under derepressing conditions. In all cases, there was no detectable *inv1*$^+$ mRNA in cells grown in high glucose, showing that the 5′ UTR is not required for repression. Thus, the *inv1*$^+$ 5′ UTR is required for full induction of *inv1*$^+$ mRNA under derepressing conditions.

The *inv1*$^+$ TATA is required for normal *inv1*$^+$ mRNA levels. Although TATA elements were once believed to be hallmarks of eukaryotic promoters, consensus TATA elements actually occur in only a minority of eukaryotic promoters (3, 40). At *S. pombe* *inv1*$^+$, there is a consensus TATA element (TATATATA) located at −35 to −28 bp 5′ of the *inv1*$^+$ transcription start site. To test whether this sequence is required for *inv1*$^+$ expression, we mutated the *inv1*$^+$ TATA element to GCGGCCGC. Northern analysis showed that this mutation greatly decreases *inv1*$^+$ mRNA levels (Fig. 2, lanes 3 and 4). These results demonstrate that the TATA element at *inv1*$^+$ is required for normal *inv1*$^+$ RNA levels under inducing conditions.

***inv1*$^+$ has several redundant *cis*-regulatory regions.** To identify additional *cis*-acting sequences that regulate *inv1*$^+$ transcription, we constructed a set of deletions 5′ of the *inv1*$^+$ TATA element (diagrammed in Fig. 2) and measured *inv1*$^+$ mRNA levels by Northern blot analyses. Surprisingly, a deletion of the entire intergenic region, from the 3′ end of the upstream ORF (SPCC191.10) to 22 bp 5′ of the TATA, had only a modest effect on *inv1*$^+$ mRNA levels or regulation (*inv1-prΔ1*; Fig. 2, lanes 5 and 6). Consistent with this finding, a set of smaller deletions, each removing approximately one-third of the intergenic region, also had modest effects on *inv1*$^+$ regulation (Fig. 2, lanes 7 to 12). These results demonstrate that the *inv1*$^+$ 5′ intergenic region is not strongly required for normal *inv1*$^+$ transcriptional regulation.

To identify the region required for *inv1*$^+$ expression, we constructed and tested larger deletions. First, we constructed a deletion that removes the entire *inv1*$^+$ 5′ intergenic region and the next ORF, SPCC191.10 (*inv1-prΔ2*). Northern analysis showed that this deletion greatly reduces *inv1*$^+$ mRNA levels, suggesting that it removes the *cis*-acting elements required for *inv1*$^+$ induction (Fig. 2, lanes 13 and 14). SPCC191.10 encodes a potential protein of 149 amino acids that is not conserved even in other *Schizosaccharomyces* strains. Northern analysis shows that it is transcribed at low but detectable levels (S. Ahn and F. Winston, unpublished data). In this deletion, then, the weak SPCC191.10 promoter is likely directing expression of *inv1*$^+$, possibly accounting for the greatly reduced levels of *inv1*$^+$ mRNA. Therefore, we also made an even longer deletion that extends beyond the SPCC191.10 promoter to the next gene, *gst1*$^+$, which lies in the opposite orientation from SPCC191.10 (*inv1-prΔ4*; Fig. 2, lanes 17 and 18). In this mutant, *inv1*$^+$ was also not induced in low glucose, again implicating 191.10 in *inv1*$^+$ regulation. These two deletions, then, suggest that sequences within SPCC191.10 are sufficient for *inv1*$^+$ expression and regulation.

To test whether SPCC191.10 is necessary for *inv1*$^+$ expression, we constructed a deletion that removes only the SPCC191.10 ORF (*inv1-prΔ3*). Northern analysis showed that this deletion causes only a small effect on *inv1*$^+$ mRNA levels and regulation (Fig. 2,

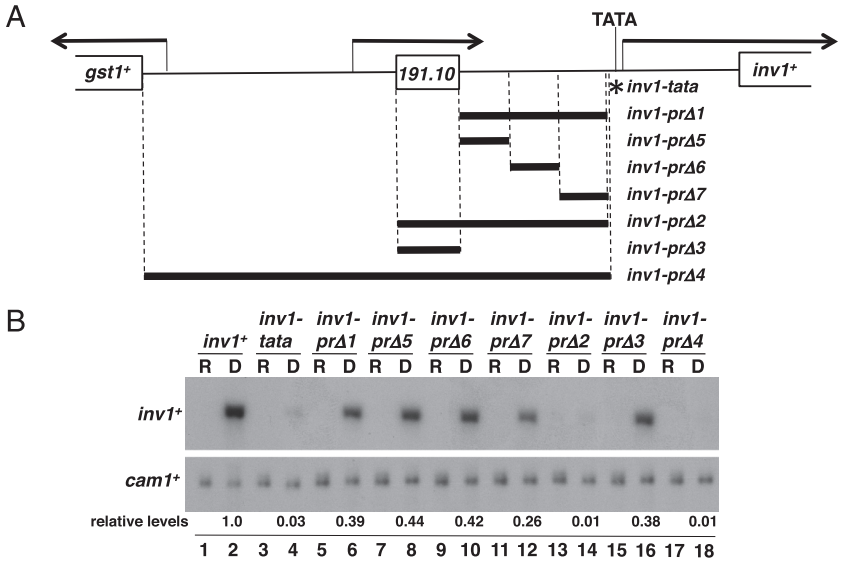

FIG 2 Analysis of the *inv1*$^+$ regulatory region. (A) Diagrammed are several deletions that were made and that remove different portions of the *inv1*$^+$ regulatory region 5′ of the *inv1*$^+$ TATA element. The arrows at the top indicate the direction of transcription of the three genes shown. (B) Northern analysis of *inv1*$^+$ intergenic deletion mutants. Strains were grown under either repressing (R) or derepressing (D) conditions, and Northern blot analyses were done as described in Materials and Methods. *cam1*$^+$ serves as a loading control. The strains used were FWP449 and FWP457 to FWP463. Quantitation of the Northern blot shown in the figure was performed as described in Materials and Methods. Values are presented for the derepressed (D) lanes only, and the values are normalized to 1.0 for the wild-type strain (lane 2).

lanes 15 and 16), suggesting that there are additional regulatory elements, likely in the intergenic region between *inv1*$^+$ and SPCC191.10, that can regulate *inv1*$^+$. Taken together, these results suggest that at least two redundant elements, one in the SPCC191.10 ORF and another in the *inv1*$^+$ intergenic region, that can each confer glucose-repressible transcription of *inv1*$^+$ exist.

Several *inv1*$^+$ regulatory regions can confer glucose-regulated transcription. Given the apparent redundancy of *inv1*$^+$ regulatory regions, we tested whether specific sequences 5′ of *inv1*$^+$ are sufficient to activate another gene. To do this, we moved specific fragments of the *inv1*$^+$ 5′ regulatory region to an ectopic site, the *his5*$^+$ locus, replacing the *his5*$^+$ regulatory region (diagrammed in Fig. 3). Transcription of *his5*$^+$ is normally not significantly controlled by glucose levels (data not shown). We then tested each construct for *his5*$^+$ mRNA levels in high and low glucose. As controls, we compared these strains to a strain where the *his5*$^+$ ORF replaces the *inv1*$^+$ ORF at the *inv1*$^+$ locus (*inv1-his5*$^+$; diagrammed in Fig. 3). As expected, the *inv1-his5*$^+$ strain showed a glucose repression pattern similar to that of *inv1*$^+$ (Fig. 3, lanes 1 and 2 in all three panels).

For our analysis of the *inv1*$^+$ regulatory region, we divided the region into three arbitrary segments: two regions that cover the *inv1*$^+$ intergenic region (regions A and B, shown in green and red in Fig. 3) and one region that contains SPCC191.10 (region C, shown in light blue in Fig. 3). For each region, the entire segment, as well as subsegments, was integrated in place of the entire *his5*$^+$ intergenic region and tested for *his5*$^+$ mRNA levels. From these experiments, it was found that the *inv1*$^+$ 5′ UTR is not sufficient for expression (Fig. 3A, lanes 3 and 4) and region A is able to confer only weak expression and glucose repression (Fig. 3A, lanes 9 and 10). In contrast, regions B and C are each able to confer a high level of expression and glucose repression to *his5*$^+$ (Fig. 3B and C), although the repression conferred by region B or C in high glucose is not quite as tight as that for *inv1*$^+$ or for *inv1-his5*$^+$ (for example, compare lanes 1 and 2 to lanes 9 and 10 in Fig. 3C). For both regions B and C, our results suggest that there are multiple elements within each that contribute to the overall level of transcription, since longer regions generally show stronger repression and induction than shorter regions. Furthermore, since strong regulation is conferred by a region of SPCC191.10 that does not contain its promoter (Fig. 3C, lanes 7 and 8), transcription of SPCC191.10 is unlikely to be required for *inv1*$^+$ activation. These results strongly support the idea that multiple regulatory elements regulate *inv1*$^+$.

SPCC191.10 can activate *inv1*$^+$ transcription over a long distance and in either orientation. While transcriptional activation distance has not been directly studied in *S. pombe*, intergenic regions are generally larger in *S. pombe* than in *S. cerevisiae* (22, 39), suggesting that activation may be able to occur over longer distances. Indeed, SPCC191.10, which is able to activate *inv1*$^+$, is located over 1 kb from the *inv1*$^+$ transcription start site. However, our test for the ability of SPCC191.10 to activate *inv1*$^+$ moved SPCC191.10 adjacent to the *inv1*$^+$ transcription start site (Fig. 2). To verify that SPCC191.10 is sufficient to activate *inv1*$^+$ from its normal distance and to test even greater distances, we constructed three additional strains, diagrammed in Fig. 4. In these constructs, we integrated different lengths of the plasmid pKS in place of the *inv1*$^+$ intergenic region, resulting in SPCC191.10 being 1, 2, and 3 kb 5′ of the *inv1*$^+$ transcription start site. Our results (Fig. 4, lanes 1 to 10) show that SPCC191.10 is able to activate *inv1*$^+$ transcription even from 3 kb away, albeit more weakly, and that this activation is greater in low glucose. The diminished strength of glucose repression in these constructs may be caused by sequences within pKS plasmid sequences.

Two characteristics of mammalian enhancers are that they are able to activate from great distances and their ability to activate is

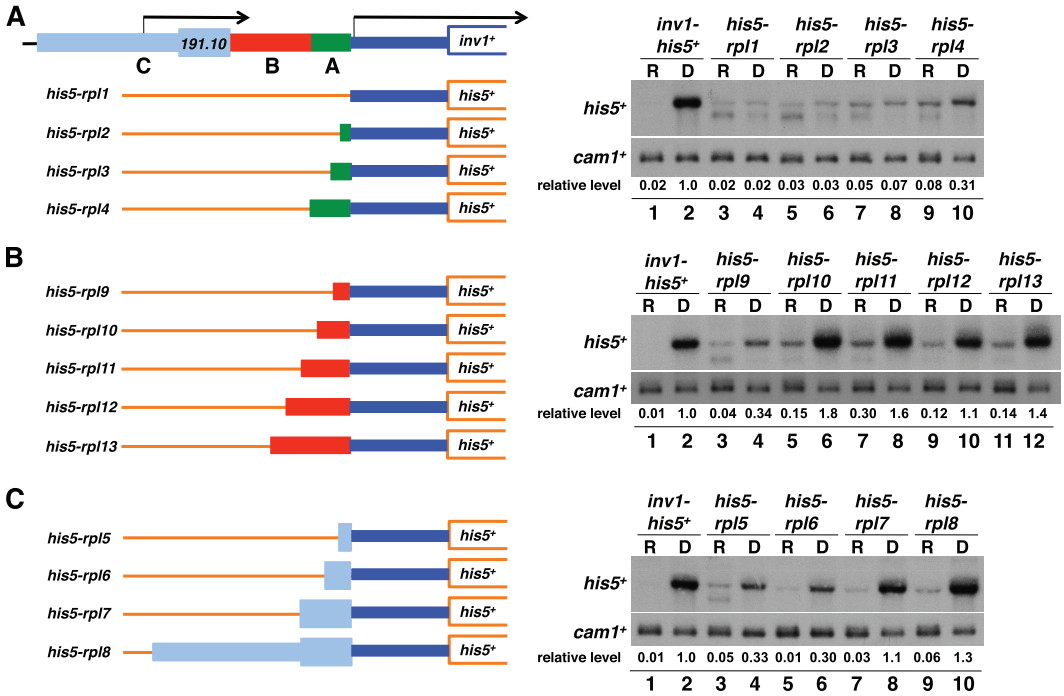

FIG 3 Activation by *inv1*$^+$ regulatory sequences of a reporter gene. (A) On the left are diagrammed constructs in which different portions of *inv1*$^+$ regulatory region A, along with the *inv1*$^+$ 5′ UTR, have replaced the *his5*$^+$ regulatory region. The smallest portion comes from the 3′ end of region A, and the larger regions extend toward the 5′ end of this region. On the right is shown a Northern blot of the *inv1*$^+$ intergenic deletion mutants. Strains were grown under either repressing (R) or derepressing (D) conditions, and Northern blot analyses were done as described in Materials and Methods. *cam1*$^+$ serves as a loading control. The strains used were FWP449 and FWP464 to FWP468. Quantitation of the Northern blot shown in the figure was performed as described in Materials and Methods. Values are normalized to 1.0 for the wild-type strain grown under derepressing conditions (lane 2). (B) Same as for panel A, except that the constructs analyze regulatory region B. The strains used were FWP449, FWP464, and FWP469 to FWP473. (C) Same as for panel A, except that the constructs analyze regulatory region C. The strains used were FWP449, FWP464, and FWP474 to FWP477.

orientation independent. To test the latter characteristic, we constructed a strain that contains SPCC191.10 in the opposite orientation from that found at wild-type *inv1*$^+$. Our results (Fig. 4, lanes 11 to 12) showed that even in this flipped orientation, SPCC191.10 is able to confer normal regulation to *inv1*$^+$. We also placed SPCC191.10 at a position 3′ of the *inv1*$^+$ ORF and in that case did not see any activation of *inv1*$^+$ (data not shown). This negative result suggests that this region may not be able to activate from 3′ of a gene. However, SPCC191.10 appears to have some characteristics of enhancer elements of larger eukaryotes.

SAGA and Swi/Snf are directly required to activate *inv1*$^+$. To identify factors that are required for *inv1*$^+$ activation, we screened the *S. pombe* deletion set (21) and we also tested a small number of candidate mutants. Our screen of the *S. pombe* deletion set for mutants that grow poorly on raffinose as a carbon source (see Materials and Methods) identified *spt7*$^+$, which encodes a core component of the SAGA coactivator complex. In addition, our direct test by Northern blot analyses showed that *snf22*$^+$, encoding the ATPase of Swi/Snf, is also required for normal *inv1*$^+$ mRNA levels (data not shown). As SAGA contains 19 components and carries out different activities in transcription (7), we tested additional SAGA deletion mutants by real-time PCR to measure *inv1*$^+$ mRNA levels. Our results (Fig. 5) show that among those tested, only *spt7*Δ, which completely abolished SAGA function, causes a large decrease in *inv1*$^+$ mRNA levels. Loss of Gcn5, the SAGA histone acetyltransferase, or Ubp8, the SAGA histone deubiquitylase, had only a small effect on *inv1*$^+$ mRNA levels. These results suggest that multiple functions within SAGA contribute to activate *inv1*$^+$ transcription under inducing conditions. Our analysis also showed that *snf22*Δ causes a decrease in *inv1*$^+$ mRNA levels. Thus, as for the *S. cerevisiae inv1*$^+$ orthologue, *SUC2*, both SAGA and Swi/Snf are required for activation (34).

To test whether SAGA and Swi/Snf act directly at *inv1*$^+$, we performed ChIP assays, testing Spt7 of SAGA and Snf22 of Swi/Snf, using epitope-tagged versions of each (Spt7-myc and Snf22-TAP). Given the large size of the *inv1*$^+$ regulatory region, we performed the assays using five different primer pairs. Our results (Fig. 6) show that both SAGA and Swi/Snf are recruited across the *inv1*$^+$ regulatory region, but only under derepressing conditions. Recruitment was greatest when assayed with primer pair 1, within the SPCC191.10 activating region, and primer pair 3, which spans regions also shown to confer activation (Fig. 3), although some recruitment was also observed with primer pairs 2 and 4. To test whether SAGA recruitment is dependent upon Swi/Snf or vice versa, we repeated the Spt7 ChIP assay in an *snf22*Δ mutant and the Snf22 ChIP assay in an *spt7*Δ mutant. Our ChIP results (Fig. 6A) suggested that there is a mutual dependency between SAGA and Swi/Snf for their recruitment to the *inv1*$^+$ regulatory region. However, our Western analysis (Fig. 6B) suggested that there is a reduced level of Spt7-myc in the *snf22*Δ mutant compared to that in wild-type strains; thus, this reduced level of protein may at least partially explain the reduced Spt7-myc ChIP signal. For Snf22-TAP, the level of protein is the same between the wild-type and *spt7*Δ strains; therefore, we conclude that SAGA is required for

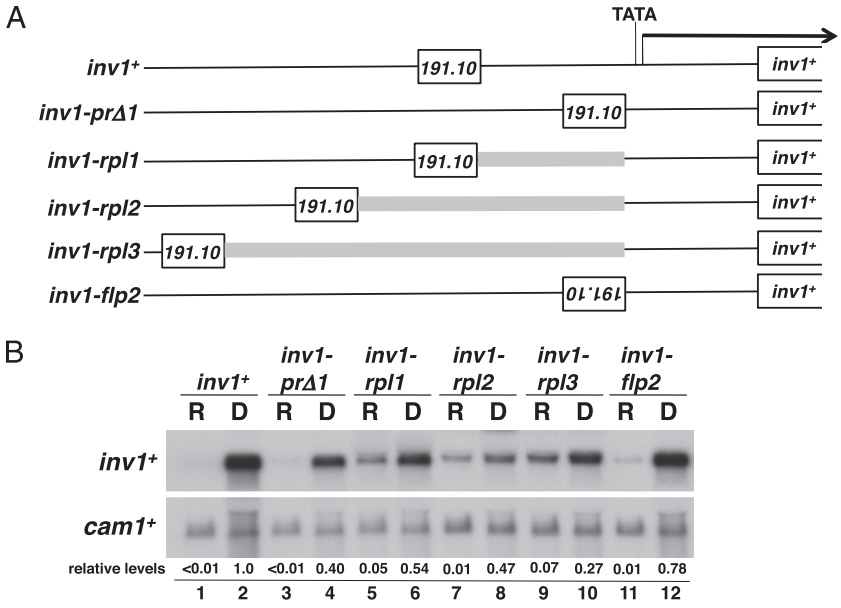

FIG 4 Analysis of effects of distance and orientation on activation by SPCC191.10. (A) Diagrammed are three constructs that place SPCC191.10 at different distances from *inv1*$^+$ and one construct that places SPCC191.10 in an inverted orientation with respect to *inv1*$^+$. (B) Northern analysis of the strains diagrammed in panel A. Strains were grown under either repressing (R) or derepressing (D) conditions, and Northern blot analyses were done as described in Materials and Methods. *cam1*$^+$ serves as a loading control. The strains used were FWP449 and FWP478 to FWP482. Quantitation of the representative Northern blot shown in the figure was performed as described in Materials and Methods. Values are normalized to 1.0 for the wild-type strain grown under derepressing conditions (lane 2). In the *inv1-flp2* construct, 191.10 is drawn upside down to indicate that it is in the reverse orientation.

Swi/Snf recruitment to *inv1*$^+$ under derepressing conditions. Interestingly, the level of Snf22-TAP appears to be glucose regulated, as Snf22-TAP levels are higher when cells are grown under inducing conditions. Overall, our analysis shows that both SAGA and Swi/Snf are required for full induction of *inv1*$^+$ and that both SAGA and Swi/Snf are recruited to the *inv1*$^+$ regulatory region, specifically under inducing conditions.

DISCUSSION

In this work, we have analyzed the *cis*-acting elements required for regulation of the *S. pombe inv1*$^+$ gene. Previous studies had demonstrated that *inv1*$^+$ is transcriptionally repressed in high glucose and induced in low glucose (19, 35) and that glucose repression depends upon Scr1 and Ssn6/Tup (9, 35). Our results show that *inv1*$^+$ is under the control of a complex set of regulatory elements, spread over approximately 2 kb, a size considerably larger than that normally observed for yeast genes. These elements appear to function in a redundant fashion, as distinct segments are able to confer glucose repression when placed 5′ of another gene, *his5*$^+$, that is not normally regulated by glucose repression. Furthermore, multiple regions in the *inv1*$^+$ regulatory region are able to recruit both the Swi/Snf and SAGA coactivators, both of which are required for *inv1*$^+$ transcription under inducing conditions. Finally, our results demonstrate that one region that can strongly activate *inv1*$^+$ is within an annotated open reading frame and that this region can activate *inv1*$^+$ transcription from a distance of at least 3 kb and in either orientation.

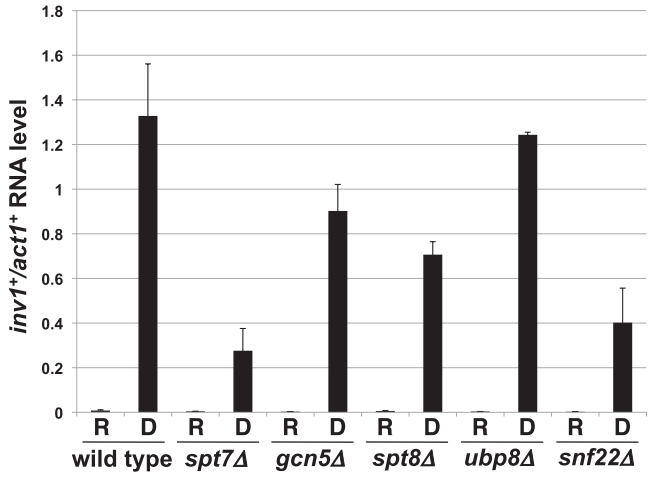

FIG 5 Swi/Snf and SAGA are required for normal levels of *inv1*$^+$ RNA. RNA was prepared as described and analyzed by real-time PCR. Each experiment was performed at least three times, and the error bars represent the standard error. The strains used were FWP165, FWP229, FWP422, FWP308, FWP314, and FWP316.

Relatively few promoter regions have been analyzed in *S. pombe*, but among these, some appear to be complex. For example, the *mei3*$^+$ promoter, while considerably smaller than that of *inv1*$^+$, also contains several redundant regions that contribute to *mei3*$^+$ transcription (36). The *fbp1*$^+$ promoter, which, like *inv1*$^+$, is glucose regulated, is also complex, but in a different way, as this promoter is regulated by a cascade of noncoding RNAs whose transcription is required to repress transcription in high glucose (14). On the basis of this small number of cases, it seems likely that continued analysis of *S. pombe* promoters will reveal additional layers of complexity for transcriptional regulation.

One particularly interesting aspect of *inv1*$^+$ regulation is that one of the strongest activating regions, SPCC191.10, is distant from the transcription start site, about 1 kb away, and it is within a transcribed open reading frame. Furthermore, this region can confer glucose-mediated regulation in the absence of the rest of

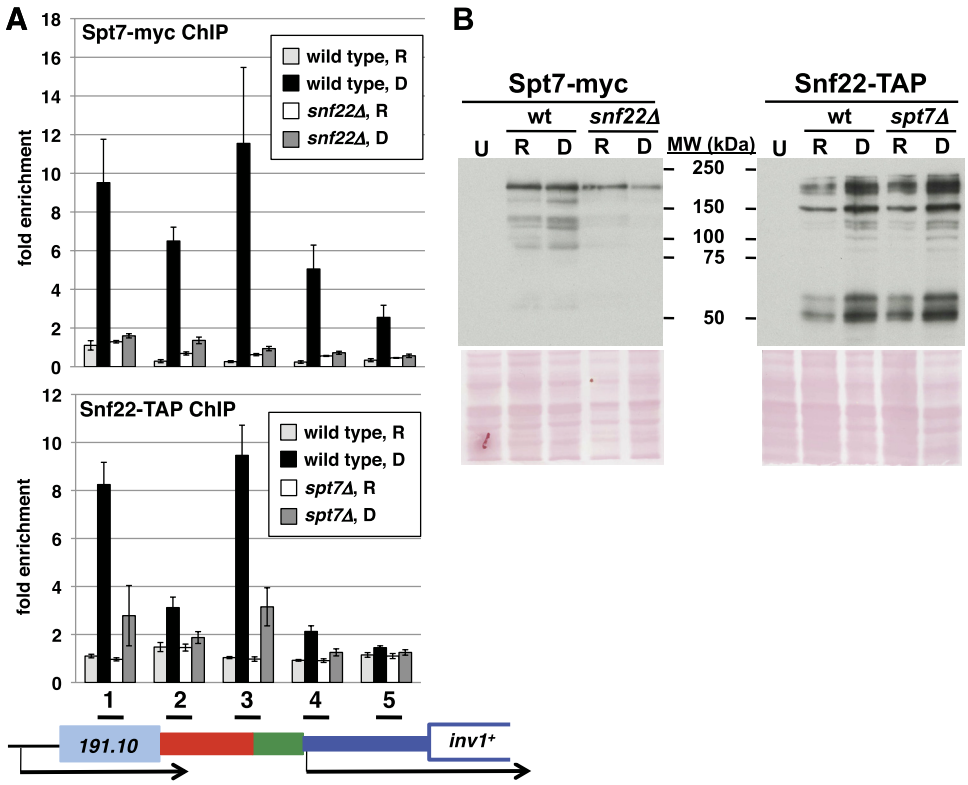

FIG 6 (A) Swi/Snf and SAGA physically associate with the *inv1*$^+$ regulatory region. Shown are the results of ChIP experiments for Snf22-TAP (top) and Spt7-myc (bottom). The five regions tested are diagrammed at the bottom. The strains used were FWP317, FWP331, FWP491, FWP492, FWP165, FWP219, and FWP495. (B) Western analysis of epitope-tagged protein levels in the ChIP experiments. Ponceau red staining (bottom) serves as a loading control for the Western blot analyses. wt, wild type.

the *inv1*$^+$ regulatory region, even when it is moved to 3 kb away or when it is in an inverted orientation. Consistent with our findings that SPCC191.10 confers glucose repression, an Scr1 consensus binding site was previously identified in this region, although no functional tests have yet been done (19, 35). On the basis of our other analyses of SPCC191.10, it does not need to be transcribed to activate, and it likely contains multiple sites that contribute to regulation. SPCC191.10 is not conserved, even in other *Schizosaccharomyces* strains (30). The lack of conservation and our finding that a deletion grows normally suggest that SPCC191.10 is unlikely to encode an important function and may serve only to regulate *inv1*$^+$ transcription. Our results suggest the possibility that other *cis*-regulatory elements in *S. pombe* are not restricted to intergenic regions.

Some factors that regulate *inv1*$^+$ transcription have been identified, but it seems likely that several more exist. With respect to repression, Scr1, an orthologue of *S. cerevisiae* Mig1, has previously been shown to be required for *inv1*$^+$ repression (35). Furthermore, the Ssn6-Tup11/12 corepressor complex, previously shown to be required for repression of several *S. pombe* genes (9), is also required for *inv1*$^+$ repression (9; our unpublished results). Finally, several other mutants that express *inv1*$^+$ constitutively have been isolated, although the mutant genes have not yet been identified (20). With respect to activation, one recent report identified Ssp2, an Snf1-like kinase, to be required for *inv1*$^+$ derepression via phosphorylation and relocalization of Scr1 (26). Given the large size of the *inv1*$^+$ regulatory region, we considered the possibility that noncoding RNAs might play a regulatory role, as has been previously shown in other cases (for example, see references 5 and 25). One noncoding RNA, SPNCRNA1223, which initiates in the *inv1*$^+$ intergenic region, has been identified in this region and is transcribed in the opposite direction as *inv1*$^+$ (30). However, since deletion of the *inv1*$^+$ intergenic region has no detectable effect on *inv1*$^+$ regulation, it seems unlikely that this RNA plays a major regulatory role. It will be of great interest to identify the remaining *inv1*$^+$ regulatory factors and to elucidate their mechanisms of activation across the large *inv1*$^+$ regulatory region.

ACKNOWLEDGMENTS

We thank Dominique Helmlinger for strains and helpful advice.

This work was supported by NIH grant GM45720 to F.W. S.A. was supported in part by a Samsung Scholarship.

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