Reciprocal interferences between nucleosomal organization and transcriptional activity of the yeast *SNR6* gene

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Recent work has demonstrated a repressive effect of chromatin on the transcription of the yeast *SNR6* gene in vitro. Here, we show the relations between chromatin structure and transcriptional activity of this gene in vivo. Analysis of the *SNR6* locus by micrococcal nuclease digestion showed a protection of the TATA box, nuclease-sensitive sites around the A and B blocks, and arrays of positioned nucleosomes in the flanking regions. Analysis of a transcriptionally silent *SNR6* mutant containing a 2-bp deletion in the B block showed a loss of TATA-protection and rearrangement or destabilization of nucleosomes in the flanking regions. Hence, *SNR6* organizes the chromatin structure in the whole region in a manner dependent on its transcriptional state. Transcriptional analysis was performed by use of maxi-gene *SNR6* constructs introduced into histone-mutated strains. Chromatin disruption induced by histone H4 depletion stimulated the transcription of promoter-deficient, but not of wild-type *SNR6* genes, revealing a competition between the formation of nucleosomes and the assembly of Pol III transcription complexes that was much in favor of transcription factors. On the other hand, amino-terminal mutations in histone H3 or H4 had no effect (H4) or only a moderate stimulatory effect (H3) on the transcription of promoter-deficient *SNR6* genes.

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A large number of studies carried out both in vitro and in vivo have established a globally repressive effect of chromatin on transcription, even if in some cases (Thomas and Elgin 1988; Cullen et al. 1993; Lu et al. 1993; Schild et al. 1993) a positive role of chromatin has been suggested through positioned nucleosomes bringing regulatory sequences closer to each other. In vivo, inhibition of transcription via positioned nucleosomes has been suggested for several genes, but the mechanisms are not known. In yeast, activation of the *PHO5* promoter is accompanied by disruption of four positioned nucleosomes, including the nucleosome that covers the TATA box (Almer et al. 1986). Similarly, it was proposed that repression of α -cell-specific genes by the α 2-MCM1 complex acts by positioning of a nucleosome over the

TATA box (Roth et al. 1990, 1992; Ganter et al. 1993). However, it was reported recently that transcription remains repressed irrespective of whether the TATA box is located on a nucleosome or in an internucleosomal linker region (Patterson and Simpson 1994). A more global repression effect of chromatin (which could not be due to precisely positioned nucleosomes) on RNA polymerase II (Pol II) transcription in vivo in yeast has been demonstrated by Grunstein and co-workers, who showed that partial loss of nucleosomes activated reporter genes linked to basal promoters derived from genes as different as *PHO5*, *CYC1*, *GAL1* (Han and Grunstein 1988), *CUP1*, and *HIS3* (Durrin et al. 1992).

Numerous studies have also focused on chromatin-mediated repression of RNA polymerase III (Pol III) genes in vivo. The 5S rRNA genes have been subjected to intense investigation, especially the *Xenopus* oocyte-type 5S genes whose transcription was found to be repressed by chromatin in somatic cells, with histone H1 playing a

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key role in this process (Schlissel and Brown 1984; Wolffe 1989). In vitro studies with *Xenopus* egg extracts, which mimic in vivo conditions in terms of transcription, DNA replication, and chromatin assembly, have demonstrated a competition between the formation of transcription complexes and of nucleosomes on replicating 5S templates, resulting in the establishment of selective repression of 5S gene transcription (Almouzni et al. 1990). Yeast 5S genes do not seem to be under the same constraints, because in vivo nucleosome disruption was not found to stimulate their transcription (Kim et al. 1988). Yeast tRNA genes also appear to be very resistant to chromatin-mediated repression. Even if a yeast tRNA gene could be repressed by insertion into the silent *HMR* mating-type locus (Schnell and Rine 1986), the *SUP4* tRNA^{Tyr} gene was found able to override two different nucleosome positioning signals in vivo (Morse et al. 1992), and, as for the 5S genes, no overall transcriptional stimulation of tRNA genes was triggered by nucleosome loss in yeast (Kim et al. 1988).

Burnol et al. (1993a) demonstrated recently that the yeast U6 RNA gene, *SNR6*, was repressed in vitro after chromatin assembly in *Xenopus* egg extracts or after nucleosome reconstitution with purified histones. This yeast Pol III gene presents some unusual features in its promoter elements: Its A block, at position +21, is degenerate when compared with the consensus tRNA gene element; its B block is located downstream of the termination signal, 202 bp away from the A block; and it has a TATA box at position -30 (Brow and Guthrie 1990). Naked DNA templates of *SNR6* gene can be transcribed effectively in vitro with purified transcription factor (TF) TFIIB and Pol III, and the presence of the extragenic B block is not required for this process (Moenne et al. 1990; Margottin et al. 1991; Joazeiro et al. 1994). In contrast, the B block was found to be an essential promoter element in vivo (Brow and Guthrie 1990). This paradox was resolved by the demonstration that the chromatin repression observed in vitro was relieved by the interaction of TFIIC with the B block, establishing a new, antirepressor function for this transcription factor (Burnol et al. 1993a).

In this paper we present an investigation of the relation between chromatin structure and transcriptional activity of wild-type and mutant *SNR6* genes in vivo. The nucleosomal organization of the *SNR6* locus was determined at nucleotide resolution and was shown to undergo dramatic changes upon the inactivation of the *SNR6* gene by a 2-bp deletion in its B block. The competition between the establishment of chromatin structure and the binding of transcription factors was demonstrated by an increase in the transcript levels of *SNR6* mutant genes following in vivo disruption of nucleosomes. In these experiments, the transcriptional activity of the wild-type *SNR6* gene remained unchanged, indicating that class III transcription factors can compete successfully with histones. Finally, we show that mutations in the amino-terminal domain of histone H3, but not histone H4, can enhance the transcription of mutant *SNR6* genes.

Results

Chromatin structure of the SNR6 locus

The *SNR6* locus, shown schematically in Figure 1A, comprises the transcribed sequence of the *SNR6* gene and its promoter elements, the TATA box, and the A and B blocks. In the 5' region of the *SNR6* gene, spanning base pairs -425 to -91 relative to *SNR6* transcription start site, lies a solo δ element with its TATA box and upstream activating sequences (UASs). δ elements are the long terminal repeats (LTRs) of Ty1 or Ty2 retrotransposons and are responsible for initiation of Ty transcription by Pol II (the 5' LTR) and for polyadenylation of the transcript (the 3' LTR). However, short RNA transcripts representing initiation and termination within the same LTR (or solo δ element) are not found in yeast cells (for review, see Boeke and Sandmeyer 1992).

To investigate whether the inactivation of the *SNR6* gene by a 2-bp deletion in its B block would affect chromatin structure, chromatin of the wild-type *SNR6* locus and of the mutated locus were analyzed with respect to nucleosome organization by use of micrococcal nuclease (MNase) digestions. MNase preferentially attacks linker DNA between nucleosomes and leads to double-strand cuts (Noll 1974), but it may also introduce single-strand nicks on the nucleosome surface (Cockell et al. 1983). To define nucleosome positions unambiguously, double- and single-strand cutting information is required. Genomic chromatin and deproteinized DNA were digested with different amounts of MNase. Double-strand cutting sites were displayed by indirect end-labeling (low resolution mapping; Fig. 1B, C). Primer extension was used to display the cutting sites on the bottom and top strands at high resolution (Fig. 2). An interpretation of the results is summarized in Figure 3.

The chromatin studies of the wild-type *SNR6* locus were done in yeast strains of different origins: GCY5 was derived from YM262 (Johnston and Davis 1984) and was used previously for chromatin studies (Cavalli and Thoma 1993). FTY113 and FTY114 were derived from YPH500 α (Sikorski and Hieter 1989). The chromatin structures of the wild-type locus were indistinguishable in these different strains (Fig. 1B,C; Fig. 2). With MNase mapping (Figs. 1 and 2), the bands in the D lanes represent the preferential cutting sites for MNase in deproteinized DNA. Some of these sites were protected by chromatin (indicated on the left side of Fig. 1B), whereas others were accessible (indicated on the right side of Fig. 1B and Fig. 2, A-D). A protected region of 140–200 bp was interpreted as a positioned nucleosome (open box) (Thoma et al. 1984).

At low resolution, the chromatin structure of the wild-type *SNR6* upstream region shows a series of nucleosomes positioned on the solo δ sequences (nucleosomes -1, -2, -3) and on the flanking genomic DNA (nucleosome -4) (Fig. 1B,C). At high resolution, nucleosomes -1 to -4 can be clearly identified as protections in the top strand (bars in the CHR lanes of Fig. 2A,B) and on the bottom strand (bars in Fig. 2C,D). The major cutting sites recorded at high resolution agree very well with the sites

Figure 1. A 2-bp deletion in the B block disrupts chromatin structure in the *SNR6* gene and in the flanking regions. (A) A schematic view of the *SNR6* locus (according to Brow and Guthrie 1988, 1990). The *SNR6* and solo δ regions, the TATA boxes (T), the solo δ UAS and potential initiation site (I), the A and B blocks (A, B), the transcribed region of *SNR6* (arrow), the relevant restriction sites (*Ssp*I, *Hpa*I, *Pst*I), and the DNA fragment used to generate a probe (bar) are indicated. Distances (in base pairs) are from the *SNR6* transcription start site (1). (B) Chromatin structure of the wild-type *SNR6* locus (strain GCY5). Chromatin (lanes C) and deproteinized DNA (lanes D) were digested with various concentrations of MNase. The cutting sites were mapped by indirect end-labeling from the *Hpa*I site (position 630). The numbers represent averaged measurements of two gels with a total of nine chromatin lanes and four deproteinized DNA lanes. The standard deviations were below ± 10 bp for cutting sites between 466 and -334, and below ± 10 –20 bp for sites between -367 and -711. Protected and accessible sites are labeled on the left and right, respectively. An interpretive diagram is shown at right. *SNR6* and solo δ promoter elements are denoted by the same abbreviations as in A, except for the solo δ UAS, which is designated by U. Protected regions of 140–200 bp are interpreted as positioned nucleosomes (open boxes, -1, -2, -3, -4, 1). Partial protection of sites at 85 and 206 may suggest a nucleosome on the wild-type *SNR6* gene (hatched box; for details see text). The protection over the *SNR6* TATA box is marked (○). (C) Chromatin structure of the wild-type (strain FTY113, wt) and mutant *SNR6* locus with a 2-bp deletion in the B block (strain FTY115, $\Delta 2$). The cutting sites in chromatin (lanes C) and deproteinized DNA (lanes D) were mapped by indirect end-labeling from the *Pst*I site located about position 830. The major accessible sites and interpretive diagrams of the wild-type and mutated loci are shown at right and left, respectively. The naked DNA pattern of FTY115 was indistinguishable from that of FTY113 (not shown). Nucleosome positioning is lost in the $\Delta 2$ mutant, which is denoted by the absence of open boxes, and the *SNR6* TATA region becomes hypersensitive to MNase (arrowhead). Moreover, the solo δ TATA box becomes protected (stippled circle), and a putative nucleosome could be present in the 3' end of the mutated *SNR6* locus (stippled box). Note that the chromatin structure inferred by this method always reflects the average structure of a population.

mapped at low resolution (Fig. 3). With respect to the putative functional elements of the solo δ , the UAS was found in the linker region between nucleosomes -1 and -2. In contrast, the TATA box of solo δ was localized in nucleosome -2. On the basis of the double-strand cuts (Fig. 1), this nucleosome was placed on a position be-

tween -214 and -367. At high resolution, the cutting sites in the bottom and top strands did not overlap precisely, in particular each strand showed additional cutting on the 5' end of the putative nucleosome -2. Major cuts in the top strand were at -220 and -368, with additional cuts around -345 (Figs. 2B and 3). The bot-

Figure 2. Chromatin structure at high resolution of wild-type and mutant *SNR6* loci. Strains FTY113 and FTY114 harbor a wild-type chromosomal *SNR6* locus (wt) whereas FTY115 has a 2-bp mutation in the *SNR6* B block that inactivates the gene ($\Delta 2$). The cutting sites of MNase in chromatin (CHR) and deproteinized DNA (D) were displayed by linear primer extension for the top strand (A,B) and the bottom strand (C,D) (lanes 5–19). Increasing levels of digestion are shown (<). Lanes 1–4 represent the genomic sequence of the mutated *SNR6* locus (C, T, A, and G refer to the sequence on the template strand). The promoter elements of the solo δ region and of the *SNR6* gene are marked by vertical bars at lane G. The major sites accessible in wild-type chromatin and interpretive diagrams of the wild-type *SNR6* locus are shown right. Abbreviations (T, A, B, U) and symbols are as in Fig. 1; positioned nucleosomes are indicated by open boxes. The *SNR6* TATA box is accessible in the mutant (arrowhead on the diagram and in the CHR lanes) and protected in the wild-type gene (open ellipse). Major protected regions are also indicated by bars at the CHR lanes. A dot at position 236 (Fig. 2A) illustrates an altered sequence specificity for MNase resulting from the $\Delta 2$ deletion.

2C), whereas the 5' end showed a series of cuts between -220 and -255. Hence, the TATA box, which is located

Figure 3. Chromatin changes at the *SNR6* locus. The diagrams summarize the positions of the MNase sites at low and nucleotide resolutions, and provide a global interpretation of the nucleosomal structure of both the wild-type (wt) and mutant ($\Delta 2$) *SNR6* loci. The major diagnostic cutting sites for MNase are illustrated: Arrows mark double-strand cuts at low resolution and top and bottom strands (TS and BS) cuts at nucleotide resolution. Positioned nucleosomes are indicated by open ellipses. Transparent ellipses indicate weak protection, compatible with the presence of a nucleosome but not strongly supporting it, overlapping ellipses suggest a loss of nucleosome positioning or a loss of nucleosomes. A hatched ellipse in the *SNR6* gene indicates that a nucleosome might lead to the partial protection observed at 85 and 206. Protection of the TATA boxes in the *SNR6* gene and the *solo delta* element are marked by circles.

around -255 , was protected in the top strand but accessible on the bottom strand.

The *SNR6* gene is characterized by a protection of the TATA box and hypersensitive sites around the A block and close to the B block. Three chromatin-specific cutting sites were found around the TATA box and the A block (Fig. 1, hypersensitive sites -46 , 7 , 50). This observation was substantiated by a series of cuts at these positions on the top and bottom strands (Fig. 2). Most dramatically, the TATA box, which showed up as a band in naked DNA (Fig. 1, lanes D, site -16), was protected by chromatin. This was also evident at high resolution on the top and bottom strands (arrowheads in Fig. 2). In contrast to the TATA box, no protection of the A block was obvious. Two chromatin-specific hypersensitive sites were also observed 3' of the B block (Fig. 1B,C, hypersensitive sites 267 , 312). The B block DNA itself was not a preferred substrate for MNase (no double-strand cuts in lanes D) nor was it cut in chromatin. The region between the A and the B blocks was difficult to interpret. No strong cutting sites for MNase are available between 85 and 206 on deproteinized DNA that would allow an unambiguous estimate of their protection by chromatin.

Downstream of the B block, a site at 403 was protected and a site at 466 was accessible (Fig. 1B,C). This suggests that a nucleosome is positioned between 312 and 466 . Analysis of the bottom strand revealed at least two positioned nucleosomes downstream of *SNR6* (Fig. 2D, nucleosomes 1, 2).

The structure of the *SNR6* locus is summarized in Figure 3. *SNR6* characteristics include protection of the TATA box, hypersensitive sites close to the A and B blocks, and a series of positioned nucleosomes in the 3'- and 5'-flanking regions.

A 2-bp deletion in the B block disrupts protection over the TATA box of the SNR6 gene

FTY115 is a yeast strain in which the *SNR6* gene was inactivated by a 2-bp deletion in the B block. The strain was rescued by a wild-type gene placed on a plasmid. Chromatin analysis by MNase revealed a dramatic loss of protection in the TATA region of the silent chromosomal gene. Although a double-strand site at -16 was protected in the wild-type *SNR6* gene (Fig. 1B,C, GCY5 and FTY113), it was heavily cut in the $\Delta 2$ mutant FTY115 (arrowhead, Fig. 1C). The enhanced accessibility

was confirmed at high resolution on the top and bottom strands (arrowheads in Fig. 2B,D; cf. chromatin lanes of the wild-type gene and the $\Delta 2$ mutant).

Because relief of repression by TFIIC was required for in vitro transcription only when the DNA was packaged in chromatin (Burnol et al. 1993a), we expected that a lack of TFIIC binding to the mutated B block would allow nucleosome formation on the *SNR6* gene. However, neither a protection of the TATA box, the A block, nor the B block region by a nucleosome was observed on the silent gene (Figs. 1 and 2). No substantial differences were recorded at low and high resolution in the chromatin structure of the wild-type and mutant *SNR6* genes, except for the TATA region.

In conclusion, the major effect of the mutation in *SNR6* B block was the loss of protection in the TATA box, suggesting that TFIIC is required to recruit and stabilize the TATA-binding initiation complex in genomic chromatin.

A 2-bp deletion in the B block disrupts chromatin structure in the 5'- and 3'-flanking regions of SNR6

To our surprise, the effects of the B block mutation on chromatin structure were not confined to the TATA region of the *SNR6* gene but extended on both sides into the flanking sequences. Downstream of the *SNR6* gene, the protection that characterized nucleosomes 1 and 2 was lost in the mutant (Fig. 2D), indicating that nucleosomes, if still present, might have been rearranged. Furthermore, site 312 was partially protected in the mutant and site 403 became more accessible (Fig. 1C). This suggests that at least a fraction of nucleosomes might be positioned between 267 and 403 (stippled box, Fig. 1C). Hence, in this case, the downstream nucleosome 1 would have changed its position.

Upstream of the *SNR6* gene, the chromatin structure of the solo δ region was severely affected by the mutation in the B block. Instead of the clear nucleosomal pattern observed in the wild-type locus, more cleavage sites were accessible in the mutant, but their positions were still different from the cutting pattern in deproteinized DNA (Figs. 1C and 2B,C). This indicated that nucleosomes were disrupted or rearranged (the presence of nucleosomes in this region could not be directly tested by hybridization of a nucleosomal repeat with solo δ sequences, because solo δ sequences are highly repetitive in the genome). The TATA box of the solo δ element is located in a protected region (Fig. 1C, lanes 1–3, between –214 and –334). This region is unlikely to be covered by a positioned nucleosome, because it is only ~120 bp long and shorter than the nucleosome footprint observed in the wild-type locus (Fig. 1C, lane 4, between –214 and –367). In contrast to the protection over the TATA box of the wild-type *SNR6* gene, the protection over the solo δ TATA box in the mutant *SNR6* locus was revealed only by mapping the double-strand sites, whereas the top and the bottom strands mapped separately showed substantial cutting. Hence, whatever the component that created that protection, it was associated with the DNA

in a way that allowed the individual strands to be cut separately.

Chromatin structure of the wild-type SNR6 gene and of the $\Delta 2$ mutant in centromeric plasmids

Gene structure and function are frequently and efficiently tested on plasmid constructs. Because gene expression can be affected by the local chromatin structure and because chromatin structure can be altered by new flanking regions, we tested whether and to what extent chromatin structures were maintained when the (–140; +314) region of the wild-type and mutant *SNR6* genes was placed on plasmids. The chromatin structure of the wild-type *SNR6* gene and the $\Delta 2$ B block mutant, harbored on the centromeric plasmid pRS314, were tested by MNase digestion and indirect end-labeling (Fig. 4).

In the vector regions (pRS314), the cutting pattern of MNase was very similar in deproteinized DNA (lanes D) and in chromatin (lanes C). No protection was obvious that might indicate the positioning of nucleosomes, which suggests that nucleosomes were arranged randomly on bacterial sequences. With respect to the wild-type *SNR6* gene, cutting at the TATA box (site –16) was clearly protected and the chromatin-specific sites at –46 and 7 were accessible (e.g., Fig. 4B, lane 6). On the other hand, cutting at the hypersensitive sites at 50, 85, and 267 was less pronounced in plasmid chromatin and site 206 was more accessible (Figs. 1B and 4B, cf. sites 267 and 206 in plasmid and genomic DNA). When the B block deletion mutant was analyzed, the TATA box was accessible to MNase (Fig. 4, lanes 3,4,9) demonstrating a similar loss of protection as was observed in the genomic mutation. Furthermore, the chromatin pattern was similar to the deproteinized DNA pattern, indicating that nucleosomes, if present, were not positioned.

In conclusion, the chromatin structure was less clear on the plasmids than in the genome, but it showed the characteristic features and the mutation-dependent transitions. It must be pointed out that no effect of the *SNR6* gene on the flanking plasmid regions was observed, in contrast to the genomic chromatin organization.

Nucleosome disruption leads to an increase in the transcript level of mutant but not wild-type SNR6 genes

To investigate the effect of chromatin on *SNR6* transcription in vivo, and the part that the different promoter elements of *SNR6* could play in relieving it, we used the in vivo nucleosome disruption system developed by Grunstein and co-workers (Han and Grunstein 1988; Han et al. 1988; Kim et al. 1988). They constructed a strain, UKY403, whose two genes encoding histone H4 (H4-1 and H4-2) have been disrupted, and which survives with a unique histone H4 gene placed under control of the *GAL1* promoter on a centromeric plasmid (Kim et al. 1988). H4 mRNA synthesis is thus activated by galactose and repressed by glucose. After a shift to a glucose-containing medium, the UKY403 cells undergo about one round of cell division before arresting in G₂, at which

Figure 4. Chromatin structure of wild-type and $\Delta 2$ mutant *SNR6* genes in centromeric plasmids. (A) The wild-type *SNR6* gene (wt) and the mutant $\Delta 2$ gene ($\Delta 2$) were placed on pRS314 plasmids and introduced into the YPH500 α strain. The relevant part of the constructs is illustrated: the *SNR6* TATA box (T), A block (A), B block (B), and transcribed region (arrow) are indicated, as well as the fragments used as probes (bars). (B) Chromatin (lanes C) and deproteinized DNA (lanes D) were digested with various concentrations of MNase. The DNAs in lanes 1–2, 5–6, 8, and 10–11 are from the pRS314 plasmid harboring the wild-type (wt) *SNR6* gene, and the DNAs in lanes 3–4 and 9 from the plasmid harboring the *SNR6* $\Delta 2$ mutant ($\Delta 2$). A DNA ladder consisting of multiples of 256 bp was used for calibration (M, lane 7). The cutting sites were mapped from the *NaeI* site with a *NaeI*–*PvuII* probe (lanes 1–6) and from the *PvuII* site with a *PvuII*–*EcoRI* probe (lanes 8–11). Relevant cutting sites for MNase and diagrams are shown at sides. The TATA box was protected in the wild-type *SNR6* gene (○) and accessible in the $\Delta 2$ mutant (arrowhead). Almost no difference was observed between lanes C and D in the pRS314 regions, indicating that nucleosomes were randomized.

stage the density of nucleosomes on the DNA is approximately half the normal level (Kim et al. 1988). These events are referred to as nucleosome disruption or nucleosome loss. To analyze the relationship between chromatin structure and *SNR6* transcription, various *SNR6* genes harboring wild-type or mutated promoter elements were introduced into UKY403 cells.

We analyzed several mutant *SNR6* genes, which are described in Figure 5B. The mutations called TATA-down, Adown and B $\Delta 2$ (which was also used for the chromatin structure analysis) destroy, respectively, the TATA box, the A block, and the B block. Two constructs

were truncated at position +122 and lacked the B block, B $\Delta 1$ and (B $\Delta 1$ + Aup). The mutation Binv has the B block inverted, whereas the Aup mutation replaces the degenerate A block by a consensus A block derived from the sequences of tRNA A blocks (Burnol et al. 1993b). The (–140; +314) *SNR6* construct, used previously to analyze nucleosomal structure (Fig. 4), was mutated as described above and inserted into the multicopy YEp352 plasmid. An unrelated 59-bp DNA fragment was introduced into the transcribed region of the gene, at position +73, so as to distinguish the corresponding transcripts from the endogenous, wild-type U6 RNA.

UKY403 cells containing the various *SNR6* constructs were grown on galactose and then transferred to a glucose-containing medium for 6 hr. Figure 5A shows the steady-state transcript levels of both endogenous and episomal *SNR6* genes monitored by Northern blots, before and after the glucose shift. It can be noted that the *SNR6* maxi-genes, harbored on multicopy plasmids, had relatively much lower levels of transcripts than the wild-type, chromosomal gene, probably because of the 59-bp insertion in their transcribed sequences, which could alter either their rates of transcription or the half-life of their transcripts. In vivo, the A and B blocks are essential promoter elements of the *SNR6* gene, whereas the TATA box is dispensable and contributes with the A block to the selection of the transcription start site (Brow and Guthrie 1990; Burnol et al. 1993b; Chalker and Sandmeyer 1993; Eschenlauer et al. 1993). Accordingly, with a normal chromatin structure (before glucose shift), the transcripts produced from the constructs B $\Delta 2$, B $\Delta 1$, or Adown were hardly detectable on Northern blots (Fig. 5A, lane 9), even after a long exposure (lanes 11,13,15). The Binv and (B $\Delta 1$ + Aup) constructs, which are affected in their TFIIC-binding sequences, were also transcribed less efficiently (~15 and 7% of the wild-type level, respectively, lanes 5,7). The TATA-down construct (lane 3) generated about half as many (40%) transcripts as the wild-type construct (WT, lane 1); and, in that case, the transcripts were slightly shorter, in agreement with previous observations on the role of the TATA box in the selection of the transcriptional start site.

After nucleosome disruption, the chromosomal gene and the plasmid-borne wild-type construct showed no difference in transcriptional activity. In contrast, all of the gene constructs with mutated promoter elements presented a marked increase in transcript levels upon repression of histone H4 synthesis. For the TATA-down construct, nucleosome loss restored an accumulation of transcripts equivalent to the wild type (Fig. 5A, lane 4), and the transcript levels of the Binv and the (B $\Delta 1$ + Aup) constructs were increased strongly to 80% and 52% the level of the wild-type construct, respectively (lanes 6,8). The amount of transcripts generated from the B block-less genes, B $\Delta 2$, and B $\Delta 1$, was also increased after glucose shift, even though the transcriptional activity of these genes remained very low (lanes 12,14). Finally, the presence of transcripts derived from the Adown construct became detectable following nucleosome disruption (lane 16). The size of the transcripts was the same

Figure 5. Activation of mutant *SNR6* genes by depletion of histone H4 and nucleosomes. (A) The Northern blot illustrates the transcriptional activation of *SNR6* genes harboring a 59-bp insert in the transcribed region and different mutations. These genes are on multicopy plasmids and generate transcripts (U6 maxi-RNA) easily distinguishable from the endogenous U6 RNA produced from the wild-type, chromosomal *SNR6* gene. Plasmids harboring the *SNR6* mutant genes were introduced into the yeast strain UKY403, which contains a unique histone H4 gene under the control of the *GAL1* promoter. Cells were grown in galactose-based medium until they reached an OD_{660} of 0.3 (indicated as glucose shift -) and then transferred to a glucose medium for 6 hr (glucose shift +). (Right; lanes 11–16) The autoradiography of the gel was about four times longer. (B) Mutants analyzed. All plasmids contain the entire *SNR6* RNA gene, from position -140 to +314, except for the Bdel and the (Bdel + Aup) plasmids, which harbor a truncated fragment (from -120 to +122), without the B block. The mutations affecting the *SNR6* promoter elements (the TATA box and the A and B blocks) are described (the scheme is not drawn to scale). The nucleotides that have been mutated are underlined, and all positions refer to the transcriptional start site (+1).

before and after the glucose shift, suggesting that the activation of the *SNR6* genes by nucleosome disruption did not alter the transcription start site.

To check that these effects were related to nucleosome depletion and not to the galactose to glucose metabolic change and the consecutive modification of the cell growth rate, similar experiments were performed with the control strain MHY308. MHY308 is a strain isogenic to UKY403 except that its sole histone H4 gene is under control of its own wild-type promoter (Han and Grunstein 1988). After a similar shift from galactose to glucose, no significant increase in the transcript level of any *SNR6* construct could be observed in this strain (data not shown).

We conclude that the transcriptional activation of promoter-deficient *SNR6* genes was attributable to depletion of histone H4. The wild-type *SNR6* genes, on the other hand, were apparently insensitive to the chromatin state.

Mutations in the amino-terminal domain of histone H3, but not H4, enhance the transcriptional activity of mutated SNR6 genes

Grunstein and co-workers have demonstrated that deletions or mutations of the amino-terminal domains of

histones H3 and H4 had specific effects on the transcriptional activation of Pol II genes (Durrin et al. 1991; Johnson et al. 1992; Mann and Grunstein 1992; Roth et al. 1992). Because we had some evidence of chromatin-mediated effects on the transcription of the *SNR6* gene, we investigated whether these mutations could also affect in vivo the transcription of wild-type or mutant *SNR6* genes.

Grunstein and co-workers (Durrin et al. 1991; Mann and Grunstein 1992) have constructed two series of yeast strains lacking either histone H3 or H4 genes that survive with centromeric plasmids harboring wild-type or mutated versions of either the histone H3-2 or H4-2 genes under control of their own promoter. The wild-type, TATA down, Bin, and BΔ2 constructs were introduced into yeast strains variously mutated on histone H3 or H4 genes, and the corresponding transcripts were monitored by Northern blots.

Three yeast strains mutated in the histone H4-2 gene were tested: PKY817 and PKY813, which contain amino-terminal deletions of this gene (residues 4–14 and 4–28, respectively), and LDY107, in which the first four lysine residues of histone H4-2 were substituted by glutamine (Durrin et al. 1991). None of these mutations was found

to induce significant changes in the transcript levels of wild-type or mutant *SNR6* genes (Fig. 6A; data not shown).

Four yeast strains mutated in the histone H3-2 gene were analyzed. No significant change in the transcriptional activity of the *SNR6* genes could be observed in yeast strains RMY410 and RMY430, which contain amino-terminal deletions of the H3-2 gene (residues 4–10 and 4–30, respectively; Fig. 6B; data not shown). In contrast, we found that two mutations affecting the acetylation sites of histone H3-2 could enhance the transcriptional levels of *SNR6* genes mutated in their B blocks. The histone H3 mutations, which either replaced the first three acetylation sites (lysine residues) by three glycine or changed the first four acetylation sites to arginine, allowed some increase in the transcriptional activity of the *SNR6* constructs affected in the B block, B inv, and BA2 (Fig. 6B, lanes G and R, respectively).

Discussion

The wild-type SNR6 gene functions as an organizer of local chromatin structure

In the wild-type situation, the transcriptionally active U6 RNA gene is flanked on both its 5' and 3' regions by arrays of positioned nucleosomes. This structure is lost on the transcriptional inactivation of the gene by a 2-bp deletion in the distant B block, which prevents TFIIC binding. This result represents the first evidence that a transcriptionally active Pol III gene can exert a boundary effect and organize the chromatin structure of its flanking regions. The effect is original in that it is linked to the integrity of *SNR6* B block. The yeast $\alpha 2$ repressor is

a protein that positions nucleosomes by factor-mediated contacts with histones (Roth et al. 1990; Shimizu et al. 1991; Ganter et al. 1993; Cooper et al. 1994). By analogy to the $\alpha 2$ repressor, it is proposed that TFIIC might organize the nucleosomal structure of *SNR6* 5' region by recruiting the components that induce a strong protection around the TATA box (likely TFIIB, see below) and that could spread nucleosome positioning in the upstream region. Similarly, (transient) binding of TFIIC to the B block could establish nucleosome positioning on the 3' side. Alternatively, we cannot exclude that the transcription process itself could affect nucleosome positioning in *SNR6*-flanking regions. Whatever the mechanisms involved in this process, and the specific interactions between the components of the Pol III machinery and the adjacent nucleosomes, the *SNR6* boundary effect was not observed when the gene was inserted in the centromeric vector pRS314: The bacterial sequences of the plasmid were not organized into sets of positioned nucleosomes, which suggests the presence of either sequence-specific effects or episomal constraints different from the chromosomal ones and incompatible with *SNR6*-induced chromatin organization.

In yeast, tRNA genes that are transcribed by Pol III are frequently found near the upstream control regions of genes transcribed by Pol II or downstream of Ty retrotransposons or free solo δ regions. It was shown recently that a tRNA gene may inhibit transcription from adjacent Pol II promoters (Hull et al. 1994). This inhibition required active transcription of the tRNA gene but was independent of its orientation. It was considered that repression might occur by competitive interactions of transcription factors, by steric interference, or by chro-

Figure 6. *SNR6* mutant RNA levels in yeast strains with wild-type or amino-terminally mutated histones H4-2 (A) or H3-2 (B). Mutant *SNR6* genes, harbored on multicopy plasmids, were introduced into wild-type and variant yeast strains, and the accumulation of the corresponding transcripts (U6 maxi-RNA) was monitored by Northern blot. The mutant *SNR6* genes (TATAdown, Binv, BA2) are described in Fig. 5B. The variant yeast strains mutated in the histone H4-2 or H3-2 genes are indicated as follows: (A) Wild-type (WT) yeast strain PKY501 with wild-type histone H4-2; (Δ), yeast strain PKY813 with histone H4-2 deleted from residue 4 to 28; (Q) yeast strain LDY107 with the first four acetylation sites (positions 5, 8, 12, and 16) substituted for by glutamine (Durrin et al. 1991). (B) Wild-type (WT) yeast strain RMY200 with wild-type histone H3-2; (G) yeast strain RMY247 with the first three acetylation sites of H3-2 (positions 9, 14, and 18) changed in glycine; (R) yeast strain RMY253 with the first four acetylation sites of histone H3-2 (positions 9, 14, 18, and 23) substituted for by arginine; (Δ) yeast strain RMY430 with histone H3-2 deleted from residue 4 to 30 (Mann and Grunstein 1992). (BA2) The autoradiography was three times longer.

matin structure (Hull et al. 1994). The *SNR6* locus represents a similar situation with a solo δ element immediately upstream of *SNR6*. In this case, it is shown clearly that the wild-type, active *SNR6* gene organizes the nucleosome arrangement in the solo δ region. Although the UAS of solo δ overlapped the ends of nucleosomes -1 and -2 , the TATA box of solo δ was located within nucleosome -2 (Fig. 3). In the $\Delta 2$ mutant, nucleosome positioning was lost but the solo δ TATA box was occupied by something different from a nucleosome. These results support chromatin repression as a mechanism by which tRNA genes could affect flanking Pol II promoters. It must be noted that the effect on chromatin structure was observed on both sides of *SNR6*, which is consistent with the observation that the repression was independent of the orientation of the tRNA genes (Hull et al. 1994). RNA transcripts representing initiation and termination within a solo δ element are normally not found in yeast cells. However, it would be interesting to test whether transcription from the solo δ element is indirectly stimulated by the $\Delta 2$ mutation in the *SNR6* gene.

Analysis of protein/DNA contacts provides insights into the mechanism of SNR6 transcription

In vitro, on naked *SNR6* DNA, the binding of TFIIC to the B block leads via DNA looping to a weak, secondary interaction between TFIIC and the A block region, observed by electron microscopy (Burnol et al. 1993b). Once bound, TFIIC stimulates the formation of TFIIB-*SNR6* complexes but the TATA box provides sufficient information for proper, TFIIC-independent binding of TFIIB (Joazeiro et al. 1994).

Remarkably, MNase digestions of chromatin revealed a protection of the TATA box, but not of the A and B blocks, on the wild-type *SNR6* gene. This result was confirmed by DNase I digestions (not shown). The protection of the TATA box and of the 5' region was strictly linked to the transcriptional activity of the *SNR6* gene. Furthermore, the extent of this protection (from about -46 to 7 , MNase, Fig. 3; -41 to -3 , DNase I, not shown) was comparable to TFIIB footprinting on naked *SNR6* DNA in vitro (from -40 to -10 ; Joazeiro et al. 1994), which strongly suggests that it is attributable to the binding of TFIIB. The increased nuclease sensitivity of the TATA box in the mutant *SNR6* gene might then reflect a loss of TFIIB or at least altered binding properties of the factor. In contrast, no substantial differences were recorded in the region of the A or B blocks between the wild-type and mutant *SNR6* loci, and we found no evidence for the continuous presence of TFIIC on the active U6 RNA gene. This suggests that TFIIC does not stay permanently bound to the U6 RNA gene. These results can be compared with in vivo footprinting experiments performed with tRNA or 5S rRNA genes. Except for the potentially active somatic 5S RNA genes of the transcriptionally quiescent *Xenopus* erythrocytes, where a strong protection of the internal control region (ICR), presumably by TFIIA, was observed (Chipev and Wolffe 1992), no evidence was found for the permanent binding

of the assembly factors TFIIA or TFIIC on active 5S RNA or tRNA genes (Huibregtse and Engelke 1989; Engelke and Gottesfeld 1990). In contrast, the proximal upstream region of the same genes was clearly protected, indicating the presence of TFIIB (Huibregtse and Engelke 1989; Chipev and Wolffe 1992). These observations support the hypothesis that TFIIA and TFIIC might be only temporarily bound to their promoter sequences, so as to recruit TFIIB, and might dissociate hereafter leaving TFIIB alone to direct the initiation of transcription by Pol III, as suggested by in vitro experiments (Kassavetis et al. 1990).

Another aspect of *SNR6* transcription that we wanted to investigate was the potential presence of a nucleosome between the A and the B blocks. The unusual location of *SNR6* B block 202 bp away from the A block [whereas the optimal length of A-B spacing in terms of transcriptional efficiency for tRNA and VA RNA genes was found to be 30–60 bp (Cannon et al. 1986; Baker et al. 1987; Fabrizio et al. 1987)] had led several investigators (Burnol et al. 1993b; Eschenlauer et al. 1993) to propose the existence of a DNA loop stabilized or structurally organized by a nucleosome positioned between the A and B blocks (static loop model). Our data do not allow us to unambiguously conclude about the presence of a nucleosome between the A and B blocks, which cannot be completely ruled out. However, chromatin does not appear to have a positive role in U6 RNA transcription because nucleosome loss via histone H4 depletion did not reduce the transcriptional activity of the wild-type *SNR6* gene (Fig. 5A).

Chromatin-mediated repression of SNR6 transcription

Consistent with the demonstration by Burnol et al. (1993a) of a repressive effect of nucleosome assembly on the transcription of the *SNR6* gene in vitro, we found that nucleosome disruption induced an increase in the transcript levels of plasmid-born U6 RNA genes mutated either at the TATA box or at the A or B blocks. However, the wild-type gene, with intact promoter elements, remained apparently insensitive to chromatin changes. Although we cannot rule out indirect effects of nucleosome loss on transcription of mutant U6 RNA genes, the consistency of these results with the in vitro data leads us to assume that they reflect a competition between nucleosomal organization and the binding of transcription factors on the *SNR6* gene. This competition is largely in favor of the transcription factors and becomes apparent only when the efficiency of the transcription machinery is weakened through mutations of promoter elements. It is not clear how the repressive effect of the nucleosomes evidenced by these experiments works. The analysis of the chromatin structure of the mutated *SNR6* gene, either on the pRS314 plasmid or in the genome, demonstrated that the inactivation of the gene did not lead to the incorporation of its promoter or transcribed sequences into positioned nucleosomes. The repressive effect of chromatin on *SNR6* transcription thus could occur through variably or randomly placed nucleosomes. This also seems to be the case for the *Xenopus* oocyte-

specific 5S rRNA in kidney-derived cell lines (Engelke and Gottesfeld 1990), whereas in erythrocyte nuclei the majority of the oocyte 5S DNA repeats appears to be assembled into weakly positioned nucleosomal arrays (Chipev and Wolffe 1992).

The effect of nucleosome depletion on the transcriptional efficiency of the various *SNR6* constructs allows us to evaluate the main steps of the transcriptional complex assembly that are competed for by nucleosome formation in this plasmidic context. For both the Binv and the (Bdel + Aup) constructs, the only parameter that can be improved following nucleosome disruption is the binding of TFIIC to the A block. Indeed (Bdel + Aup) lacks the B block and the B block of Binv is merely inverted without alteration of sequence; besides, both constructs have wild-type 5' sequences. Accordingly, the increase in Binv and (Bdel + Aup) transcript level upon nucleosome loss reflects the competition between TFIIC and the nucleosomes at the level of the A block. Similarly, the increase of TATA_{down} transcripts reflects the competition between nucleosomes and TFIIC-dependent binding of TFIIB at the 5' end of the gene.

The A and B block-less genes (BΔ2, Bdel, and A_{down}) stand apart in that, whatever the chromatin structure, they probably cannot be bound productively by TFIIC on the A block so as to recruit TFIIB. Therefore, we believe that the low amount of transcripts generated by the BΔ2, Bdel, and A_{down} constructs upon nucleosome loss is due to TFIIC-independent binding of TFIIB, similar to the TFIIC-independent positioning of TFIIB on naked DNA in vitro (Burnol et al. 1993b, Joazeiro et al. 1994). These mutant *SNR6* genes can be compared with Grunstein and co-workers' constructs containing the basal promoters of *CYC1* and *GAL1* fused to the *lacZ* reporter gene (Durrin et al. 1992). Both these Pol II constructs and the mutant A or B block-less *SNR6* genes are devoid of enhancer or enhancer-like sequences and have their levels of transcription strongly increased by nucleosome disruption. However, in both cases, the transcription levels induced by chromatin disruption remain 25- to 50-fold lower than the levels of transcription induced by the corresponding full promoters. Accordingly, TFIIB recruitment via protein-protein interactions, rather than chromatin anti-repression, would seem to be the predominant function of TFIIC regarding the *SNR6* gene in vivo. In vitro data, however, suggested that TFIIC could function essentially as an antirepressor with an accessory role of enhancing the recruitment of TFIIB (Moenne et al. 1990; Burnol et al. 1993a; Joazeiro et al. 1994). These observations can be conciliated if one considers that the nucleosome loss induced by histone H4 depletion leads only to a partial, probably nonhomogeneous, disruption of chromatin, which remains a template very different from the naked DNA used for the in vitro experiments.

Having established the antirepressor function of TFIIC for the *SNR6* gene in vivo, we searched for mutations in the histone H3 or H4 genes that could affect, positively or negatively, the interactions between the Pol III transcription machinery and the nucleosomes.

Durrin et al. (1991) demonstrated that mutations in the amino-terminal domain of yeast histone H4 reduced considerably the activation of several but not all Pol II genes (*GAL1*, -7, -10 but not *GAL4* or *PRC1*). However, none of the mutations tested affected the transcript levels of wild-type or mutant *SNR6* genes. In contrast, we found that two mutations modifying the acetylation sites of histone H3 significantly enhanced the transcriptional activity of *SNR6* genes mutated in their B blocks. The same mutations also allowed hyperactivation of the *GAL1* gene (but not of the *PHO5* or *PRC1* genes, Mann and Grunstein 1992). It is not clear why these mutations have that effect on the *SNR6* gene, which was not exhibited by the deletion of the residues 4–30. These results can be interpreted in two ways: Either the two mutations slightly modify the structure of the nucleosomes, allowing them to be more easily, passively displaced from the *SNR6* initiation site, or these mutations somehow specifically enhance the interactions between the chromatin and the Pol III transcription machinery, leading to a more efficient displacement of the nucleosomes from the transcriptional start site. The precise interactions between histones and transcription complexes remain to be determined.

Material and methods

Plasmids

All plasmids contain the region of the *SNR6* gene spanning base pairs –140 to +314 relative to *SNR6* transcription start site, except for the Bdel and (Bdel + Aup) constructs, which harbor a truncated fragment from –120 to +122, lacking the B block (Burnol et al. 1993b). These fragments were mutated as described (Fig. 5B; Burnol et al. 1993b). To study the transcriptional activity of wild-type, and mutant *SNR6* genes in yeast strains mutated on their histone H3 or H4 genes, a 59-bp *Xba*I–*Hinc*II DNA fragment from the Bluescript SK vector (BSSK, Stratagene) was inserted into the transcribed region, at the *Eco*NI site (position +73). This insertion maps at the beginning of the third loop of the predicted RNA secondary structure (Fortner et al. 1994). Once generated in BSSK plasmids, the constructs were transferred to multicopy vectors, YEp352 (Hill et al. 1986) and pRS424 (Christianson et al. 1992).

For chromatin studies of wild-type and mutant *SNR6* genes on plasmids, a DNA fragment encompassing the *SNR6* gene (–140; +314) with either a wild-type or a mutated B block (Δ2) was introduced into the centromeric plasmid pRS314 (Sikorski and Hieter 1989) to give pRS314-U6 and pRS314-Δ2 respectively.

To create the 2-bp deletion in the B block of the *SNR6* chromosomal locus, the *SNR6* DNA fragment (–140; +314) containing the BΔ2 mutation (Fig. 5B) was cloned into the integrative vector pRS306 (Sikorski and Hieter 1989) to give pRS306-Δ2. All mutations were controlled by DNA sequencing.

Strains

The *Saccharomyces cerevisiae* strains used for these studies were derived from YM262 (GCY5) or YPH500α (FTY113, FTY114, FTY115). FTY113 and FTY114 are transformants of YPH500α, containing the pRS314-U6 and pRS314-Δ2 plasmids respectively. FTY115 corresponds to the strain YPH500α with the BΔ2 mutation at its *SNR6* chromosomal locus, which is rescued by the centromeric plasmid pRS314-U6. This strain was

constructed as follows: YPH500 α was first transformed with the integrative plasmid pRS306- Δ 2, and a clone with pRS306- Δ 2 integrated at the *SNR6* locus was selected following *EcoRI*-*HpaI* analysis of genomic DNA (the Δ 2 mutation creates a *HpaI* site). This clone was transformed with the pRS314-U6 plasmid, and recombination events at the *SNR6* locus were then selected by plating of the cells on 5-fluoro-orotic acid (5-FOA) plates. The genomic DNA of the 5-FOA-resistant clones was analyzed by *EcoRI*-*HpaI* digest, and FTY115 was a clone that had conserved the mutated gene at the *SNR6* locus. This restriction analysis was finally confirmed by direct genomic sequencing (Fig. 2A).

UKY403, MHY308, PKY501, PKY813, PKY817, LDY107, RMY200, RMY247, RMY253, RMY410, and RMY430 have been described by Kim et al. (1988), Han and Grunstein (1988), Durrin et al. (1991), and Mann and Grunstein (1992).

Chromatin analysis by MNase

Preparation of genomic chromatin, digestion with MNase and mapping of nucleosome positions were done as described (Thoma et al. 1984; Bernardi et al. 1991). The DNA was cut to completion with a restriction endonuclease, fractionated on 1% agarose gels, blotted to nitrocellulose, and hybridized to radioactively labeled probes as indicated in the legend to Figure 1. Radioactive probes were made by random priming using a labeling kit (Pharmacia). A DNA ladder consisting of multiples of 256 bp was used for calibration (Thoma et al. 1984).

Mapping chromatin structure at nucleotide resolution MNase cleavage sites were analyzed by primer extension with *Taq* DNA polymerase (Shimizu et al. 1991; Roth et al. 1992). The digested DNA was purified on ELUTIP-d columns (Schleicher & Schuell, D-3354 Dassel, Germany). The primer used for mapping the top strand was primer 716 5'-CGTACCATTGCAT-AGCTGTAACAATATTC-3' (from base pairs 363 to 335). The primer used for bottom strand mapping was primer 717 5'-AATTTATATTGCTACCATGACTGTCTGAG-3' (from base pairs -449 to -421). The primers were labeled at the 5' end with polynucleotide kinase and [γ - 32 P]ATP (Maniatis et al. 1982). Ten microliters ($\sim$ 1 μ g) of the DNA was mixed with 9 μ l of 0.3 pmole 5' end-labeled primer (5×10^5 to 1×10^6 cpm), 5 μ l of 5 $\times$ *Taq* buffer (50 mM Tris-HCl pH 8.3, 250 mM KCl, 15 mM MgCl₂). After the samples were heated at 95°C for 5 min and chilled in ice water, 1.2 μ l of nucleotide mixture (5 mM each dGTP, dATP, dTTP, and dCTP) and 1 unit of *Taq* polymerase (Cetus, Perkin Elmer; in 5 μ l 1 $\times$ *Taq* buffer) were added. The sample was overlaid by $\sim$ 25 μ l paraffin oil. Thirty cycles were run at 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min. The water phase was transferred to a new tube, and the DNA was precipitated with one-tenth volume of 3 M sodium acetate and three volumes of ethanol. DNA samples were electrophoresed at 1800 V for 2 hr (short run) or 3.5 hr (long run) on 4% polyacrylamide gels (31 cm $\times$ 38.5 cm, width by height, BRL model S2) containing 50% urea and 1 $\times$ TBE (Maniatis et al. 1982). Sequencing of minichromosomes for comparison was done by the chain termination method with *Taq* polymerase.

RNA analysis

The multicopy plasmids YEp352 or pRS424, harboring the various *SNR6* constructs, were introduced into yeast strains mutated on their histone H3 or H4 genes. Yeast transformation procedures, RNA extraction, and Northern blot analysis were performed as described (Burnol et al. 1993b), by use of a body-labeled DNA fragment encompassing the U6 RNA-coding sequence (-120 to +122). For the glucose shift experiments with the UKY403 and MHY308 strains, transformant cells contain-

ing various plasmids with mutant *SNR6* genes were first grown overnight in a selective, galactose-containing medium until they reached an OD₆₆₀ of 0.3. An aliquot was then taken and the remaining cells were shifted to a glucose-based medium and allowed to grow for another 6 hr before being harvested.

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