Continuous and widespread roles for the Swi–Snf complex in transcription

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Chromatin presents a significant obstacle to transcription, but two means of overcoming its repressive effects, histone acetylation and the activities of the Swi–Snf complex, have been proposed. Histone acetylation and Swi–Snf activity have been shown to be crucial for transcriptional induction and to facilitate binding of transcription factors to DNA. By regulating the activity of the Swi–Snf complex *in vivo*, we found that active transcription requires continuous Swi–Snf function, demonstrating a role for this complex beyond the induction of transcription. Despite the presumably generalized packaging of genes into chromatin, previous studies have indicated that the transcriptional requirements for the histone acetyltransferase, Gcn5, and the Swi–Snf complex are limited to a handful of genes. However, inactivating Swi–Snf function in cells also lacking *GCN5* revealed defects in transcription of several genes previously thought to be *SWI–SNF*- and *GCN5*-independent. These findings suggest that chromatin remodeling plays a widespread role in gene expression and that these two chromatin remodeling activities perform independent and overlapping functions during transcriptional activation.

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Introduction

The precise mechanisms by which chromatin represses transcription remain unclear. Attention has focused primarily on the ability of histones to inhibit transcriptional initiation since the formation of nucleosomes on DNA templates blocks initiation by RNA polymerase II and reduces the ability of the transcriptional activators to bind DNA (Lorch *et al.*, 1987; Matsui, 1987; Workman and Roeder, 1987; Knezetic *et al.*, 1988; Pina *et al.*, 1990; Taylor *et al.*, 1991; Workman *et al.*, 1991). Allowing RNA polymerase and activators to bind DNA prior to nucleosome assembly permits transcription in the presence of histones, suggesting that chromatin restricts access to binding sites in DNA. This model of nucleosome repression seems to hold for promoters *in vivo*, at which the binding of transcriptional factors to critical promoter elements appears to be blocked by nucleosomes (Almer *et al.*, 1986; Axelrod *et al.*, 1993; Wolffe, 1994).

Activities that disturb nucleosome structure or position to permit transcription factor binding would be expected

to facilitate transcriptional activation from promoters repressed by chromatin. Consistent with this hypothesis, transcriptional activation from several promoters is accompanied both by nucleosome disruption and by binding of transcription factors to previously inaccessible sites in the promoters (Almer *et al.*, 1986; Axelrod *et al.*, 1993; Wolffe, 1994). The identification of transcription factors whose activities appear to be dedicated to disrupting chromatin during transcriptional activation has confirmed the notion that nucleosome disruption facilitates transcription. Two major types of these chromatin remodeling activities have been identified: multisubunit ATPases such as the Swi–Snf complex and histone acetyltransferases such as Gcn5.

The yeast *SWI* and *SNF* genes originally were identified in yeast screens for genes required for expression of *HO* (Stern *et al.*, 1984) and *SUC2* (Neugeborn and Carlson, 1984). A subset of *SWI–SNF* genes encode subunits of a complex believed to remodel chromatin during transcriptional activation. *swi–snf* mutations impair transcription and alter chromatin structures at promoters *in vivo* and, consistent with a role for *SWI–SNF* in chromatin remodeling, mutations in histone genes suppress these defects (Sternberg *et al.*, 1987; Kruger and Herskowitz, 1991; Hirschhorn *et al.*, 1992; Kruger *et al.*, 1995). In addition, the purified Swi–Snf complex alters nucleosome structure *in vitro* (Cote *et al.*, 1994, 1998).

The mechanisms by which chromatin remodeling by Swi–Snf stimulates transcription remain unresolved. Several *in vitro* studies suggest that the Swi–Snf complex functions to augment DNA binding by transcription factors (Cote *et al.*, 1994; Owen-Hughes *et al.*, 1996), and the enhanced affinity of transcription factor binding persists even after Swi–Snf has been inactivated (Cote *et al.*, 1998; Schnitzler *et al.*, 1998). These results suggest a sequence of events in which Swi–Snf acts to remodel chromatin, and transcriptional activators subsequently bind their enhancer sites. In support of this model, augmenting transcription factor binding by increasing the number of activator-binding sites or the affinities of these sites relieves the *SWI–SNF* dependence of promoters *in vivo* (Laurent and Carlson, 1992; Burns and Peterson, 1997). Contrary to this model, however, the Swi–Snf complex appears to act after activator binding at some Swi–Snf-dependent promoters since transcription factor binding at these promoters is impaired minimally in *swi–snf* mutants (Burns and Peterson, 1997; Ryan *et al.*, 1998). Thus, the physiological function of chromatin remodeling has not been determined conclusively.

Acetylation of the N-termini of histones represents another means of altering chromatin structure during transcriptional activation. Several studies have established a correlation between histone acetylation and transcription (Turner, 1991), and multiple transcription factors have

been found to possess histone acetyltransferase activity (Kuo *et al.*, 1996; Mizzen *et al.*, 1996; Candau *et al.*, 1997; Pennisi, 1997).

The cloning of a histone acetyltransferase from *Tetrahymena* revealed that this protein had significant homology to the transcription factor Gcn5 (Brownell *et al.*, 1996). Gcn5 functions together with Ada2 and Ada3 to mediate activation by certain transcriptional activators (Berger *et al.*, 1992; Marcus *et al.*, 1994). Further experimentation confirmed that Gcn5 possesses histone acetyltransferase activity and that this activity is essential for the function of Gcn5 as a transcriptional co-activator (Kuo *et al.*, 1996; Candau *et al.*, 1997). Furthermore, multisubunit complexes containing Gcn5 (referred to here as Gcn5–Ada complexes) are capable of acetylating nucleosomal histones (Grant *et al.*, 1997).

The Swi–Snf and Gcn5–Ada complexes both appear to facilitate transcription by remodeling nucleosomes. Similar to Swi–Snf activity, histone acetylation facilitates binding of transcription factors to sites within nucleosomes (Lee *et al.*, 1993; Juan *et al.*, 1994; Vettese-Dadey *et al.*, 1996), and Swi–Snf and Gcn5–Ada share at least some common target genes (Pollard and Peterson, 1997). However, the functional relationship between Swi–Snf and Gcn5–Ada remains unknown. Understanding whether the two complexes act in the same pathway to activate transcription or function independently is critical to elucidating the mechanisms of chromatin remodeling. We used genetic approaches to explore the interactions between Swi–Snf and Gcn5–Ada, and also to dissect the physiological functions of the Swi–Snf complex *in vivo*. Interestingly, Swi–Snf function was required continuously for transcription of several genes. Furthermore, inactivating Swi–Snf in *gcn5–ada* mutants revealed that the two chromatin remodeling activities act in independent and redundant pathways to activate transcription.

Results

Conditional alleles of *SWI2* regulate Swi–Snf function *in vivo*

To explore the physiological functions of chromatin remodeling complexes in more detail, we generated temperature-sensitive alleles of *SWI2*. *SWI2* encodes a component of the yeast Swi–Snf complex that is required for Swi–Snf function (Laurent *et al.*, 1993). Conditional *Swi2* function would therefore allow the temporal coordination of Swi–Snf inactivation with different stages of transcriptional activation. This means of regulating *Swi2* activity would also permit the growth of strains requiring *SWI2* for viability, and thereby afford the opportunity to make precise assessments of the roles of the Swi–Snf complex in transcription in these strains.

Swi2 contains a consensus ATPase domain, and mutations that reduce *Swi2* ATPase activity debilitate transcription of *SWI–SNF*-dependent genes *in vivo* and abolish chromatin remodeling by a purified mutant complex *in vitro* (Laurent *et al.*, 1993; Cote *et al.*, 1994). Thus, *Swi2* ATPase activity is crucial for Swi–Snf function. Temperature-sensitive alleles of *SWI2* were generated using PCR to mutagenize the consensus ATPase domain of *Swi2* within the context of the otherwise wild-type

gene. Mutants were isolated on the basis of temperature-sensitive growth on glycerol media.

Further analysis of one temperature-sensitive allele, *swi2-6.3*, suggested that temperature sensitivity was limited to the ATPase domain. At the restrictive temperature, the growth phenotype of *swi2-6.3* mimics that of *swi2K798A*, a mutant allele of *SWI2* specifically lacking ATPase activity, instead of behaving like a *SWI2* deletion (Figure 1A). Consistent with these results, cells carrying either *swi2-6.3* or wild-type *SWI2* contained similar *Swi2* protein levels at the permissive and restrictive temperatures (Figure 1B).

The distinct growth phenotypes associated with the *swi2Δ* allele and the *swi2-6.3* and *swi2K798A* alleles demonstrated that the latter mutations did not eliminate *SWI2* function completely. However, *SWI2* deletions and mutants lacking ATPase activity display similar transcriptional defects, suggesting that *Swi2* ATPase activity is crucial for transcriptional activation by Swi–Snf (Laurent *et al.*, 1993). To confirm that *swi2-6.3* conferred temperature-sensitive transcriptional defects, we measured expression from the *HO* and *SUC2* promoters, for which transcription depends on the Swi–Snf complex (Neigeborn and Carlson, 1984; Stern *et al.*, 1984). Secreted invertase activity in yeast corresponds to the rate of *SUC2* transcription (Carlson and Botstein, 1982) and, therefore, *SUC2* expression was assessed by measuring invertase activity in cells carrying different alleles of *SWI2*. *HO* promoter activity was analyzed by measuring β-galactosidase activity in cells carrying different *SWI2* alleles and an integrated *HO–LacZ* fusion that joins the *HO* promoter with the β-galactosidase open reading frame. At the permissive temperature, *swi2-6.3* drove *HO* and *SUC2* expression at 41% and 56%, respectively, of wild-type levels. At the restrictive temperature, however, *swi2-6.3* cells expressed *HO* and *SUC2* at levels similar to those seen in cells carrying *swi2K798A* (Figure 1C). Expression from a third *SWI–SNF*-dependent promoter, a variant of the *GAL1* promoter (see below for details on this promoter), was also temperature sensitive in *swi2-6.3* strains (see Figure 5B). Thus *swi2-6.3* provided a means of using temperature to control the transcriptional activity of *Swi2*, and thereby the Swi–Snf complex, *in vivo*. In these experiments, *HO* expression demonstrated significant temperature sensitivity in wild-type *SWI2* cells. The minimal effects of temperature on *SUC2* expression in a wild-type *SWI2* background suggested that the effects of temperature on *HO* expression reflected alterations in activities other than the Swi–Snf complex at the *HO* promoter.

Continuous role for *Swi2* in transcription

Many, if not all, *SWI–SNF*-dependent promoters identified to date (including *HO*, *SUC2* and *INO1*) share the property of rapid transcriptional induction in response to specific signals. This observation suggests that the Swi–Snf complex may be dedicated to initiating gene expression. Consistent with this possibility, transient Swi–Snf activity imparts long-term changes to chromatin structure *in vitro* (Owen-Hughes *et al.*, 1996; Logie and Peterson, 1997; Cote *et al.*, 1998; Schnitzler *et al.*, 1998). Swi–Snf establishes structural alterations in nucleosomes that persist for up to 4 h following Swi–Snf inactivation. To determine whether the Swi–Snf complex plays a role in

Fig. 1. (A) *swi2K798A* and *swi2-6.3* both supported wild-type growth rates. CY120 carrying the indicated alleles of *SWI2* was plated on minimal glucose media at 30 or 37°C for 3 days. (B) Swi2 protein levels were not altered by temperature in *SWI2* and *swi2-6.3* strains. CY120 carrying *SWI2* or *swi2-6.3* was grown at 26.5°C and then either shifted to 37°C or maintained at 26.5°C for 2 h. Whole-cell extracts were then prepared and Swi2p levels were determined by Western blotting. (C) Temperature-sensitive transcriptional activity of *swi2-6.3*. CY120 carrying *SWI2* (wt), *swi2-6.3* (ts) or *swi2K798A* (K798A) was grown at 24 or 37°C and then assayed for *HO-LacZ* or *SUC2* expression at the same temperatures by measuring β -galactosidase or invertase activity, respectively. Fold activation relative to *swi2K798A* cells is plotted, and standard error is shown.

maintaining transcription beyond initiation *in vivo*, we examined the effects of inactivating Swi2 after transcription had been activated.

We initially focused on *SUC2* transcription. In wild-type cells, glucose represses *SUC2* expression (Carlson and Botstein, 1982). Growth in media lacking glucose induces *SUC2* expression, but the extremely long half-life (>180 min) of the *SUC2* mRNA under these conditions precludes straightforward measurements of the rate of *SUC2* transcription. However, the *SUC2* message has a very short half-life in glucose media (≤ 10 min; Cereghino and Scheffler, 1996) and, therefore, measuring message levels in cells grown in glucose would provide accurate assessments of on-going rates of transcription. Glucose represses *SUC2* transcription through the two repressors, Mig1 and Mig2 (Lutfiyya and Johnston, 1996). Therefore, to permit *SUC2* expression in glucose media, our experiments were conducted using yeast carrying *MIG1* and *MIG2* deletions. In cells lacking *MIG1* and *MIG2*, expression of *SUC2* in glucose media depended on Swi2 activity (Figure 2A). To determine the role of Swi2 in on-going transcription, *SUC2* expression was measured following a shift to the restrictive temperature in *mig1Δ mig2Δ* cells carrying either wild-type *SWI2* or *swi2-6.3*. In *SWI2* cells, a shift to 37°C had little effect on *SUC2* expression (Figure 2A). However, when *swi2-6.3* cells were shifted to 37°C, *SUC2* message was reduced rapidly to the level seen in *swi2K798A* cells. These data demonstrate that an activated promoter still requires Swi-Snf activity for its expression. *SUC2* mRNA levels dropped 8-fold after 1 h at 37°C. Given the half-life of the *SUC2* message (~10 min) and the fact that a period of 15–20 min was required for the temperature of the media to reach 37°C, these results

establish *SUC2* as a direct transcriptional target of Swi-Snf activity.

To extend this analysis, we examined the effects of inactivating Swi2 on transcription from a variant of the *GAL1* promoter. Unlike the *SUC2* promoter, for which no classical transcriptional activators have been identified, expression from the *GAL1* promoter depends on the prototypical DNA-binding transcriptional activator, Gal4. Interestingly, although expression from the full-length *GAL1* promoter, which has four Gal4-binding sites, proceeds independently of Swi-Snf function, transcription from a truncated version of the promoter, which contains only two Gal4-binding sites, displays pronounced transcriptional defects in *swi-snf* mutants (Burns and Peterson, 1997; Gaudreau *et al.*, 1997). The truncated *GAL1* promoter is referred to here as GALp*. To assess the effects of inactivating Swi2 on on-going transcription from GALp*, the ORF and 3'-untranslated region (3'-UTR) of *STE3* were fused downstream of this promoter to generate a GALp*-*STE3* fusion. Since the half-life of the *STE3* mRNA is short (≤ 4 min; Herrick *et al.*, 1990), measuring *STE3* message levels permitted accurate assessments of on-going rates of transcription from GALp*. The GALp*-*STE3* transcript exceeded the length of the endogenous *STE3* message by ~150 bases but failed to produce full-length Ste3 protein.

Consistent with the physiological regulation of the *GAL1* promoter, expression of the GALp*-*STE3* gene remained repressed in glucose media and was strongly induced in galactose (Figure 2B). Furthermore, GALp*-*STE3* expression required Swi2 function (Figure 2B). In wild-type *SWI2* cells grown in galactose, a shift to 37°C reduced GALp*-*STE3* mRNA levels (Figure 2B).

Fig. 2. (A) Swi2 activity was required continuously to maintain ongoing transcription of *SUC2*. Top: *SUC2* expression was compared in *mig1Δ mig2Δ* strains (YSB16) carrying *SWI2* or *swi2K798A* and grown in glucose media at 30°C. Total RNA was isolated and *SUC2* mRNA levels were determined by RNase protection. Bottom: *mig1Δ mig2Δ* strains carrying *SWI2* or *swi2-6.3* were grown in glucose media at 26.5°C and then shifted to 37°C. Total RNA was isolated at the indicated times following the shift to 37°C and *SUC2* mRNA levels were determined by RNase protection. Levels of the cytoplasmic invertase message (inv), whose expression does not depend on *SWI-SNF* (Neigeborn and Carlson, 1984), were measured as a control. (B) Swi2 activity was required continuously to maintain ongoing transcription of a *GALp*-STE3* fusion. Left: CY120 carrying an integrated *GALp*-STE3* reporter gene (YSB21) and either *SWI2* or *swi2K798A* was grown in glucose, and then shifted into galactose (gal) for 8 h or maintained in glucose (glu). Total RNA was isolated and *STE3* mRNA levels were determined by Northern blotting. The bands corresponding to the *GALp*-STE3* and the endogenous *STE3* messages are indicated. The *STE3*-specific probe also recognized a slow migrating band (marked by an asterisk) whose presence correlated with *GALp** inactivity. Right: YSB21 carrying either *SWI2* or *swi2-6.3* was grown in glucose at 26.5°C and then moved into galactose media at 26.5°C for 8 h. The cells were then shifted to 37°C. Total RNA was isolated at the indicated times following the shift to 37°C and *STE3* mRNA levels were measured by Northern blotting. The bands corresponding to the *GALp*-STE3* and endogenous *STE3* messages are indicated, as is the band corresponding to *GALp** inactivity (*). The graph shows densitometric quantification of the data from the right side of the figure. mRNA levels from cells grown under non-inducing conditions in the absence of galactose are also shown.

However, a reduction in the levels of the endogenous *STE3* message was also seen following a shift to 37°C. The similarity between the changes in the expression of endogenous *STE3* at 37°C in *SWI2* and *swi2-6.3* backgrounds suggests that the changes in *STE3* and *GALp*-STE3* mRNA levels in wild-type cells represented temperature effects that were independent of changes in Swi-Snf activity. In *swi2-6.3* cells grown in galactose, a shift to 37°C rapidly extinguished *GALp*-STE3* expression (Figure 2B). These results confirmed that the maintenance of on-going transcription from active promoters depends on continuous Swi2 activity. Furthermore, similar to *SUC2*, the rapid reduction in transcription from *GALp** suggests that this promoter is a direct target of Swi-Snf function.

Swi2 and the histone acetyltransferase, Gcn5, function in independent pathways to activate transcription

We were interested in exploring the functional relationship between the Swi-Snf complex and the Gcn5-Ada complex during transcription. Similar to the Swi-Snf complex, the Gcn5-Ada complex has been proposed to facilitate transcription by remodeling chromatin (Pollard and Peterson, 1997). The ability of Gcn5 to acetylate nucleosomes may be responsible for, or facilitate alterations in chromatin structure necessary for gene expression (Kuo *et al.*, 1996; Candau *et al.*, 1997; Grant *et al.*, 1997). Histones acetylated by Gcn5, for example, may become substrates for the Swi-Snf complex or vice versa, or

these two complexes may act independently to activate transcription. Mutations in *SWI-SNF* genes are synthetically lethal with mutations in *GCN5-ADA* genes (Pollard and Peterson, 1997; Roberts and Winston, 1997), suggesting that these two complexes may perform independent functions. However, given the profound growth defects in *swi-snfΔ* mutants and the mild growth defects in *gcn5-adaΔ* mutants (Figure 1A and data not shown), the inviability of *swi-snfΔ gcn5-adaΔ* double mutants may represent simply the combined growth defects of the individual mutants. Also, the inviability of cells lacking the functions of both complexes precludes critical analysis of the transcriptional defects in cells lacking both Swi-Snf and Gcn5-Ada. Thus the question of whether the Swi-Snf and Gcn5-Ada complexes serve in the same or distinct pathways during transcriptional activation remains unresolved. The conditional allele of *SWI2* provided a unique opportunity to analyze quantitatively the functional relationship between the Swi-Snf and Gcn5-Ada complexes.

swi2-6.3 gcn5Δ strains failed to grow at 37°C on glucose media, whereas *SWI2 gcn5Δ* strains grew under both permissive and restrictive conditions (Figure 3A). Neither *SWI2 GCN5* nor *swi2-6.3 GCN5* strains demonstrated temperature sensitivity on glucose (see Figure 1A). Since *swi2-6.3* supports wild-type growth rates on glucose at 37°C in wild-type *GCN5* strains, the inviability of *swi2-6.3 gcn5Δ* mutants does not represent merely the accumulation of growth defects, but instead suggests that the

Fig. 3. (A) Temperature-sensitive growth of *swi2-6.3 gcn5*Δ double mutants. *swi2-6.3 gcn5*Δ and *SWI2 gcn5*Δ mutants were grown at 26.5 or 37°C on rich glucose media. (B) Swi2 and Gcn5 act independently to activate *SUC2* expression. Cells with the indicated *GCN5* alleles and either wild-type *SWI2* (wt) or *swi2-6.3* (ts) were grown in 2% glucose at 26.5°C, shifted to 37°C for 1 h, and then transferred into 0.05% glucose (lo) media at 37°C for 1.5 h. Wild-type *SWI2 GCN5* cells were also maintained in 2% glucose (hi) throughout the experiment. Total RNA was isolated and *SUC2* mRNA levels were determined by RNase protection. Levels of the cytoplasmic invertase message (*inv*) were measured as a control. Relative *SUC2* mRNA levels are based on message levels from *SWI2 GCN5* cells maintained in 2% glucose.

activities of Swi–Snf and Gcn5–Ada complexes combine to perform some function(s) critical for cell growth.

To address the functional interactions between the Swi–Snf and Gcn5–Ada complexes during transcriptional activation, we compared the abilities of wild-type cells, and single and double mutants to activate *SUC2* transcription (Figure 3B). Relative to wild-type cells, *swi2-6.3* mutants expressed reduced levels of *SUC2* at the restrictive temperature, consistent with the role of *SWI2* in *SUC2* expression (see Figure 1C). *gcn5*Δ mutants also displayed mild transcriptional defects. *swi2-6.3 gcn5*Δ double mutants, however, expressed *SUC2* at lower levels than the *gcn5*Δ single mutant. Thus, inactivating Swi2 in a *gcn5*Δ background produced pronounced transcriptional defects, showing that Swi2 plays a role in transcription independent of *GCN5*. These data demonstrate that Swi–Snf and Gcn5–Ada act in distinct pathways to activate *SUC2* transcription. If these two complexes functioned exclusively in the same pathway, then the transcriptional defects in the *swi2-6.3 gcn5*Δ double mutants should have matched the defects seen in the *SWI2 gcn5*Δ single mutants. The observation that *swi2-6.3 GCN5* and *SWI2 gcn5*Δ mutants drive such different levels of *SUC2* transcription further supports the conclusion that the Swi–Snf and Gcn5–Ada complexes operate in distinct pathways. Inactivating Swi2 in both *GCN5* and *gcn5*Δ backgrounds produced apparently similar reductions in *SUC2* mRNA levels. However, in

both cases, message levels approximate mRNA levels under conditions that repress *SUC2* expression. Thus, for *SUC2* transcription, the nearly complete requirement for Swi2 activity in the presence of *GCN5* limited the extent of the effects of inactivating Swi2 in the absence of *GCN5*. We therefore explored the roles of Swi2 and Gcn5 in the expression of other genes.

Redundant roles for Swi2 and Gcn5 in *GAL1* expression

To explore further the functional interactions between the Swi–Snf and Gcn5–Ada complexes, we compared the abilities of wild-type cells, and single and double mutants to activate transcription of the endogenous *GAL1* gene. Expression from the full-length *GAL1* promoter proceeds at nearly wild-type levels in *swi–snf* mutants (Burns and Peterson, 1997; Gaudreau *et al.*, 1997), and transcriptional activation by Gal4, which drives *GAL1* expression, displays minimal defects in *gcn5–ada* mutants (Marcus *et al.*, 1994). However, the observations that expression from a truncated version of the *GAL1* promoter depends on Swi–Snf (Burns and Peterson, 1997; Gaudreau *et al.*, 1997), and that the Gal4 activation domain physically interacts with Ada2 (Melcher and Johnston, 1995), suggest that Gal4 may utilize both of these complexes to activate *GAL1* expression.

For our experiments, cells grown in glucose media at

Fig. 4. Loss of *GCN5* revealed a requirement for *Swi2* in *GAL1* expression. Cells with the indicated *SWI2* and *GCN5* alleles were grown in glucose at 26.5°C, shifted to 37°C for 1 h, and then either maintained in glucose (glu) or transferred into galactose (gal) for 6 h at 37°C. Total RNA was isolated, and *GAL1* mRNA and 25S rRNA levels were measured by Northern blotting. *GAL1* mRNA levels were quantitated after correcting for relative rRNA levels. Relative *GAL1* message levels were plotted on a logarithmic scale and standard error is shown. Similar results were seen following 2 and 4 h inductions in galactose.

the permissive temperature were shifted to 37°C for 1 h and then transferred into galactose media at 37°C to induce *GAL1* expression. Relative to wild-type cells, cells lacking either *Swi2* or *Gcn5* activity had mild defects in *GAL1* induction; 1.5- and 2.7-fold, respectively (Figure 4). Cells lacking both *Swi2* and *Gcn5* activity, however, showed a significantly greater transcriptional defect; 12.8-fold. Gal4 levels were similar in all strains under these conditions (data not shown). Thus *swi2-6.3 gcn5Δ* double mutants exhibited pronounced transcriptional defects relative to *SWI2 gcn5Δ* single mutants, confirming the conclusion that *Swi-Snf* and *Gcn5-Ada* act in distinct pathways to activate transcription.

Interestingly, the effects of inactivating *Swi2* depended on the *GCN5* background of the yeast. In *gcn5Δ* mutants, inactivating *Swi2* revealed significant defects in transcription of *GAL1* (4.7-fold). However, inactivating *Swi2* in a wild-type *GCN5* background produced only minimal defects in transcription (1.5-fold). These findings indicate that *Swi-Snf* and *Gcn5-Ada* ultimately perform redundant functions during transcription, because one complex is dispensable unless cells lack the other complex.

***Gal4* utilizes both *Swi-Snf* and *Gcn5-Ada* to activate transcription**

The defects in *GAL1* induction in *swi2-6.3 gcn5Δ* mutants strongly support the idea that *Gal4* utilizes both *Swi-Snf* and *Gcn5-Ada* to activate transcription. The differential *SWI-SNF* dependence of the full-length and truncated *GAL1* promoters can then be explained if, at full-length

promoters containing four binding sites, *Gal4* provides an excess of chromatin remodeling capabilities. At promoters containing only two binding sites (*GALp**), the ability of *Gal4* to recruit remodeling activities may become limiting and, therefore, inactivating either complex would cripple transcriptional activation from this promoter. Consistent with this proposal, inactivating either *Swi2* or *Gcn5* significantly reduced transcription from *GALp** (Figure 5A).

Glucose represses *GAL1* transcription in part through an upstream repressing sequence that binds transcriptional repressors whose activities require the presence of glucose in the media (Flick and Johnston, 1990). Transcription from the *GAL1* promoter further requires galactose to unveil the activation domain of *Gal4*. Since the induction of *GAL1* expression involves inactivation of transcriptional repressors as well as transcriptional activation by *Gal4*, the defects in *GAL1* induction seen in *swi2-6.3 gcn5Δ* mutants could have reflected defects in derepression or activation, or both. Several lines of evidence argue that the defects lie in activation. First, the *Gal4* activation domain requires *SWI2* to activate transcription from a promoter lacking the *GAL1* upstream repressing sequence (Laurent and Carlson, 1992). Secondly, synthesis of *Gal4*, which involves the same derepression phenomenon that occurs at the *GAL1* promoter, occurred normally in *swi2-6.3 gcn5Δ* mutants (data not shown). Thirdly, on-going transcriptional activation of *GALp** by *Gal4* required the continuous activity of *Swi2* (see Figure 2B). Lastly, *swi2-6.3* cells grown at the permissive temperature in either glucose (promoter-repressed and not activated) or raffinose (promoter-derepressed but not activated) had nearly the same defects in transcription from *GALp** after being shifted to the restrictive temperature for 1 h and then induced in galactose (promoter-derepressed and activated) (Figure 5B). These results demonstrated that derepression prior to *Swi2* inactivation failed to restore high levels of transcription.

Widespread role for *Swi2* and *Gcn5* in transcription

Given that many genes in the genome are presumably assembled into chromatin, and that deleting *GCN5* revealed a role for *SWI2* in *GAL1* transcription, we examined the role of *SWI2* in the expression of other genes in *gcn5Δ* yeast. Relative to *SWI2 gcn5Δ* cells, shifting *swi2-6.3 gcn5Δ* cells to 37°C reduced the expression of at least two additional genes, *ACT1* and *PGK1* (Figure 6). Minimal changes in transcription of these genes were seen when *swi2-6.3 GCN5* cells were compared with wild-type *SWI2 GCN5* cells at 37°C. Furthermore, growth at 26.5°C restored transcription in *swi2-6.3 gcn5Δ* mutants. Given the half-lives of the *PGK1* and *ACT1* messages [45 and 30 min at 37°C, respectively (Herrick *et al.*, 1990)], the reductions in expression of these genes were likely to have been direct effects of inactivating *Swi2* in a *gcn5Δ* background. These results also reinforce the conclusion that *Swi-Snf* plays a continuous role in transcription.

PGK1 and *ACT1* expression was also measured in cells carrying a temperature-sensitive allele of *SRB4*, a subunit of the RNA polymerase holoenzyme whose activity is essential for RNA polymerase II transcriptional activity *in vivo* (Thompson and Young, 1995) (Figure 6). Transcriptional defects in *srb4* mutants were 2- to 3-fold greater than the

Fig. 5. (A) Expression from a truncated *GAL1* promoter depended on both *SWI2* and *GCN5*. *SWI2* or *swi2K798A* cells carrying an integrated *GALp**-*LacZ* reporter (YSB22) were grown in glucose media, induced in galactose media for 8 h at 30°C and then assayed for β-galactosidase activity. *GCN5* or *gcn5Δ* cells carrying an integrated *GALp**-*LacZ* reporter (YSB23 and YSB24, respectively) were treated similarly. β-Galactosidase activities are shown as relative percentages of the levels in wild-type cells. Standard error was <1%. (B) Derepression of *GALp** failed to overcome its requirement for *Swi2* activity. *SWI2* or *swi2-6.3* cells carrying an integrated *GALp**-*LacZ* reporter (YSB22) were grown in glucose or raffinose media at 26.5°C, shifted to 37°C (left) or maintained at 26.5°C (right) for 1 h, and then transferred into galactose media at the same temperatures for 8 h. Cells were then assayed for β-galactosidase activity. Standard error is shown.

defects in *swi2-6.3 gcn5Δ* mutants. These results suggest that a low level of *PGK1* and *ACT1* expression proceeds in the absence of *Swi2* and *Gcn5* activities, possibly due to other chromatin remodeling activities.

Similar to *GAL1*, the effects of inactivating *Swi2* on *PGK1* and *ACT1* expression depended on the *GCN5* background of the yeast. In *gcn5Δ* mutants, inactivating *Swi2* revealed significant defects in transcription of *PGK1* and *ACT1* (4.3-fold for *PGK1* and 3.8-fold for *ACT1*). However, inactivating *Swi2* in a wild-type *GCN5* background produced only mild defects in transcription of the same genes (1.7-fold for *PGK1* and 1.0-fold for *ACT1*). These findings support the conclusion that *Swi-Snf* and *Gcn5-Ada* ultimately perform overlapping functions during transcription.

We noted that not all genes showed equivalent defects in the absence of *Swi-Snf* and *Gcn5-Ada* functions. Shifting *swi2-6.3 gcn5Δ* cells to 37°C did not significantly alter *TUP1*, *TCM1*, *TUB2* and uninduced *HIS3* expression relative to *SWI2 gcn5Δ* cells (Figure 6 and data not shown).

Transcriptional defects in *swi2-6.3 gcn5Δ* mutants are not due to loss of viability

One potential explanation for the observed transcriptional defects in *swi2-6.3 gcn5Δ* double mutants could have been

a rapid loss of viability of these cells after a shift to the restrictive temperature. However, comparison of the growth characteristics of *swi2-6.3 gcn5Δ* and *SWI2 gcn5Δ* cells after various times at 37°C showed that both cell types retained viability throughout the course of the experiments (Figure 7). *SWI2 gcn5Δ* cells continued to grow and divide at 37°C, as shown by the increasing OD₆₀₀ of the cultures and their ability to form increasing numbers of colonies after being plated at the permissive temperature. *swi2-6.3 gcn5Δ* cells also continued to grow at 37°C, as shown by the increasing OD₆₀₀ of the cultures but, instead of dividing, these cells grew in size relative to *SWI2 gcn5Δ* cells (data not shown). Consistent with this observation, *swi2-6.3 gcn5Δ* cells formed similar numbers of colonies at all times following a shift to 37°C (Figure 7). In addition, staining *swi2-6.3 gcn5Δ* and *SWI2 gcn5Δ* cells with methylene blue (Iida *et al.*, 1990) failed to reveal any significant loss of viability of either cell type at 37°C (data not shown).

Discussion

Continuous role for *Swi-Snf* in transcription

The *SWI-SNF* genes are crucial for initiating transcription of inducible genes such as *HO*, *SUC2* and *INO1* (Peterson

Fig. 6. Loss of *GCN5* revealed a requirement for *SWI2* in the expression of *PGK1* and *ACT1*. *SWI2 GCN5*, *swi2-6.3 GCN5*, *SWI2 gcn5Δ* and *swi2-6.3 gcn5Δ* strains grown in rich glucose media at 26.5°C were shifted to 37°C for 2.5 h. Similarly, *srb4-138* and *SRB4* cells grown in rich glucose media at 26.5°C were shifted to 37°C for 2.5 h. *SWI2 gcn5Δ* and *swi2-6.3 gcn5Δ* strains were also maintained at 26.5°C throughout the experiment. Total RNA was isolated, and *PGK1*, *ACT1* and *TCM1* mRNA and 25S rRNA levels were determined by Northern blotting. The relative mRNA levels of wild-type and temperature-sensitive strains within a given *GCN5* background are shown for *PGK1*, *ACT1* and *TCM1*. These values were corrected using relative 25S rRNA levels.

and Tamkun, 1995). Our studies further demonstrate that the Swi-Snf complex plays a role in maintaining active transcription. *In vitro* studies suggest that the Swi-Snf complex stably disrupts nucleosomes and provides transcription factors with opportunities to bind nucleosomal DNA (Cote *et al.*, 1998; Schnitzler *et al.*, 1998). However, inactivating Swi2 *in vivo* rapidly silenced on-going transcription from the *SUC2* promoter and a *SWI-SNF*-dependent variant of the *GAL1* promoter, GALp*. These results demonstrate that Swi-Snf function is not limited to the initiation of gene expression and suggest that transcription continuously requires chromatin remodeling. If the biochemical results demonstrating stable nucleosome disruption by Swi-Snf (Owen-Hughes *et al.*, 1996; Logie and Peterson, 1997; Cote *et al.*, 1998; Schnitzler *et al.*, 1998) reflect the physiological activity of this complex, then Swi-Snf must perform additional, transitory functions during transcriptional activation. Alternatively, yeast may possess additional activities capable of rapidly restoring nucleosomes to their base states in the absence of Swi-Snf activity.

The continuous requirement for Swi2 activity indicates that Swi-Snf accomplishes more than just providing transcriptional activators with access to their binding sites on promoters. Once bound to DNA, transcription factors stimulate transcription even in the presence of histones (Matsui, 1987; Workman and Roeder, 1987; Knezetic *et al.*, 1988; Workman *et al.*, 1990, 1991), suggesting that, once histones are disrupted and activators bind DNA, further chromatin remodeling should be unnecessary for transcription. Consistent with a role for Swi-Snf beyond activator binding, *swi-snf* mutations minimally diminish Gal4 binding at *SWI-SNF*-dependent promoters such as

Fig. 7. *swi2-6.3 gcn5Δ* mutants retained viability at the restrictive temperature. *SWI2 gcn5Δ* and *swi2-6.3 gcn5Δ* strains were grown to mid-log phase at 26.5°C and then shifted to 37°C. The OD₆₀₀ of the cultures, and the number of colonies (c.f.u.) formed on plates at 26.5°C (expressed as percentages of colony numbers formed prior to shifting to 37°C at *t* = 0 h) were determined at the indicated times following the shift to 37°C. Standard error is shown for the colony-forming assay.

GALp* (Burns and Peterson, 1997; Gavin and Simpson, 1997).

Components of the Swi-Snf complex have been found associated with the RNA polymerase holoenzyme (Wilson *et al.*, 1996). Though this interaction is a controversial one (Kim *et al.*, 1994; Cairns *et al.*, 1996), other components of the holoenzyme are also crucial for on-going transcription (Thompson and Young, 1995; Moqtaderi *et al.*, 1996). The continuous requirement for Swi-Snf activity could be explained if, as a component of the holoenzyme, Swi-Snf acts during each round of transcriptional initiation to disrupt nucleosomes and facilitate transcriptional initiation by RNA polymerase. Alternatively, Swi-Snf function may be essential for transcriptional elongation. In some studies, RNA polymerases appear to proceed unimpeded through nucleosomes *in vitro* (Lorch *et al.*, 1987; Studitsky *et al.*, 1994, 1997), but physiological chromatin structures may pose more substantial challenges to polymerase elongation *in vivo*. Also, other studies have identified activities necessary for polymerases to elongate through nucleosomes (Orphanides *et al.*, 1998).

Swi-Snf and Gcn5-Ada perform overlapping functions during transcription

Both Swi-Snf and histone acetyltransferases have been proposed as means of remodeling nucleosomes (Lee *et al.*, 1993; Pollard and Peterson, 1997; Cote *et al.*, 1998). However, the functional relationship between these activities had not been addressed. By comparing expression from several distinct promoters in cells lacking the activities of one or both complexes, we found that inactivating Swi2 in cells lacking *GCN5* significantly reduced transcription. These results show that Swi-Snf plays a role in transcription that is independent of *GCN5*, thereby demonstrating that the two complexes act in distinct pathways to activate transcription. Thus, histone acetylation by Gcn5 does not simply serve to stabilize nucleosomes that have been disrupted by Swi-Snf, or to label nucleosomes as future targets of Swi-Snf activity. Our data do not exclude a role for Gcn5 in Swi-Snf function, but clearly Swi-Snf retains significant transcriptional activity in the absence of *GCN5*.

Our results further suggest that Swi-Snf and Gcn5-Ada perform redundant functions during transcription. The transcriptional requirements for Swi2 function

depended on the *GCN5* background of the yeast, such that the absence of *GCN5* revealed novel functions for Swi2. Inactivating Swi2 in a wild-type *GCN5* background produced minimal defects in *GAL1*, *PGK1* and *ACT1* transcription (1.0- to 1.7-fold), but inactivating Swi2 in a *gcn5Δ* background reduced transcription of these genes 3.9- to 4.7-fold. These findings indicate that Swi–Snf and Gcn5–Ada perform overlapping functions since one complex is dispensable for transcription unless cells lack the other complex. Thus, the Swi–Snf and Gcn5–Ada complexes represent two independent means of accomplishing the same step during transcription, namely chromatin remodeling.

Transcriptional activators use both Swi–Snf and Gcn5–Ada to activate transcription

DNA-binding transcriptional activators often employ additional mechanisms to stimulate gene expression. Our results suggest that Gal4, a prototypical transcriptional activator, utilizes the Swi–Snf and Gcn5–Ada complexes as co-activators of transcription. The induction of *GAL1* transcription depends on Gal4 and involves changes in nucleosome positions on the promoter (Axelrod *et al.*, 1993). Consistent with a role for chromatin remodeling during *GAL1* transcription, we found that cells lacking the activity of both Swi–Snf and Gcn5–Ada showed profound defects in *GAL1* expression. We propose a model of *GAL1* transcriptional activation in which Gal4 engages the functions of both Swi–Snf and Gcn5–Ada to disrupt nucleosomes and activate transcription.

GAL1 expression involves separable events of derepression and transcriptional activation. Repression at the *GAL1* promoters depends on the transcriptional co-repressor composed of Ssn6 and Tup1 (Flick and Johnston, 1990). Ssn6–Tup1 positions nucleosomes on target promoters, and the disruption of these structures accompanies transcriptional activation (Roth *et al.*, 1992; Cooper *et al.*, 1994). Although these data suggest a possible functional interaction between Ssn6–Tup1 and Swi–Snf (Gavin and Simpson, 1997), we found that Swi–Snf function at the *GAL1* promoter is dedicated to transcriptional activation by Gal4 instead of counteracting repression by Ssn6–Tup1. Transcriptional activation by Gal4 under derepressing conditions required continuous Swi–Snf activity, and derepression prior to Swi2 inactivation failed to restore transcription from *GALp**. Consistent with our results, chromatin remodeling and transcription from the *SUC2* promoter require *SWI1* even in strains lacking *SSN6* or *TUP1* (Gavin and Simpson, 1997).

Widespread role for Swi–Snf in transcription

Our results revealed that, in the absence of *GCN5*, Swi–Snf activity is required for transcription of multiple, distinctly regulated genes. The general packaging of genes into chromatin, along with the demonstration that histone depletion appears to activate promoters non-specifically (Han and Grunstein, 1988; Han *et al.*, 1988), suggests that transcription of many, if not all, genes should require chromatin remodeling. Until now, however, the roles for chromatin remodeling complexes, including the Swi–Snf, Gcn5–Ada and Rsc complexes, were thought to be limited to a handful of promoters. The finding that *swi2-6.3 gcn5Δ* double mutants display widespread transcriptional defects

substantiates the notion that chromatin remodeling is a general requirement for gene expression, and establishes the Swi–Snf and Gcn5–Ada complexes as major activities fulfilling this role.

Our results define three general classes of genes based on their requirements for Swi–Snf and Gcn5–Ada functions. Expression of one class, including *HO* and *SUC2*, depends on both complexes (Pollard and Peterson, 1997). A second category consists of genes whose expression depends on either the Swi–Snf or the Gcn5–Ada complex. Transcription of these genes persists unless both complexes are inactivated. Examples of this class include genes whose expression is regulated (*GAL1*) and genes that are constitutively expressed (*PGK1* and *ACT1*).

The third category consists of genes whose expression is affected minimally or indirectly in strains lacking both Swi–Snf and Gcn5–Ada (e.g. *TCM1*). This class of genes may not require chromatin remodeling activities due to the presence of sequences in the promoters, such as nucleosome positioning elements, that prevent nucleosomes from repressing transcription. Alternatively, these promoters may use chromatin remodeling activities other than Swi–Snf and Gcn5–Ada to disrupt nucleosomes and activate transcription. The Rsc complex, for example, contains many Swi–Snf-related proteins and remodels nucleosomes *in vitro* (Cairns *et al.*, 1996), but its role in transcription awaits further study.

In summary, we have used a conditional allele of Swi2 to regulate the function of the Swi–Snf complex *in vivo* and explore the physiological function of this chromatin remodeling complex. We found that Swi–Snf plays a continuous role in transcription and that Swi–Snf and the histone acetyltransferase, Gcn5–Ada, function in independent and redundant pathways to stimulate transcription. Furthermore, in the absence of *GCN5*, Swi–Snf activates transcription of several, distinctly regulated genes, indicating that these chromatin remodeling activities play widespread roles in the expression of many yeast genes.

Materials and methods

Yeast strains

Yeast were grown and manipulated using standard protocols (Guthrie and Fink, 1991). CY120 (Mat α *swi2Δ::HIS3 HO-LacZ leu2-Δ1 his3-Δ200 ura3-52 lys2-801 ade2-101*) was transformed with pRS-SWI2, pRS-6.3 or pRS-K798A, which contained the 6 kb genomic *BglII*–*SalI* fragments encompassing the promoter and ORF of these *SWI2* alleles in pRS315 (Sikorski and Hieter, 1989). The *swi2Δ* strains carried pRS315 without any *SWI2* sequences.

YSB16 (Mat α *swi2Δ::HIS3 mig2Δ::URA3 leu2*) was generated by crossing CY120 with YM4801 (Lutfiyya and Johnston, 1996) and selecting *swi2Δ mig1Δ mig2Δ* haploids. The *swi2Δ::HIS3* and *mig2Δ::HIS3* alleles were identified by PCR. Note that the alleles present at the Mat *HO-LacZ GAL80 TRP1 MET CAN1* loci have not been determined.

YSB21 (CY120 *URA3::GALp*-STE3*) was generated by transforming CY120 with pYSB112 cut with *NcoI*.

swi2-6.3 gcn5Δ (YSB17) and *SWI2 gcn5Δ* (YSB18) strains were generated by crossing CY120 carrying either pRS-6.3 or pRS-SWI2, respectively, with GMy22 (Marcus *et al.*, 1994). Diploids were sporulated and haploids were selected that contained the *SWI2* plasmids and *gcn5Δ::URA3*. Strains also carrying the *swi2Δ::HIS3* allele were identified by PCR. Note that the alleles at the Mat *ADE2, HIS3, HIS4, TRP* and *ADE1* loci have not been determined. Congenic strains in the S288C background were also generated and yielded quantitatively similar results. The congenic strains (YSB25, 26) will be provided in response to requests for these yeast lines.

YSB22 (CY120 *URA3::GALp*-LacZ*) was generated by transforming CY120 with pEG28 (Burns and Peterson, 1997) cut with *ApaI*. YSB23 and YSB24 strains were generated by transforming BWG-17a (*GCN5*) (Marcus *et al.*, 1994) and GMy23 (*gcn5Δ*) (Marcus *et al.*, 1994) with pEG28 cut with *ApaI*.

The *srb4-138* (Z628) and *SRB4* (Z579) strains have been described (Thompson and Young, 1995).

Plasmids and *swi2* mutant isolation

Temperature-sensitive alleles of *SWI2* were generated using PCR to amplify the 1.6 kb *NcoI*-*BglII* fragment encoding the ATPase domain. The products were cloned into the *NcoI* and *BglII* sites of pRS-SWI2 to replace the wild-type ATPase domain. A mutant library was transformed into CY120 at 30°C. The transformants were replica-plated onto YPGlycerol at 30 and 37°C. Fifty-seven percent of the library was screened, and strains that grew at 30°C but not 37°C were selected for further analysis. The *swi2* alleles from clones failing to grow specifically at 37°C were isolated. Linkage of temperature sensitivity to the ATPase domain of these alleles was confirmed by subcloning this domain back into the context of the otherwise wild-type gene and showing that the reconstituted alleles possessed temperature-sensitive Swi2 function (data not shown).

Sequence analysis of *swi2-6.3* revealed four base pair changes in the ATPase domain of Swi2, one of which was a silent mutation and three of which generated the following amino acid changes: T909A, P943L and E1077G. P943 is conserved among Swi2-related proteins from various species, including Sth1 from yeast, Brm from *Drosophila* and Brg1 from mammals. T909 is conserved in Sth1 and the structurally related ATPase domain of Rad54. E1077 does not show any conservation, and the corresponding residue in Sth1 is a glycine, similar to the change in *swi2-6.3*. Which of these changes, or combination of changes, are responsible for temperature sensitivity has not been determined, and the contributions of these residues to the structure and function of Swi2, specifically its ATPase activity, awaits the determination of the crystal structure of this or a related ATPase domain. Sequence analysis of another temperature-sensitive allele, *swi2-7.8*, revealed five amino acid changes: L820I, K885E, T912A, D1008G and L1091P. All of these residues except T912 are conserved among Swi2, Sth1 and Brm.

The *GALp*-STE3* plasmid, pYSB112, carried the truncated *GAL1* promoter from p121-Δ10 (West *et al.*, 1984) upstream of the *STE3* coding region and 3'-UTR. A *HindIII*-*EcoRI* fragment containing *URA3* and the truncated *GAL1* promoter sequences was cloned into the same sites in pBluescript (Stratagene). The *STE3* gene (−3 to +1798) was then cloned as a *BamHI* PCR product into the *BamHI* site to generate pYSB112. Note that this fusion fails to generate Ste3 protein due to a frameshift at the *Gal1*-*Ste3* junction.

Protein analysis

Whole-cell extracts were prepared as described (Fiorentino and Crabtree, 1997). Proteins were separated on 6% SDS-PAGE gels and Swi2p levels were determined by Western blotting.

Gene expression analysis

β-Galactosidase was measured in logarithmically growing cells as described in Ho *et al.* (1996). For expression measurements in YSB22 derivatives, levels of β-galactosidase activity in 0.05% glucose media were subtracted from levels in galactose to determine induced activity. *SUC2* expression was induced for 3 h in minimal raffinose media, and secreted invertase activities were then measured as described (Vallier and Carlson, 1994).

SUC2 mRNA levels were measured by RNase protection (Melton *et al.*, 1984) using a probe that protects a 190 base fragment from the secreted invertase message and a 90–100 base fragment from the cytoplasmic invertase message. Total RNA was prepared as described (Schmitt *et al.*, 1990).

STE3, *GAL1*, *PGK1*, *ACT1* and 25S RNA levels were measured by Northern blotting. Total RNA was prepared as described (Schmitt *et al.*, 1990). A 10 μg aliquot of total RNA was separated on 1% agarose, 50 mM borate (pH 8.3), 3% formaldehyde gels. Gels were washed with H₂O, soaked in 0.05 M NaOH for 20 min, rinsed with H₂O and soaked in 10× SSC. RNA was transferred to GeneScreen (Dupont) membranes with 10× SSC. Membranes were hybridized with 5×10⁵ c.p.m./ml of probe at 68°C in 0.5 M NaPO₄ (pH 7.2), 1% bovine serum albumin (BSA) and 7% SDS, and washed twice in 1× SSC and once in 0.2× SSC at 65°C. All probes were antisense RNA probes labeled with [³²P]UTP.

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