Chemically Regulated Transcription Factors Reveal the Persistence of Repressor-resistant Transcription after Disrupting Activator Function*

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Control of gene expression often requires that transcription terminates rapidly after destruction, inactivation, or nuclear export of transcription factors. However, the role of transcription factor inactivation in terminating transcription is unclear. We have developed a means of conducting order of addition and co-occupancy experiments in living cells by rapidly exchanging proteins bound to promoters. Using this approach, we found that, following specific disruption of activator function, transcription from active promoters decayed slowly, persisting through multiple cell divisions. This persistent transcriptional activity raised the question of what mechanisms return promoters to inactive states. By exchanging or directing co-occupancy of protein complexes bound to a promoter, we found that the transcriptional inhibitor, Ssn6-Tup1, lost its effectiveness as a repressor following activator dissociation. Similar experiments with another repressor, the histone deacetylase Sin3-Rpd3, reinforced this distinction between repression in the presence and absence of an activator. These results suggest that although repressors such as Ssn6-Tup1 and Sin3-Rpd3 prevent activation of gene expression, other mechanisms of repression return promoters to inactive states following the dissociation or inactivation of a transcriptional activator.

Although many mechanisms of transcriptional regulation have been proposed, perhaps the most general and well documented is the recruitment of transcriptional activators to sites on DNA (for review see Ref. 1). The effectiveness of recruitment seems surprising; because the mammalian genome encodes perhaps 10,000 transcriptional activators (61), random interactions of activators with promoters would be expected based on a high cumulative concentration of activators within the nucleus. Furthermore, activators often require only short stretches of DNA for stable binding in vitro, and therefore a background of transcription would be expected because of non-specific DNA binding by activators. The fact that such a background is not generally observed suggests that repressors and/or chromatin prevents transcription resulting from non-specific functions of the many activators in the nucleus. Alternatively, recruitment or displacement of an activator might actually be remarkably precise regulatory mechanisms, and repressors and chromatin serve other purposes.

Controlling the proximity of an activator to DNA may also be important in inactivating transcription in response to external stimuli. In many cases, activators are displaced from DNA after post-translational modification of the activator, for example, by phosphorylation. In other cases, transcription factors are exported from the nucleus (NF-ATc and Pho4) or degraded (Swi5, NFκB) following the termination of signaling (62–64). In each case, the promoter is left without the function of a critical activator. Disrupting activators coincides with transcriptional down-regulation over fairly short periods of time. These rapid responses generate the tightly controlled patterns of expression of cell cycle-regulated genes and direct a well ordered sequence of gene expression in lymphocytes following receptor activation. A priori, mechanisms that lead to the termination of transcription could be as simple as removal of the activator or may include additional repressor mechanisms dedicated to returning genes to their inactive states. We set out to establish whether specifically disrupting activator function sufficed to rapidly silence gene expression.

Transcriptional repressors inhibit gene expression. Interestingly, promoters lacking binding sites for transcriptional activators fail to drive expression in vivo, suggesting that the presence or absence of activators, and not transcriptional repressors, determines which genes are turned on and off. Instead of preventing gene expression, repressors may expedite the silencing of active promoters. Consistent with this possibility, stimuli that silence ongoing expression lead to the simultaneous displacement of an activator and recruitment of a repressor at several promoters (2–4). In these examples, repressors are functionally positioned to quickly shut off transcription. Thus we wanted to explore this potential role of transcriptional repressors in more detail.

The activities of transcription factors require, in many cases, the simple act of localizing these proteins to sites within promoters (5). We exploited this principle to develop methods of regulating the activities of transcriptional activators and repressors by controlling their interactions with DNA. The interactions generated by cell-permeable compounds such as FK506, and rapamycin permitted the use of these drugs to control transcription factor activity in living cells (1). In the present study we have made use of the reversibility of FK506-driven interactions to activate and inactivate the function of a transcriptional activator in vivo. The properties of FK506 and rapamycin allowed us to further develop this system and perform experiments in which activators and repressors acted either sequentially or simultaneously at the same promoter. Using these approaches we showed that transcription shuts off slowly following the disruption of activator function. The remaining transcriptional activity was relatively insensitive to

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suppression by Sin3-Rpd3 and Ssn6-Tup1, indicating that these repressors are dedicated to preventing activation from a promoter but do not serve to reset promoters to inactive states after activator displacement.

MATERIALS AND METHODS

Yeast Strains and Plasmids—YPD6 (Mata fpr1::ADE2 TOR1-1 leu2d-3, 112 uro3-52 trp1-901 his3-D200 ade2-101 gal4Δ gal80Δ URA3::GAL-LacZ GAL-Leu2 ZY2::GAL-HIS3), and the plasmids expressed Gal4BD-FKBP, pSE-CN2b, and pACT-FRAP, respectively. Vogel and Crabtree (1995) described previously (1, 56). YSB7 (Mata leu2-3, 112 uro3-52 trp1-901 his3-D200 ade2-101 gal4Δ gal80Δ fpr1::ADE2 TOR1-1 LY2::GAL-HIS3 GAL-LacZ) was derived from a cross between YDF6 and YJO-Z (59). The resulting diploids were sporulated and haploids carrying the fpr1::ADE2, TOR1-1, and LY2::GAL-HIS3 alleles were selected. YSB6 (Mata TOR1-1 ade2-1 ade3-222 his3-11, 15 leu2-3, 112 trp1-1 can1-100 uro3-1 GAL4 GAL80 MIG1) was generated by transforming YM111 (Mata ade2-1 ade3-222 his3-11, 15 leu2-3, 112 trp1-1 can1-100 uro3-1 GAL4 GAL80 MIG1) with the Nco-KpnI containing the TOR1-1 mutation and selecting rapamycin-resistant colonies. Mutations in TOR1 were distinguished from FRP1 mutations by testing the shift of a Gal4 expression plasmid to express rapamycin sensitivity. YSB9 was generated by transforming YSB7 with pMG1ΔΔ2 (26) cut with ScaI.

The FRB overexpression vector, pYSB100-FRB45, expresses the 45-kDa FRB domain of the yeast Tor1 (amino acids 1765–2158) from the yeast GPD1 promoter. The calcineurin expression plasmid, pCNA/B, expresses the murine calcineurin A subunit (amino acids 12–254) fused to the SV40 nuclear localization signal and the influenza hemagglutinin epitope tag and expressed from the GPD1 promoter. pCNA/B, a high copy number plasmid, also carries a yeast genomic fragment containing the yeast calcineurin B subunit expressed from its endogenous promoter. YcpGal4/FK is a single copy plasmid (unlike pGal4/FKBP) expressing Gal4BD-FKBP from the ADH1 promoter. The FRB-Ssn6 expression construct, pFRP-Ssn6, expressed the entire open reading frame of Ssn6 fused to the FRB domain of FRAP (amino acids 2012–2146) (8) from the GPD1 promoter on a single-copy URA3 plasmid. The FRB-Ssn3 expression construct, pFRP-Ssn3, expressed the open reading frame of SIN3 fused to the FRB domain of FRAP from the GPD1 promoter on a single-copy URA3 plasmid. Details of the construction of these plasmids are available upon request.

Analysis of Gene Expression—β-Galactosidase assays were performed as described previously (1). Total yeast RNA was isolated as described (60). To minimize the amounts of rapamycin and FK506 necessary to conduct these experiments, cells in log phase were concentrated 3-fold in fresh medium at 30 °C. Under these conditions, cells continued to divide exponentially for at least 7 h (data not shown). For unknown reasons, rapamycin competes with FK506 more efficiently at higher cell densities (data not shown). Thus a 300-fold excess of rapamycin completely eliminated FK506-induced transcriptional activation under these conditions. mRNA levels were determined by Northern blotting using antisense RNA probes as described (1). RNA levels were quantitated by PhosphorImager analysis using ImageQuant software. The HIS3 probe recognized an endogenous message whose levels corresponded well to β-actin (ACT1) message levels (data not shown). This band was therefore used a control for RNA loading.

To arrest cells in G1 phase of the cell cycle, cells were grown into mid-log phase in minimal glucose medium, spun, and resuspended in rich glucose medium (pH 6.0) containing 50 mg/ml FK506 and 40 μg/ml α-factor for 3 h at 30 °C. >95% of cells were shmoo morph for this time. Cells were washed twice with fresh YPD and split into two cultures. Fresh α-factor was added to one of the cultures, and both were treated with 15 μg/ml rapamycin. >95% of cells budded and divided after washing and growth in the absence of α-factor, whereas cells maintained in α-factor retained shmoo morphology (data not shown).

RESULTS

Ligand-regulated Protein Exchange at Promoters in Vivo—To study the roles of activators and repressors in controlling gene expression, a method of inducibly and reversibly localizing proteins to promoters in living cells was developed (1). This strategy was based on the use of synthetic ligands or chemical inducers of dimerization and takes advantage of the fact that, in many cases, recruitment of transcription factors to DNA suffices to activate their function (5, 6). The specific approach in this study employed two cell-permeable compounds, FK506 and rapamycin, which both bind the FK506-binding protein (FKBP) with nanomolar affinities (7–9). The composite surface formed by FKBP-FK506 or FKBP-rapamycin binds either calcineurin or an FKBP-rapamycin binding domain (FRB), respectively. In cells expressing the Gal4 DNA-binding domain (Gal4BD) fused to FKBP12, FK506 induces the association of FKBP12 with calcineurin (CN) at promoters containing Gal4-binding sites. By creating a version of calcineurin fused to the Gal4 activation domain (Gal4AD-CN), we generated a means of using FK506 to recruit an activator to a promoter and activate transcription (Fig. 1A) (1). Rapamycin, which dissociates FK506-mediated interactions by competing for binding to FKBP, was then used to displace the activator from Gal4BD-FKBP12 and the promoter.

Using a related approach, fusions between transcriptional repressors and the FRB domain permitted the use of rapamycin to recruit repressors and thereby inhibit transcription from promoters containing Gal4-binding sites. In yeast expressing Gal4BD-FKBP12, Gal4AD-CN, and FRB-repressor fusion proteins, FK506 and rapamycin were used to regulate the activities of both activators and repressors at the same promoter. In this way, order of addition experiments, in which an activator and a repressor act sequentially, and co-occupancy experiments, in which the activator and repressor act simultaneously, were conducted in vivo (Fig. 1B).

The use of FK506 and rapamycin afford relatively specific means of controlling protein activity in vivo. These small molecules share a nearly unique mechanism of action in that they generate large and specific protein-protein interfaces that simulate normal protein-protein interactions. Genome-wide analysis revealed that FK506 and rapamycin each altered the expression of less than 0.5% of yeast genes (10).2 By contrast, temperature shifts, which have been used traditionally to regulate protein activity in vivo, alter the expression of about 600 (~10%) yeast genes (11).

Delayed Silencing of Transcription following Recruitment and Dissociation of the Activation Domain—in cells expressing the Gal4BD-FKBP12 and Gal4AD-CN fusions, FK506 activated transcription of the endogenous GAL1 gene and an integrated GAL1-HIS3 gene over a period of about 90 min (Fig. 2, A and B). These kinetics appeared surprisingly slow but in fact closely corresponded to the rate at which galactose induces transcription from the GAL1 promoter under physiologic conditions (12, 13). Importantly, rapamycin-mediated recruitment of an activation domain showed similar kinetics of transcriptional induction, demonstrating that rapamycin rapidly enters cells and binds Gal4BD-FKBP12 (see Fig. 4 and data not shown). Other studies also suggest that rapamycin crosses membranes and forms complexes with its binding proteins within minutes of its addition to medium (14).

Once transcriptional activation by FK506 reached a steady state, treatment with rapamycin to dissociate the activation domain rapidly reduced GAL1 and HIS3 mRNA levels. Because the GAL1 and HIS3 messages have extremely short half-lives in yeast (<5 min; see Fig. 5C (15, 16), measuring the levels of these two messages provided accurate assessments of the ongoing rates of transcription of both genes. Addition of rapamycin prior to steady state activation both blocked maximal acti-

1 The abbreviations used are: FKBP, FK506-binding protein; FRB, FKBP-rapamycin-binding domain; CN, calcineurin; FRB, FK506/rapamycin binding domain of FRAP (FKBP12-rapamycin-associated protein).

2 K. Vogel and G. R. Crabtree, unpublished data.
vation and reduced mRNA levels (Fig. 2C). In this case, the accumulation of message immediately following rapamycin addition most likely reflected the time required for rapamycin to dissociate FK506-mediated interactions (see below). Thus, consistent with previous studies (1), these results confirmed that activators play a continuous role in maintaining activated transcription.

Interestingly, we consistently observed the persistence of a reduced level of transcription following extended rapamycin treatment, suggesting that promoters do not immediately return to their basal, inactive states following activation domain dissociation (Fig. 2). This phenomenon is quantitated in Fig. 2B. After treatment with rapamycin, transcription of GAL1 and HIS3 decayed slowly with a t_1/2 of approximately 50 min. The promoters returned eventually to their inactive states, but the observed declines in HIS3 and GAL1 transcriptional activity were delayed significantly relative to that expected if transcription ceased following activation domain dissociation (for the predicted curve, see dotted line in Fig. 2B). The expected kinetics, which predict a 90% reduction in mRNA levels within 30–40 min, reflect both the half-life of the messages and the known kinetics of dissociation of the FKBP-FK506-calcineurin complex (3).

To determine whether residual transcription following activator dissociation could have been the result of rapamycin-induced recruitment of Tor or other proteins, we overexpressed the FRB domain of Tor1. This domain of Tor1 (amino acids 1765–2158) renders wild-type cells resistant to rapamycin, most likely by competing with the full-length Tor proteins for binding to FKBP-rapamycin (17). The same domain of the highly homologous Tor2 protein fails to activate transcription when fused directly to the Gal4 DNA-binding domain (18).

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Given the ability of Gal4BD-FKB12-rapamycin to bind the Tor proteins, the persistence of residual transcription following rapamycin treatment could have resulted from recruitment of a cryptic activation domain(s) in these or related proteins. Indeed, assays for β-galactosidase activity, which are significantly more sensitive than Northern blotting for detecting expression, revealed a low level of transcriptional activity in cells treated with rapamycin (Fig. 3A).

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Rapamycin Rapidly Dissociated FKBP-FK506-Calcineurin Complexes at Promoters in Vivo—FK506 was chosen for activation domain recruitment because of the short half-lives of the interactions it generates. In studies with purified proteins, the half-life of the FKBP-FK506-calcineurin complex has been estimated to be less than 5 min. However, interactions with
other cellular proteins in vivo or the effects of DNA binding by Gal4BD-FKBP12 could have altered the dissociation kinetics under the conditions of our experiments. These influences on the stability of FKBP-FK506-calcineurin complexes could have unexpectedly prolonged the association of the activation domain following rapamycin treatment and thus sustained transcription following rapamycin treatment as a result of incomplete dissociation of the Gal4AD-CN fusion from Gal4BD-FKBP12. To address this possibility and confirm the half-life of FK506-driven interactions, the kinetics of dissociation of the FKBP-FK506-calcineurin complex were determined in vivo.

To measure the stability of FKBP-FK506-calcineurin complexes, Gal4BD-FKBP12 was expressed with two additional proteins: a version of calcineurin lacking an activation domain and a fusion between the Gal4 activation domain and an FKBP-rapamycin-binding domain (FRB-AD). Calcineurin was expressed in excess of Gal4BD-FKBP12 (data not shown), and high concentrations (250 ng/ml) of FK506 were used to saturate Gal4BD-FKBP12 in complexes with calcineurin. Because of the
lack of an activation domain on calcineurin, FK506 did not activate transcription. When rapamycin was added, it competed with FK506 for binding to Gal4BD-FKBP12, thereby displacing calcineurin and concomitantly recruiting FRB-AD to activate transcription. Thus, monitoring transcriptional activity following rapamycin addition provided a means of determining the rate at which rapamycin dissociated the complex of FK506, calcineurin, and Gal4BD-FKBP12.

Pretreatment with FK506 introduced an approximately 15-min delay in transcriptional induction by rapamycin (Fig. 4). This delay represented the time required for rapamycin to displace FK506 and calcineurin. These data predicted a half-life of approximately 5 min for the FKBP-FK506-calcineurin complex in vivo, consistent with the rate of dissociation of the complex measured in vitro. In cells not overexpressing calcineurin, FK506 did not detectably delay rapamycin-induced transcription (Fig. 4), consistent with the short half-life of the FKBP-FK506 interaction (<2 min). Our measurement probably overestimated the actual time required for the in vivo dissociation of calcineurin because activation domains function synergistically to activate transcription from promoters with multiple binding sites (19). Thus, before significant transcription would be induced by rapamycin-mediated activator recruitment, most of the four Gal4-binding sites in the promoter would need to be cleared of FK506 and calcineurin. The parallel nature of the two curves for cells overexpressing calcineurin indicated that essentially all Gal4 sites were occupied by FRB-AD prior to detectable transcriptional induction.

The short half-lives of these interactions are consistent with the observation that FK506 does not induce any stabilizing conformational changes in either FKBP or calcineurin (9). Furthermore, our studies demonstrated that DNA binding by Gal4BD-FKBP12 did not significantly alter the rate of dissociation the FKBP-FK506-calcineurin complex and also that endogenous yeast proteins did not stabilize this complex in vivo. Importantly, these kinetic studies utilized the same protein domains used for activator recruitment by FK506. Thus, the rate of dissociation of the FKBP-FK506-calcineurin complex in these experiments was subject to the same influences that DNA-binding proteins and/or endogenous yeast proteins might have had during activation domain recruitment by FK506. These results strongly suggest that extended periods of rapamycin treatment quantitatively displaced Gal4AD-CN from the promoters.

The experiments presented in Fig. 4 would have been unable to detect the persistence of a small fraction of calcineurin on promoters following rapamycin treatment, because sufficient FRB-AD had been recruited to fully activate transcription. A similar small amount of the Gal4AD-CN fusion could have remained associated with promoters when rapamycin was used to dissociate the activation domain. However, the synergistic nature of activator function (19) requires that most of the Gal4 sites on the promoter be occupied to induce transcription and therefore suggests that persistence of a small fraction of Gal4AD-CN at the promoter following rapamycin treatment would be insufficient to activate significant levels of transcription. Furthermore, transcription maintained by the presence of a small fraction of Gal4AD-CN would be expected to display the functional properties of activated transcription, which, as shown below, was not the case.

Persistence of transcription in a subpopulation of cells, such that a fraction of cells differentially maintained high level gene expression, is unlikely because expression of green fluorescent protein at the single-cell level varied evenly across the entire population of cells in response to treatments similar to those used in the studies described above (data not shown). Unfortunately, the prolonged half-life of green fluorescent protein precluded single-cell analysis of the rate at which transcription shuts off following rapamycin treatment.

Functional Distinction between Activated and Residual Transcription—We began to investigate the nature of the persistent transcriptional activity by comparing the abilities of glucose to repress activated and residual transcription. Adding glucose to the growth medium rapidly represses transcription from the GAL1 promoter through cis-acting sites in the upstream repressing sequence (12, 20). Thus, in the yeast strains used in these experiments, glucose directly influences the activities of promoters regulated by Gal4BD-FKBP12 and Gal4AD-CN.

In FK506-treated cells, where the activation domain was present on promoters, glucose efficiently repressed GAL1 mRNA levels (Fig. 5, A, compare lane 1 with lane 2, and B). The level of glucose-mediated repression was consistent with that
Residual Transcription Following Activator Dissociation

Glucose-mediated repression of activated but not residual transcription. A, YDF6 (MIG1) expressing Gal4BD-FKBP12 and Gal4AD-CN was grown in raffinose medium and treated with 50 ng/ml FK506 for 45 min. Half of the culture was then treated with a 300-fold weight excess of rapamycin for 50 min while the other half was not treated with rapamycin. Glucose (+) or rapamycin (−) was then added to a 2% final concentration for 30 min. The experimental protocol is shown schematically. Cells were harvested, and GAL1 and HIS3 mRNA levels were measured by Northern blotting. GAL1 and HIS3 mRNA levels were also measured in YSB9 (mig1Δ, −) that was treated similarly with the exception that cells were not treated with rapamycin (lanes 7 and 8). An endogenous HIS3 message (h) was measured to control for RNA loading. B, GAL1 message levels were quantitated by PhosphorImager analysis and corrected for RNA loading and the extent of activation by comparison with HIS3 mRNA levels. Fold repression was determined by comparing GAL1 mRNA levels in glucose- and rapamycin-treated cells. Standard error is shown. C, YSB6 (GAL4 TOR1–1Δ) was grown for >12 h in galactose. Cells were treated with 15 μg/ml rapamycin or left untreated for 50 min. Glucose was then added to a 2% final concentration (t = 0'). The experimental protocol is shown schematically. Aliquots of cells were removed at the indicated times following addition of glucose, and GAL1 mRNA levels were measured by Northern blotting.

seen in other studies (12). If, however, cells were treated with rapamycin to dissociate the activation domain, glucose was much less efficient in repressing GAL1 expression (compare lane 3 with lane 4). Consistent with inefficient glucose-mediated repression of residual transcription, the presence or absence of glucose did not significantly alter the kinetics with which transcriptional activity decayed following activation domain displacement (data not shown). As expected, transcription of the GAL1−HIS3 gene, whose promoter lacked the upstream repressing sequence, resisted repression by glucose in both the presence and absence of rapamycin.

Glucose repression occurs through a signaling pathway that activates the function of the Mig1 transcriptional repressor at the upstream repressing sequence. Glucose may have failed to repress residual GAL1 transcription because rapamycin interfered with the cellular responses to glucose. Also, rapamycin may have stabilized the GAL1 message, thereby interfering with the detection of transcriptional repression. These potential effects of rapamycin were addressed using yeast that possess the physiologic GAL1 regulatory machinery. We found that adding glucose to cells grown in galactose reduced GAL1 message levels at the same rate and to the same extent in untreated and rapamycin-treated cells (Fig. 5C). Thus, rapamycin did not alter the ability of the cells to repress GAL1 expression in response to glucose or prolong the half-life of GAL1 mRNA.

The differential repression of activated and residual transcription in response to glucose distinguishes these two types of transcriptional activity. If residual transcription merely represented low level activated transcription because of, for example, incomplete dissociation of the activation domain, then glucose would have been expected to repress both types of transcription with similar efficiencies. In fact, as will be shown below (see Fig. 7B), glucose would have repressed transcription driven by incomplete activation domain dissociation more effectively than fully activated transcription. These results demonstrate that activated and residual transcription possess different molecular properties, consistent with the conclusion that activated transcription occurs in the presence of the activation domain whereas residual transcription occurs in the absence of the activation domain.

Despite a reduced response to glucose, a small amount of repression by glucose was seen in rapamycin-treated cells (Fig. 5, A, compare lane 3 with lane 4, and B). Comparing glucose repression in wild-type (MIG1) and mig1Δ strains revealed that glucose repressed residual transcription in MIG1 cells to the same degree it repressed activated transcription in mig1Δ cells (Fig. 5A, compare lane 7 with lane 8, and B). These results indicate that this level of repression represents promoter-independent effects of glucose on GAL1 message levels. Furthermore, these data suggest that the inefficiency with which glucose repressed residual transcription stemmed specifically from the inability of Mig1 to repress this type of transcription.

Passage through the Cell Cycle Failed to Erase Residual Transcription—We also examined the effects of passage through various stages of the cell cycle on the rates of transcription following activation domain dissociation. DNA replication and mitosis influence the regulation of transcription, such that movement through these phases of the cell cycle can render promoters receptive or resistant to signals from transcription factors (21–23). If replication or mitosis erased the effects of activator functions that persist following its dissociation from promoters, then the residual transcriptional activity would be silenced at these stages of the cell cycle.

The effects of cell cycle events on residual transcription were assessed by establishing a synchronously dividing population of cells and measuring the rates of transcription as the cells divided. Cells were treated with FK506 to activate transcription and α-factor to arrest the cell cycle. Rapamycin was then added to dissociate the activation domain, and cells were either released from cell cycle arrest or held in G1 phase with α-factor. Progression through the cell cycle was monitored by measuring CLN1 mRNA levels at different times (Fig. 6). CLN1 expression is limited to the G1 and S phases of the cell cycle and peaks in late G2 (24). Cells released from α-factor generated two peaks of CLN1 expression, reflecting passage through one complete cell division and a second round of DNA replication.

Rapamycin reduced the rate of transcription with the same kinetics in cells arrested with α-factor as in cells dividing synchronously, as shown by the similar reductions in HIS3 message levels over time in both populations (Fig. 6). The dividing cells were followed through two rounds of DNA synthesis, and thus events taking place shortly after α-factor release, which may have preceded complete activation domain dissociation, were assessed during the early phases of the sec-
Residual Transcription Persisted Through DNA Replication and Mitosis

![Residual Transcription Persisted Through DNA Replication and Mitosis](image)

**FIG. 6.** Passage through the cell cycle fail to erase residual transcription. YDF6 expressing Gal4BD-FKBP12 and Gal4AD-CN was treated with 50 ng/ml FK506 to activate transcription and α-factor to arrest cell division. After 3 h, cells were washed and incubated with (+) or without (−) α-factor to maintain cell cycle arrest or release cells into synchronous progression through the cell cycle, respectively. Immediately after washing, a 300-fold weight excess of rapamycin was added to both cultures. The experimental protocol is shown schematically. Total RNA was isolated at the indicated times following rapamycin addition, and CLN1 and HIS3 mRNAs were measured by Northern blotting. Message levels were also measured in cells grown in the absence of α-factor and FK506 (asyn). An endogenous HIS3 message (h) was measured to control for RNA loading.

Control experiments confirmed that similar to treatment with rapamycin, washing FK506 away from cells generated residual transcriptional activity. Cells that had been washed or treated with rapamycin showed similar levels of residual GAL1 and HIS3 transcription, and the transcriptional activity persisting after washing was resistant to repression by glucose (Fig. 7B). Furthermore, repression by glucose was not enhanced by additionally recruiting FRB-Ssn6 with rapamycin (Fig. 7B). Similar experiments suggested that the level of repression by FRB-Ssn6 following washing in Fig. 7A was most likely because of incomplete removal of FK506 (data not shown). FRB alone did not repress transcription in response to rapamycin (data not shown).

Inverse Relationship between Strengths of Activation and Repression—An alternative explanation for the apparent activator dependence of Ssn6-Tup1 is that the repressor dictates a defined, low level of transcription. If residual transcriptional activity matches or falls below that level, Ssn6-Tup1 would fail to further repress transcription irrespective of activator status. This kind of mechanism predicts that the extent of repression would be higher with stronger activation, because weak activators would stimulate transcription at levels closer to the set point established by Ssn6-Tup1. To address this possibility, we compared the extent to which Ssn6-Tup1 repressed transcription from promoters of differing activities. Using dose-dependent transcriptional activation by FK506, we found that glucose, and therefore Ssn6-Tup1, repressed weak activation more efficiently than strong activation (Fig. 7C). These data were consistent with previous studies using promoters of different strengths (e.g. GAL1 US2 and LEU2 US2 (12)) but were inconsistent with the establishment of a transcriptional set point by Ssn6-Tup1. In addition, these data showed that if, as discussed above, residual transcription was driven simply by a fraction of activation domains remaining associated with the promoters, then Ssn6-Tup1 should have repressed residual transcription more effectively than activated transcription.

Repression by a FRB-Sin3 Fusion in the Presence and Absence of the Activation Domain—The transcriptional repressor containing Sin3 and Rpd3 appears to inhibit transcription by deacetylating histones (29, 30). Biochemical and genetic data strongly suggest that Sin3-Rpd3 and Ssn6-Tup1 exist as two distinct transcriptional repressors. Therefore, to test the generality of activator-dependent repression, we examined the ability of Sin3-Rpd3 to repress activated and residual transcription. Similar to Ssn6-Tup1, localizing Sin3-Rpd3 to promoters suffices for transcriptional repression by this complex (31, 32). Thus, SIN2 was fused to the FRB domain of FRAP, and rapamycin was used to recruit Sin3-Rpd3 to promoters.

The abilities of Sin3-Rpd3 to repress transcription in the presence and absence of the activation domain were determined by conducting co-occupancy and order of addition experiments as described above. Comparison of these two regimens demonstrated that, similar to Ssn6-Tup1, Sin3-Rpd3 repressed activated transcription more efficiently than residual transcription (Fig. 8, compare lanes 1 and 2 with lanes 3 and 4).
Thus, although both Ssn6-Tup1 and Sin3-Rpd3 rapidly repressed transcription in the presence of the activation domain (maximal repression was seen within 25 min of rapamycin treatment (data not shown)), they both depended on the activator for maximal repression.

**DISCUSSION**

**Residual Transcription following Activation Domain Dissociation**—In general, three possibilities can explain the persistence of transcription in cells treated with rapamycin to displace the promoter-bound activation domain. First, an activator other than the Gal4AD-CN fusion may have been present at the promoters under these conditions. Second, Gal4AD-CN may have remained associated with the promoters despite rapamycin treatment. Third, transcription may have persisted in the absence of any activation domain. Our experimental evidence supports the conclusion that residual transcription continued in the absence of an activation domain.

In cells treated with FK506, the simultaneous addition of rapamycin blocked FK506-induced transcription (1). Thus FK506 was not generating interactions with activators that could not be disrupted by rapamycin. The Gal4BD-FKBP12 fusion failed to activate transcription in the absence of FK506, demonstrating that this protein lacked an inherent ability to drive transcription. Furthermore, the persistence of transcription was not due to recruitment of an activation domain by rapamycin (Fig. 3). These results demonstrate that an activator other than the Gal4AD-CN fusion was not responsible for residual transcription.

FK506 and calcineurin dissociate from FKBP rapidly in vitro. We confirmed the instability of these interactions and found that the FKBP-FK506-calcineurin complex dissociated with a half-life of approximately 5 min in vivo (Fig. 4). Detailed kinetic analysis of the rate of dissociation of FK506 in mammalian cells predicts a half-life of approximately 2 min (33).

Analyses of the properties of rapamycin further suggest that rapamycin enters cells and binds Gal4BD-FKBP12 almost immediately following its addition to the growth medium (Fig. 4 and Ref. 14). Thus, several lines of evidence indicate that the half-life of FKBP-FK506-calcineurin complexes was very brief and that extended periods of rapamycin treatment quantitatively dissociated the Gal4AD-CN fusion.

Finally, the finding that glucose and Ssn6-Tup1 failed to repress residual transcription but efficiently repressed activated transcription distinguished the two types of transcription (Figs. 5 and 7). These results indicated that the residual transcription following rapamycin treatment was not simply due to the persistence of an activation domain on the promoter. Thus, disrupting the function of transcriptional activators is not sufficient to rapidly inactivate gene expression.

**Active Mechanisms to Return Promoters to Their Inactive State**

Residual Transcription following Activator Dissociation

![Graph showing activator-dependent repression by a FRB-Ssn6 fusion.](image)

**A** Activator-Dependent Transcriptional Repression by FRB-Ssn6

Thus, although both Ssn6-Tup1 and Sin3-Rpd3 rapidly repressed transcription in the presence of the activation domain (maximal repression was seen within 25 min of rapamycin treatment (data not shown)), they both depended on the activator for maximal repression.

**B** Residual Transcription After Washing FK506 Away from Cells

![Graph showing residual transcription after washing FK506 away from cells.](image)

C Ssn6-Tup1 Repressed Weak Activation More Efficiently Than Strong Activation

![Graph showing Ssn6-Tup1 repressed weak activation more efficiently than strong activation.](image)
States—Promoters are often controlled by transcription factors whose activities are regulated in response to various stimuli, and changes in gene expression frequently coincide with the inactivation of these transcriptional activators by mechanisms such as nuclear export, degradation, and post-translational modification. Our results show that disrupting the function of an activator slowly returns promoters to their inactive states over a period of multiple cell divisions. These findings suggest that tight regulation of transcription, such as occurs for cell cycle-regulated genes, demands additional mechanisms to silence transcription.

Chromatin presents one possible means of inactivating promoters. Several promoters position nucleosomes specifically over critical regulatory sequences, and in many cases the presence of these structures correlates with promoter inactivity (34–36). Transcriptional activators disrupt these nucleosomes while inducing expression, which may suffice for transcriptional activity independent of further activator function (37). In this case, rapid reversal of these structural changes in chromatin may be necessary for quickly silencing promoters. However, activities capable of disrupting chromatin during transcriptional activation appear to impart relatively stable changes to the structures of nucleosomes (38–41), suggesting that some alterations in chromatin structure may not be rapidly reversed following removal of an activator. Furthermore, the Swi-Snf and SAGA chromatin remodeling complexes remain at a promoter despite dissociation of the activator (42). However, the activity of the yeast Swi-Snf chromatin remodeling complex is required continuously to maintain ongoing transcription (43). Thus, activities that counteract the Swi-Snf complex, which functions during transcriptional activation from the GAL1 promoter (43), could mediate rapid promoter inactivation.

Transcriptional repressors are known to counteract activators and reduce gene expression. For many promoters, the combined activities of transcriptional activators and repressors control expression (2–4, 44). In several of these examples, including genes regulated by the yeast Ume6 and mammalian Max transcription factors, activators and repressors appear to exchange with each other on the promoters they regulate such that activators are dissociated and replaced by repressors (2–4). Surprisingly, our data demonstrate that the repressors, Ssn6-Tup1 and Sin3-Rpd3, lost activity in the absence of the activator. Thus these repressors are poorly suited for rapidly silencing gene expression following activator displacement. Instead, recruitment of these repressors following activator disruption may serve to prevent any further transcription driven by additional activators. For example, in the context of a “permissive” chromatin environment at the promoter of an actively transcribing gene, preventing spurious transcriptional activation by transcription factors binding to previously remodeled promoters may require monitoring by repressors.

Activator-dependent Repression by Ssn6-Tup1—The ability to recruit and dissociate the activation domain allowed us to test the role of the activation domain in Ssn6-Tup1 function. Using indirect, but physiologic means, as well as using rapamycin to directly localize Ssn6-Tup1 at the GAL1 promoter, we found that repression showed an unanticipated dependence on the presence of the activation domain. This finding suggests the possibility that activators and Ssn6-Tup1 share a common target(s) and that the mechanism of repression is to neutralize the function of a target that is recruited or otherwise activated by transcriptional activators. Thus the presence of the activator at the promoter reveals the target of Ssn6-Tup1 activity, but in the absence of the activator, this target is not available for repression. The inverse relationship between levels of activation and levels of repression (Fig. 7) further supports this kind of functional interaction between activators and Ssn6-Tup1. Increasing activator function could overwhelm repression by providing the predominant influence over a common target. This inverse relationship would not be expected if the activator and repressor worked on independent effectors of transcription; instead, the extent of repression would be constant despite varying levels of activation.

Nucleosomes represent potential targets for both activators and Ssn6-Tup1. Transcriptional repression and nucleosome positioning by Ssn6-Tup1 require the N terminus of histone H4 (45, 46). Similarly, activation of GAL1 transcription requires the H4 N terminus (47). Thus activators and Ssn6-Tup1 may convey competing signals through their interactions with histone H4.

The transcriptional mediator complex, which associates with the C-terminal domain of RNA polymerase II, is another potential target of both activators and Ssn6-Tup1. Several mediator components are crucial for efficient transcriptional activation from the GAL1/10 promoter and for efficient repression by Ssn6-Tup1 (48–51). One current model of transcriptional activation suggests that activators function by recruiting the mediator/RNA polymerase holoenzyme to promoters (52). Consistent with the mediator also being a target of repression, Ssn6-Tup1 repressed transcription activated by mediator recruitment (data not shown). The mediator complex also contains Srb10 and Srb11, which encode a kinase whose activity is required for normal levels of CTD phosphorylation. Intriguingly, point mutations that disrupt kinase activity interfere with both transcriptional activation and repression, suggesting that the level or specificity of Srb10–11 activity may be influenced by activators and Ssn6-Tup1 to regulate transcription.

In other studies, Ssn6-Tup1 has been shown to repress transcription in vitro (53, 54). These results were interpreted as
evidence that Ssn6-Tup1 represses basal transcription, which by definition occurs in the absence of any activator. However, these experiments depended on Mcm1, a transcriptional activator, being bound to the promoter and recruiting Ssn6-Tup1 to DNA. Thus, Ssn6-Tup1 may have been repressing transcription activated by Mcm1, not basal transcription.

Transcriptional repression by Sin3-Rpd3 also depended on the presence of the activator. Because Sin3-Rpd3 functions by deacetylating histones, displacing the activator and thereby inhibiting acetyltransferase function may negate the effectiveness of Sin3-Rpd3 as a repressor. The approximately 2-fold greater efficiency with which Sin3-Rpd3 inhibited residual transcription compared with Ssn6-Tup1 may reflect covalently associated acetyl groups remaining despite removal of the acetyltransferase.

**Strategy for Controlling Transcription Factors in Vivo**—By generating fusion proteins between transcription factors and either FKBP12, calcineurin, or FRB, we have demonstrated a general strategy for regulating the activities of transcription factors in living cells (1, 55, 56). This approach to the regulation of transcription compared with Ssn6-Tup1 may reflect covalently associated acetyl groups remaining despite removal of the acetyltransferase.

**REFERENCES**

1. Ho, S. N., Biggar, S. R., Spencer, D. M., Schreiber, S. L., and Crabtree, G. R. (1996) *Nature* 382, 822–826.
2. Bowdish, K. S., and Mitchell, A. P. (1993) *Mol. Cell. Biol.* 13, 2172–2181.
3. Bowdish, K. S., Yuan, H. E., and Mitchell, A. P. (1995) *Mol. Cell. Biol.* 15, 2955–2961.
4. Ayer, D. E., and Eisenman, R. N. (1993) *Genes Dev.* 7, 2110–2119.
5. Ptashne, M., and Gann, A. (1997) *Nature* 386, 569–577.
6. Fields, S., and Song, O. (1989) *Nature* 340, 245–246.
7. Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) *Cell* 66, 807–815.
8. Chen, J., Zheng, X. P., Brown, E. J., and Schreiber, S. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 4947–4951.
9. Griffith, J. P., Kim, J. L., Kim, E. E., Sintchak, M. D., Thomson, J. A., Fitzgibbon, J. M., Fleming, M. A., Caron, P. R., Hsiao, K., and Navia, M. A. (1995) *Cell* 82, 507–522.
10. Barton, M. C., and Emerson, B. M. (1994) *EMBO J.* 13, 3834–3841.
11. Neihin, J. O., Carlborg, M., and Ronne, H. (1991) *EMBO J.* 10, 1163–1171.
12. Durrin, L. K., Mann, R. K., Kayne, P. S., and Grunstein, M. (1991) *Science* 252, 659–692.
13. Leither, K. H., and Kohn, S. A. (1992) *Science* 256, 1333–1335.
14. Schmitt, M. E., Brown, T. A., and Trumpower, B. L. (1990) *Annu. Rev. Immunol.* 8, 1361–1395.
15. Henikoff, S., Greene, A. E., Pietrokovski, S., Bork, P., Attwood, T. K., and Hood, L. (1997) *Science* 276, 609–614.
16. Nasmyth, K., Adler, G., Lydall, D., and Seddon, A. (1990) *Cell* 62, 631–647.
17. Zheng, X. F., Florentino, D., Chen, J., Crabtree, G. R., and Schreiber, S. L. (1995) *Cell* 82, 121–130.