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An Enzymatic Activity in the Yeast Sir2 Protein that Is Essential for Gene Silencing

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Summary

Despite its conservation in organisms from bacteria to human and its general requirement for transcriptional silencing in yeast, the function of the Sir2 protein is unknown. Here we show that Sir2 can transfer labeled phosphate from nicotinamide adenine dinucleotide to itself and histones in vitro. A modified form of Sir2, which results from its automodification activity, is specifically recognized by anti-mono-ADP-ribose antibodies, suggesting that Sir2 is an ADP-ribosyltransferase. Mutation of a phylogenetically invariant histidine residue in Sir2 abolishes both its enzymatic activity in vitro and its silencing functions in vivo. However, the mutant protein is associated with chromatin and other silencing factors in a manner similar to wild-type Sir2. These findings suggest that Sir2 contains an ADP-ribosyltransferase activity that is essential for its silencing function.

Introduction

Gene silencing involves the formation of a specialized chromatin structure that limits access to DNA by the machineries that transcribe, replicate, and recombine the cell's genetic information. The physiological roles of silencing include the stable repression of gene expression, a process that is important for maintaining the differentiated state of cells, and repression of hyperrecombination in highly repetitive chromosome regions, a process that plays a crucial role in maintaining chromosome stability (reviewed in Loo and Rine, 1994; Lustig, 1998). In the budding yeast *Saccharomyces cerevisiae*, the silent mating–type loci and telomeric DNA regions are packaged into silent chromatin (Nasmyth et al., 1981; Rine and Herskowitz, 1987; Gottschling et al., 1990). These chromosome domains share many distinguishing properties with heterochromatin of multicellular eukaryotes and are therefore thought to be analogous to heterochromatin. Furthermore, one of the yeast silencing proteins, Sir2, is involved in repression of hyperrecombination and transcription of pol II reporter genes that are inserted within the ribosomal DNA (rDNA) repeats, suggesting that a silencing-based mechanism regulates rDNA chromatin structure (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997).

Many gene products have been identified that are required for the assembly of silent chromatin in yeast (e.g., Klar et al., 1979; Rine and Herskowitz, 1987; Shore and Nasmyth, 1987; Kayne et al., 1988; Foss et al., 1993; Straight et al., 1999). Sir2 is unique among these gene products in that it is required for all examples of silencing. We and others have shown that Sir2 is a component of two distinct complexes (Moretti et al., 1994; Moazed and Johnson, 1996; Moazed et al., 1997; Strahl-Bolsinger et al., 1997). One complex contains the Sir2, Sir3, and Sir4 proteins and mediates silencing at the silent mating–type loci and telomeric DNA regions (Moazed et al., 1997; Strahl-Bolsinger et al., 1997). A second complex contains Sir2, Net1, and Cdc14 and is required for silencing at the rDNA repeats (Shou et al., 1999; Straight et al., 1999). The general requirement for Sir2 in silencing, together with the universal conservation of a 250 amino acid domain within Sir2 (Brachmann et al., 1995; Sherman et al., 1999), strongly suggests that it is involved in a key aspect of the mechanism of silencing.

The role of Sir2 in silencing is unknown, but recent evidence suggests that the conserved core domain in Sir2-like proteins harbors an enzymatic activity that may involve the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD^+) to protein substrates (Frye, 1999). First, the *Salmonella typhimurium* Sir2-like protein, CobB, is required to compensate for the lack of CobT, a protein that functions in the cobalamine biosynthesis pathway as a nicotinate mononucleotide: 5,6-dimethylbenzimidazole phosphoribosyltransferase (Tsang and Escalante-Semerena, 1998). These results suggest that CobB may be able to perform a similar phosphoribosyltransferase reaction, explaining its apparent functional overlap with CobT. Second, two Sir2-like proteins from human and *E. coli* have been shown to catalyze the transfer of ^{32}P from ^{32}P-NAD^+ to bovine serum albumin (BSA) (Frye, 1999).

The biological roles of neither *E. coli* CobB nor any of the human Sir2-like proteins are known. Therefore, clues regarding their possible substrates and any evidence that the observed activity is functionally significant are lacking. In yeast, the primary requirement for Sir2 is in gene silencing at three distinct chromosomal loci: the silent mating–type loci, the telomeric DNA regions, and the rDNA repeats. These loci comprise all of the known silent chromatin in this organism. Studies of the possible enzymatic activity of Sir2 in yeast could therefore allow a direct examination of the role of this activity, if any, in a well-characterized cellular process. Here we show that the yeast Sir2 protein can use NAD^+ as a donor to modify histones and itself in vitro. To determine whether this enzymatic activity is required for silencing, we mutated a conserved histidine in Sir2. Mutation of the analogous histidine in SIRT2, a human

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Sir2-like protein, has been shown to abolish its enzymatic activity (Frye, 1999). We show that this point mutation abolishes the enzymatic activity of Sir2 in vitro. Significantly, the mutant Sir2 protein has no silencing function in vivo at any of the above loci, even when overexpressed from the strong *GAL1* promoter. Moreover, immunoprecipitation experiments using whole-cell extracts and soluble chromatin show that the enzymatically inactive protein is present in silencing complexes and is associated with the same DNA domains as wild-type Sir2. Consistent with these results, expression of the mutant protein disrupts silencing in *SIR2*$^+$ cells. Finally, we show that an antibody that is specific for mono-ADP-ribose can recognize a modified form of Sir2 that requires the enzymatic activity of the Sir2 protein for its generation, suggesting that Sir2 is an ADP-ribosyltransferase.

Results

SIR2 Can Transfer ^{32}P from NAD$^+$ to Proteins Including Histones and Itself

Sir2-like proteins from human and *E. coli* have been shown to catalyze the transfer of ^{32}P from NAD$^+$ to BSA (Frye, 1999). We expressed the yeast Sir2 protein as a fusion with glutathione S-transferase (GST) in *E. coli*, purified this fusion protein by chromatography on glutathione-agarose, and tested if it could use ^{32}P-α-NAD$^+$ as a donor to label BSA. As a control for these experiments, we constructed a point mutation in the *SIR2* gene that converts a conserved histidine at position 364 in Sir2 to tyrosine (hereafter referred to as Sir2-H364Y). It has previously been shown that mutation of the analogous histidine in a human Sir2-like protein abolishes its enzymatic activity (Frye, 1999).

Incubation of GST-Sir2 with BSA in the presence of ^{32}P-NAD$^+$ resulted in strong labeling of BSA. We could easily detect labeling of as little as 150 ng of BSA in these experiments (Figure 1A, lanes 2–7). No labeling of BSA was observed when GST-Sir2 was replaced with a GST-Sir2-H364Y mutant protein, indicating that the observed enzymatic activity required wild-type Sir2 (Figure 1A, lane 10). Similarly, no labeling was observed when GST-Sir2 was replaced with GST or GST-Sir2-Nterm (GST-Sir2N; Figure 1A, lanes 1, 8, and 11). In addition to labeling of BSA, we observed a very weak labeling of GST-Sir2 itself, which migrates above the BSA band at approximately 90 kDa (see Figure 1, lane 13).

Among the candidates for physiological substrates of Sir2 are other proteins that are involved in silencing. These include Sir2 itself, the other Sir proteins, and histones. In addition to being involved in silencing, histones are known to be ADP-ribosylated in the cell (Golderer and Grobner, 1991; Soman et al., 1991). We therefore tested whether Sir2 could label a mixture of histones using ^{32}P-NAD$^+$ as a donor. Incubation of GST-Sir2 with ^{32}P-NAD$^+$ and histones derived from either *Tetrahymena* (Figure 1C, lane 2; Figure 1E, lane 1) or calf thymus (Figures 1G and 1H) resulted in the modification of histones. Weak labeling of an unknown 67 kDa protein was also observed in the *Tetrahymena* histone preparation (Figure 1C, lane 2; Figure 1E, lane 1). No labeling was observed using GST or the GST-Sir2-H364Y mutant protein (Figure 1C, lanes 1 and 3; Figure 1E, lane 2). Interestingly, in the presence of histones, the self-modification activity of Sir2, mentioned above, was significantly enhanced (Figure 1C, lane 2; Figure 1E, lane 1), but histones had no effect on the labeling of BSA by Sir2 (data not shown). Moreover, histones appeared to strongly induce an NAD$^+$ breakdown activity in Sir2; in the presence of both Sir2 and histones, the free ^{32}P-NAD$^+$, which migrates near the bottom of the gel, completely disappeared (Figure 1C, lane 2). Instead of the free ^{32}P-NAD$^+$ band, we observed a ^{32}P smear throughout the lane. Extensive treatment of the reaction with proteinase K had no effect on the appearance of this smear (Figures 1B and 1C, lane 5). However, the smear could be eliminated by precipitation of the reaction with trichloroacetic acid (TCA) prior to its application to SDS polyacrylamide gels (Figure 1E, lane 1). We do not know the chemical nature of this smear but believe it unlikely that it represents the formation of protein-attached poly(ADP-ribose) chains, as its appearance was not altered by extensive treatment with proteinase K (Figures 1B and 1C, lane 5), and it did not TCA precipitate with labeled proteins (Compare Figure 1C, lane 1 with Figure 1E, lane 1).

Mono-ADP-ribosylation of proteins can also occur via a chemical (nonenzymatic) reaction that involves the conjugation of ADP-ribose to protein lysine residues through Schiff bases (Kun et al., 1976; Ueda and Hayaishi, 1985). To determine whether the observed labeling of proteins by ^{32}P-NAD$^+$ occurred through such a pathway, we performed the reactions in the presence of NADase, which hydrolyzes NAD$^+$ to nicotinamide and ADP-ribose (Ueda and Hayaishi, 1985). If the labeling of substrates in the reaction is chemical (for example, through the reaction of ^{32}P-ADP-ribose with lysine residues), then we would expect to observe an increase in labeling of lysine-rich proteins such as histones when NADase is present in the reaction. On the other hand, if labeling is enzymatic and requires intact ^{32}P-NAD$^+$, it should be diminished in the presence of NADase. Addition of NADase to the labeling reaction eliminated the free ^{32}P-NAD$^+$ band, which runs near the bottom of the gel, indicating that the enzyme was active in breaking down NAD$^+$ (Figure 1C, lane 4). NADase also eliminated both the self-labeling and the histone-labeling activity of Sir2, indicating that the observed labeling did not result from a chemical reaction of substrate proteins with ^{32}P-ADP-ribose (Figure 1C, lane 4). The labeling of both histones and Sir2 itself was also greatly diminished in the presence of snake venom phosphodiesterase or excess unlabeled NAD$^+$, but not unlabeled ATP (data not shown).

Recognition of a Modified Form of Sir2 by an Anti-ADP-Ribose Antibody

We noticed that GST-Sir2 fusion proteins purified from either *E. coli* or yeast migrated as doublets in SDS-polyacrylamide gels. These doublets were most easily detectable in long electrophoretic runs of GST-Sir2 preparations from *E. coli*, which contained a minor slower migrating species and a major faster migrating species. Western blots with anti-GST and anti-Sir2 antibodies recognized both bands, indicating the existence

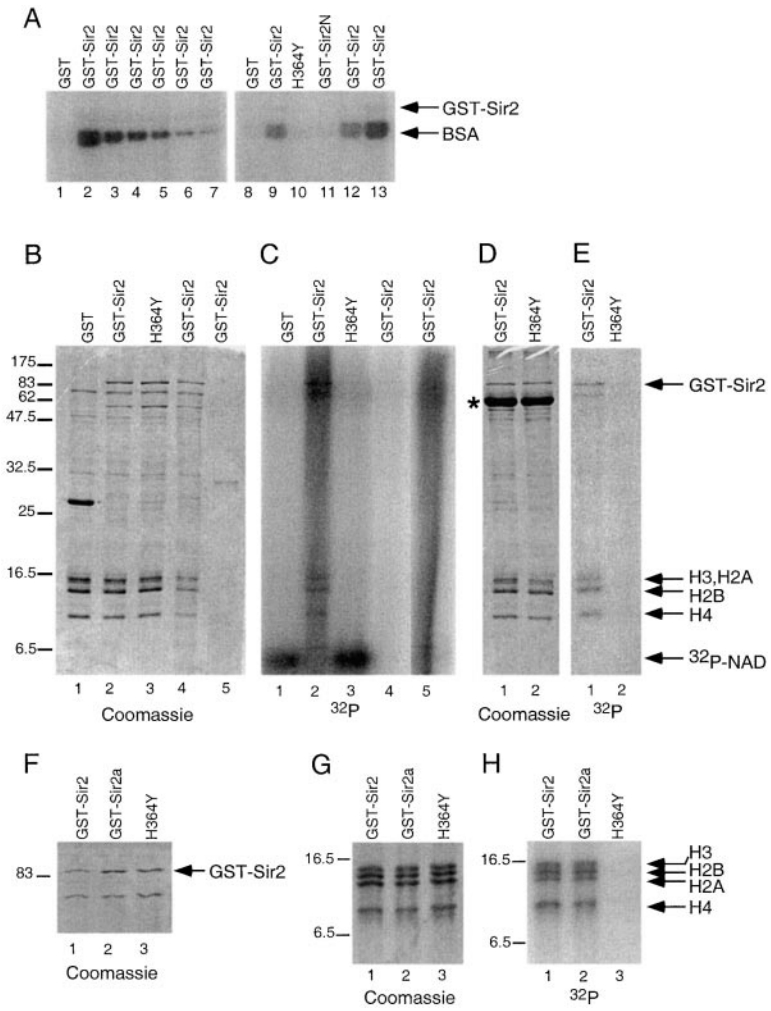


Figure 1. NAD^+-Dependent Modification of Proteins by Sir2

(A) Autoradiograms of 8.5% SDS polyacrylamide gels showing the modification of bovine serum albumin (BSA) by GST-Sir2 using ^{32}P-NAD^+ as a donor. Reactions in lanes 2–7 contain 5 μg, 2.5 μg, 1.3 μg, 0.63 μg, 0.3 μg, and 0.15 μg of BSA, respectively, and 2 μg of GST-Sir2. Lane 1 contains 5 μg of BSA and 2 μg of GST. Lanes 8–13 contain 5 μg of BSA and 0.5 μg of the indicated GST-Sir2 fusion protein except for lane 13, which contains 2.5 μg GST-Sir2.

(B–E) Modification of Sir2 and histones by GST-Sir2. (B) and (D) are Coomassie-stained 15% polyacrylamide gels showing the position of GST (B, lane 1), GST-Sir2 (B, lane 2; D, lane 1), and histones (B, lanes 1–4; D, lanes 1–2). (C) and (E) are autoradiograms of the same gels shown in (B) and (D), respectively. Reactions contained 0.5 μg of GST, GST-Sir2, or GST-Sir2-H364Y (H364Y), and 3 μg of a mixture of total histones from *Tetrahymena*. Lanes 4 (B), 5 (B), and 1 (E) show the effect of NADase, proteinase K, and TCA precipitation on the labeling reaction, respectively. TCA precipitated reactions (D and E) contained 10 μg of BSA, which was added after the completion of reactions; asterisk (*) indicates the position of BSA in the TCA precipitated reactions in (D).

(F–H) Modification of calf thymus histones by GST-Sir2. Same conditions as in (B)–(E), except that reactions contained 3 μg calf thymus histones instead of *Tetrahymena* histones. GST-Sir2 was prepared from cells containing pDM111 (lane 1, GST-Sir2) or pDM111a (lane 2, GST-Sir2a). Dash marks on the left indicate the position of prestained molecular weight markers.

of a modified form of Sir2 (Figure 2A, lane 1, and data not shown). However, the slower migrating species (GST-Sir2-s) was completely absent in Sir2-H364Y preparations that are enzymatically inactive (Figure 2A, compare lanes 1 and 2). Thus, the generation of GST-Sir2-s required the enzymatic activity of Sir2 and could result from an automodification activity of overexpressed GST-Sir2 in *E. coli*. The identity of the adduct on GST-Sir2-s may therefore indicate the nature of the enzymatic activity of Sir2. We tested whether this adduct could be recognized by an anti-mono-ADP-ribose antibody generated by Meyer and Hilz (1986). Figure 2B (lane 1) showed that an anti-ADP-ribose antiserum specifically recognized the GST-Sir2-s band but not the faster migrating GST-Sir2 band or the GST-Sir2-H364Y protein (Figure 2B, lanes 1 and 2). We note that the blot contains about 50- to 100-fold more GST-Sir2 and GST-Sir2-H364Y than GST-Sir2-s (see Figure 2A, left), verifying the specificity of the antibody for the modified form of Sir2. The anti-ADP-ribose antibody also recognized GST-Sir2 purified from yeast (Figure 2D, lane 2). The GST-Sir2 doublets in preparations from yeast were difficult to resolve on Western blots of SDS polyacrylamide gels (Figure 2C, lane 2), but could be visualized by Coomassie staining of the blots after transfer to membrane and the completion of Western detection (Figures 2C and 2D, lane 1). The band recognized by the anti-ADP-ribose antibody closely aligned with the slower migrating GST-Sir2 species on the Coomassie-stained blot (Figure 2D, lane 1). Finally, we probed Western blots containing the same GST-Sir2 fusion proteins used in the above experiments with a more sensitive and specific affinity purified anti-ADP-ribose antibody (Meyer and Hilz, 1986). This antibody also strongly recognized wild-type GST-Sir2 purified from either *E. coli* or yeast (Figure 2E, lanes 1 and 3, respectively), but did not recognize the mutant GST-Sir2-H364Y protein (Figure 2E, lane 2). Together, these results indicated that GST-Sir2-s was an ADP-ribosylated form of Sir2 and that Sir2 could auto-mono-ADP-ribosylate itself in vivo.

The Enzymatic Activity of Sir2 Is Required for Silencing at the Silent Mating–Type Loci, Telomeres, and rDNA Repeats

We next tested whether the observed enzymatic activity of Sir2 was required for its silencing function. We constructed plasmids that contained either the wild-type *SIR2* or the H364Y mutant *SIR2* gene. These plasmids were transformed into strains in which the endogenous *SIR2* gene was deleted (*sir2*Δ). We first tested whether

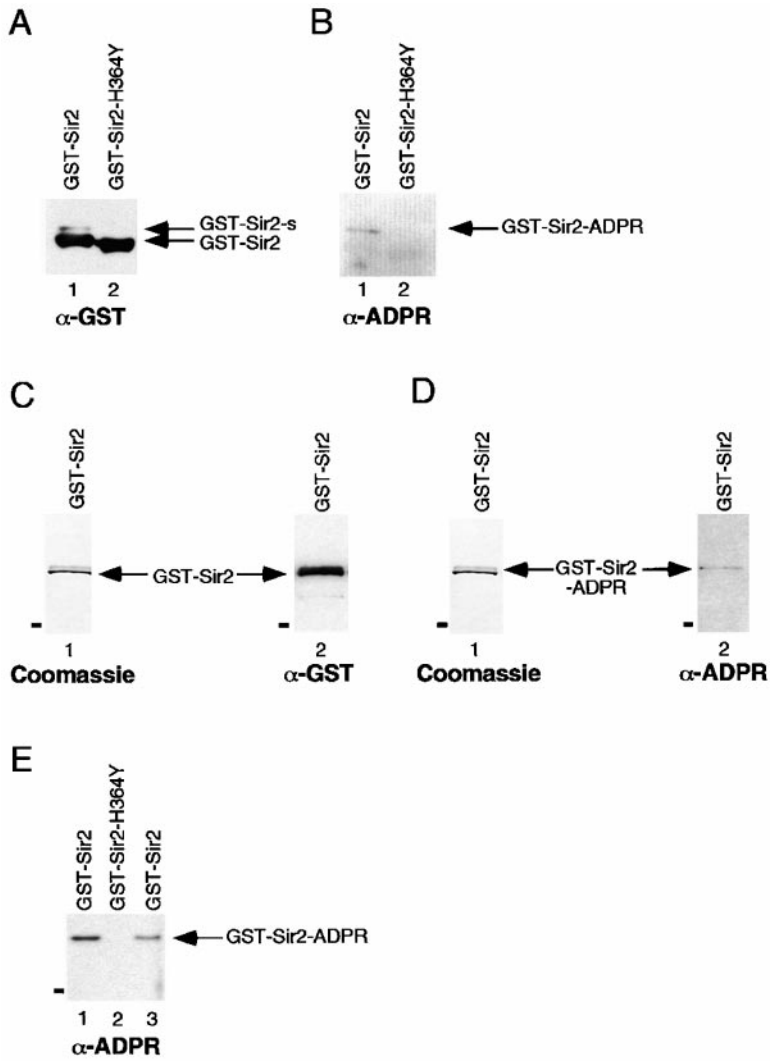


Figure 2. An Anti-Mono-ADP-Ribose Antibody Recognizes a Modified Form of Sir2

Western blots showing the detection of *E. coli* produced GST-Sir2 proteins by anti-GST (α-GST, A) and anti-ADP-ribose (α-ADPR, B) antibodies. Approximately 1 μg of each GST-Sir2 (lane 1) or GST-Sir2-H364Y (lane 2) was loaded on an 8.5% SDS polyacrylamide gel, transferred to membrane, and probed with each antibody. The slower migrating GST-Sir2-s is recognized by both α-GST (A) and α-ADPR (B), whereas the more abundant faster-migrating GST-Sir2 is only recognized by α-GST (A). In (C), GST-Sir2 was expressed in yeast under the control of the *GAL1* promoter, and after purification, 1 μg was applied to an 8.5% SDS polyacrylamide gel, blotted, and probed with either α-GST (C, lane 2) or α-ADPR (D, lane 2). The α-ADPR antibody recognized a band (D, lane 2) that runs slightly above the main GST-Sir2 band recognized by α-GST (C, lane 2) and aligns with the slower migrating Coomassie-stained GST-Sir2 band (D, lane 1). (E) Western blot showing that an affinity-purified anti-mono-ADP-ribose antibody recognizes GST-Sir2 purified from *E. coli* (lane 1) or yeast (lane 3), but not the GST-Sir2-H364Y protein (lane 2, purified from *E. coli*). In (C)–(E), "-" indicates the position of one of the prestained molecular weight markers (62 kDa) used to align Western blot signals with Coomassie-stained bands. The slight difference in the migration of the main GST-Sir2 band and the GST-Sir2-H364Y proteins (lanes 1 and 2 in A) is due to the presence of 9 additional amino acids between the GST and SIR2 open reading frames in pDM111 (see Experimental Procedures). These extra amino acids had no effect on the enzymatic activity of GST-Sir2 or its recognition by the α-ADPR antibody.

the mutant protein is expressed at a similar level as wild-type Sir2 in the cell. Western blots of yeast extracts prepared from strains containing either wild-type Sir2 or the Sir2-H364Y protein were probed with an anti-Sir2 antibody and showed that the Sir2-H364Y mutant protein was expressed to the same level as wild-type Sir2 (Figure 3A). To assess telomeric and rDNA silencing, the host strains also contained *URA3* reporter genes inserted either near a telomere or within the rDNA repeats (Gottschling et al., 1990; Smith and Boeke, 1997). In these experiments, silencing of the *URA3* reporter gene results in loss of growth on medium lacking uracil, but allows the growth of the Ura^- cells on medium that contains the compound 5-FOA, which is toxic to Ura^+ cells. While the plasmid containing the wild-type *SIR2* gene fully complemented the deletion of *SIR2* for both telomeric silencing (Figure 3B) and rDNA silencing (Figure 3C, compare *Vector* row with *pSIR2-LEU2* row), the SIR2-H364Y plasmid showed no detectable complementation activity and behaved like the vector backbone (Figures 3B and 3C, compare *Vector* row with *pH364Y-LEU2* row). These results indicated that histidine 364 was absolutely required for the telomeric and rDNA silencing functions of Sir2.

Derepression of silent mating–type information at *HML* and *HMR* results in loss of haploid cell identity and inability of haploid yeast cells to mate and form diploids (Herskowitz, 1988). The mating assay based on selection for diploid cells provides an extremely sensitive assay for assessing silencing at the silent mating–type loci. We therefore used this assay to determine whether the enzymatic activity of Sir2 is also required for mating-type silencing. The high sensitivity of the mating assay was also expected to reveal whether the Sir2-H364Y mutant protein had any residual silencing function. While the wild-type *SIR2* plasmid fully complemented the mating defect of the *sir2*Δ strain, the SIR2-H364Y plasmid showed no complementation activity (Figure 3D). Thus, histidine 364 is required for silencing at the silent mating–type loci. Together, the above experiments demonstrate that the Sir2-H364Y protein has no silencing function.

We hypothesized that the enzymatically inactive Sir2 protein may become incorporated into silencing complexes. In such a case, the mutant protein would interfere with

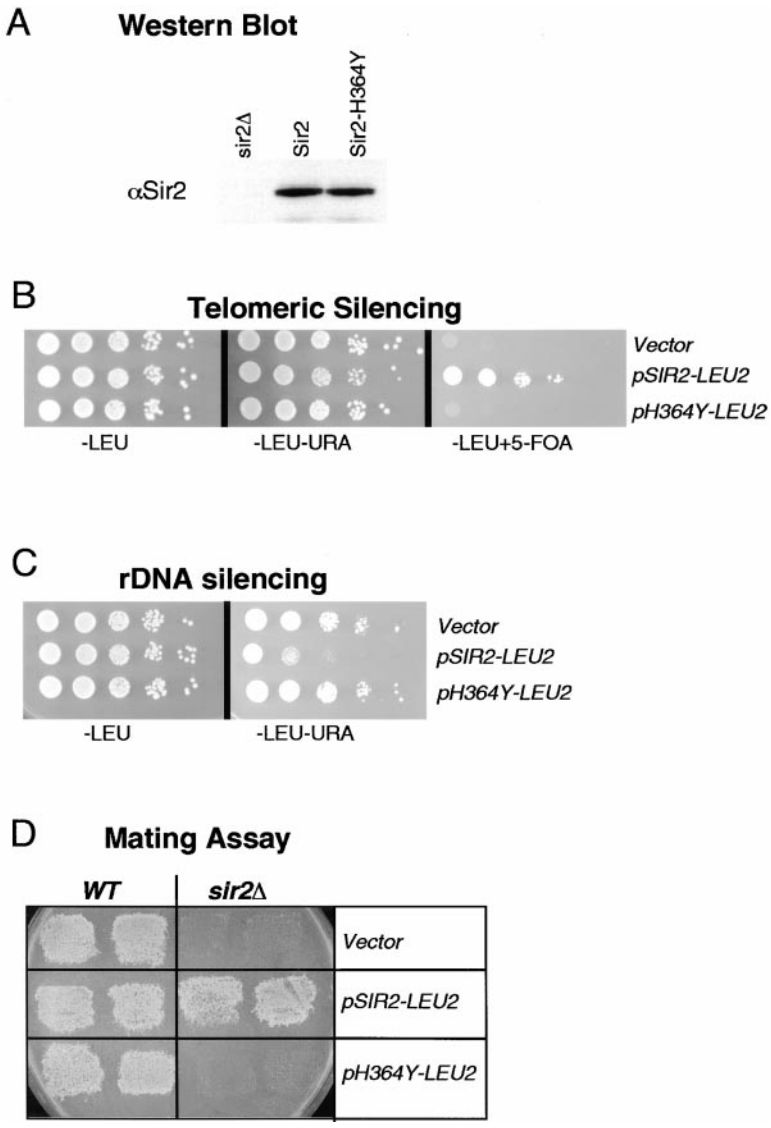


Figure 3. The Enzymatic Activity of Sir2 Is Required for Silencing at the Silent Mating Type Loci, Telomeres, and the rDNA Repeats

(A) Western blot of whole-cell yeast extracts from the same strains as those used for telomeric silencing assays in (B) probed with an anti-Sir2 antibody. *sir2*Δ, a *sir2* deletion strain transformed with a LEU2 vector; Sir2, a *sir2* deletion strain transformed pSIR2-LEU2; Sir2-H364Y, a *sir2* deletion strain transformed with pSir2-H364Y.

(B) Telomeric silencing in a *sir2*Δ strain containing a *URA3* reporter gene near a telomere (Gottschling et al., 1990). A plasmid containing the wild-type *SIR2* gene restores silencing and allows growth on 5-FOA containing media (middle row). No growth on 5-FOA medium is observed when the *sir2*Δ strain is transformed with either vector alone (top row) or the pSir2-H364Y plasmid (bottom row).

(C) Same as in (B) but with the *URA3* reporter gene located within the rDNA repeats (Smith and Boeke, 1997). The plasmid containing the wild-type *SIR2* gene restores rDNA silencing and causes loss of growth on -LEU-URA medium (C, middle row). The pH364Y-LEU2 plasmid behaves like the empty vector.

(D) A plasmid containing the H364Y mutant *SIR2* gene (pH364Y-LEU2) is unable to complement the inability of a *sir2*Δ strain to mate (bottom row).

silencing in wild-type cells by competing with wild-type Sir2 for incorporation into silencing complexes. To test this hypothesis, we expressed the Sir2-H364Y protein, either under the control of its own promoter or overexpressed from the strong *GAL1* promoter, in *SIR2*$^+$ cells. Remarkably, expression of the mutant protein under the control of its own promoter nearly abolished telomeric silencing and slightly reduced rDNA silencing in *SIR2*$^+$ cells (Figures 4A and 4B). However, rDNA silencing in *SIR2*$^+$ cells was nearly completely disrupted when Sir2-H364Y was overexpressed from the *GAL1* promoter (Figure 4C, compare *pGAL-GST-SIR2* row with *pGAL-GST-H364Y* row). This strong dominant-negative phenotype is likely to result from incorporation of an inactive Sir2 into silencing complexes, since substitution of histidine 364 with tyrosine does not interfere with the expression or stability of the mutant protein (see below, Figure 5). Furthermore, as previously reported (Holmes et al., 1997), overexpression of Sir2 from the strong *GAL1* promoter was toxic and resulted in a severe growth defect (Figure 4C, compare *Vector* row with *pGAL1-GST-SIR2* row on -LEU medium). Interestingly, overexpression of Sir2-H364Y caused only a slight growth defect, suggesting that the enzymatic activity of Sir2 contributes to its overexpression toxicity (Figure 4C).

The Enzymatically Inactive Sir2 Protein Is Incorporated into Silencing Complexes and Associates with Silent Chromatin Domains

Sir2 is associated with Sir4 and Net1 in two distinct silencing complexes that act in mating-type/telomeric and rDNA silencing, respectively (Moretti et al., 1994; Moazed et al., 1997; Strahl-Bolsinger et al., 1997; Shou et al., 1999; Straight et al., 1999). The results presented in Figure 4 suggested that Sir2-H364Y could participate in formation of both types of silencing complexes. To test this idea more directly, either wild-type or H364Y mutant Sir2 proteins were immunoprecipitated from whole-cell yeast extracts and the immunoprecipitates were tested for the presence of Sir4 and Net1. Consistent with the dominant-negative phenotype described

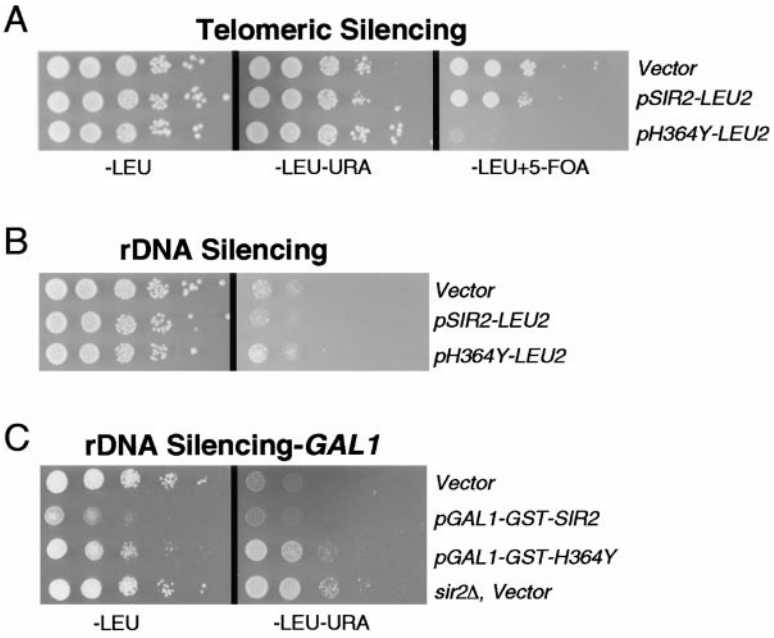


Figure 4. Disruption of Silencing by an Enzymatically Inactive Sir2 Protein

(A) *SIR2*$^+$ cells containing a *URA3* reporter gene near a telomere (Gottschling et al., 1990) were transformed with empty vector (pRS315, top row), a plasmid containing the wild-type *SIR2* gene (pSIR2-LEU2, middle row), or a *SIR2* gene encoding the H364Y mutant protein (pH364Y-LEU2, bottom row). Expression of the mutant protein disrupted telomeric silencing, resulting in loss of growth on -LEU+5-FOA medium.

(B) *SIR2*$^+$ cells containing a *URA3* reporter gene inserted within the rDNA repeats (Smith and Boeke, 1997) were transformed with the same set of plasmids as in (A) or with *GAL1-GST-SIR2* overexpression plasmids (C). Expression of H364Y Sir2 protein from its own promoter causes a slight reduction in rDNA silencing (B, compare pSIR2-LEU2 row with pH364Y-LEU2 row), but its overexpression from the *GAL1* promoter strongly disrupts rDNA silencing (C, compare pGAL1-GST-SIR2 row with pGAL1-GST-H364Y row).

above, both wild-type and Sir2-H364Y proteins immunoprecipitated Sir4 and Net1 with similar efficiencies (Figures 5A–5C). No Sir4 or Net1 was present when anti-Sir2 immunoprecipitation was carried out using an extract from a *sir2*Δ strain (Figures 5A–5C). These results indicated that the mutant protein was incorporated into silencing complexes with a similar efficiency as wild-type Sir2.

We next used the chromatin cross-linking and immunoprecipitation assay (ChIP) (Strahl-Bolsinger et al., 1997) to determine whether silencing complexes containing the enzymatically inactive Sir2-H364Y protein can associate with their usual target domains. We precipitated formaldehyde cross-linked soluble chromatin from *sir2*Δ strains expressing plasmid encoded wild-type Sir2 or mutant Sir2-H364Y proteins. *SIR2*$^+$ and *sir2*Δ strains were used as controls. As previously reported (Gotta et al., 1997; Strahl-Bolsinger et al., 1997), immunoprecipitation with an anti-Sir2 antibody followed by PCR analysis showed that Sir2 was associated with

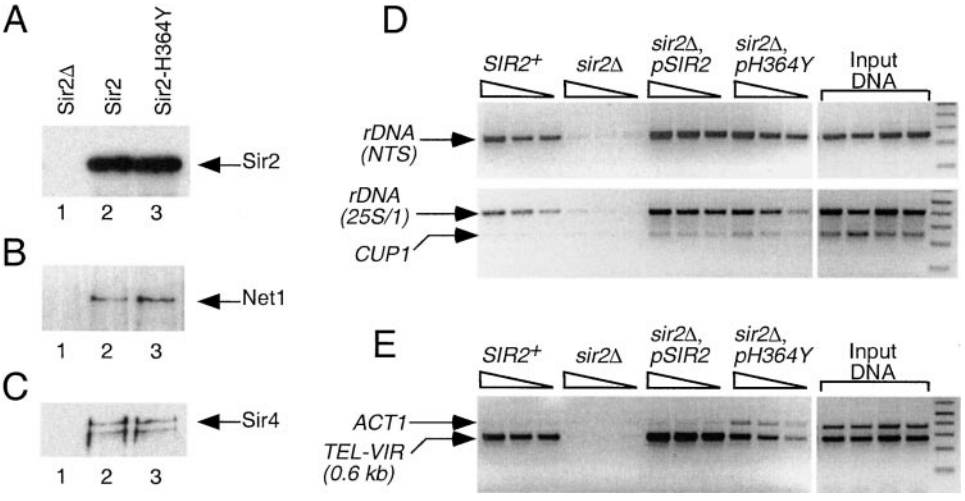
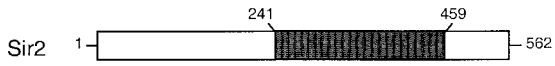


Figure 5. The Enzymatically Inactive Sir2 Is Incorporated into Silencing Complexes and Is Associated with Chromatin

(A–C) The Sir2 protein was immunoprecipitated from *sir2*Δ strains containing a plasmid backbone (lane1), a plasmid expressing the wild-type Sir2 protein (lane2), or the Sir2-H364Y mutant protein (lane 3). Western blots were probed with anti-Sir2 (A), anti-Net1 (B), and anti-Sir4 (C) antibodies.

(D and E) Immunoprecipitation of chromatin from formaldehyde cross-linked cells using an anti-Sir2 antibody showing the association of Sir2 and Sir2-H364Y proteins with rDNA (D) and telomeric DNA regions (E). PCR amplification was performed with 2.5-fold serial dilution of immunoprecipitated DNA corresponding to 1/50th, 1/125th, and 1/250th of immunoprecipitated DNA. Input DNA represents PCR amplification of approximately 1/10,000th of crude chromatin used for each immunoprecipitation reaction.



^{255}**R**KILV**L**T**GAG**V**S**TSL**GI**PD**FR**^{275}.....^{343}**TQNID**N**L**ESY**AG**ISTDKLVQC**HG**S^{366}

Figure 6. Schematic Diagram Showing the Location of the Conserved Core Domain of Sir2

Shaded region, amino acids 241 to 459, is conserved among Sir2-like proteins (see Brachmann et al., 1995 and Sherman et al., 1999 for detailed alignments). The highly conserved region between amino acids 255 to 275 has weak similarity to part of the NAD^+ binding domain in 6-phosphogluconate dehydrogenases. The asterisk (*) indicates the location of the invariant histidine 364; mutation of this residue abolishes the enzymatic activity of Sir2 and its silencing function. Bold letters indicate residues that are invariant or highly conserved in all Sir2-like proteins. See text for further discussion.

silent chromatin regions within the rDNA cluster and near a telomere, but not with active genes such as *ACT1* and *CUP1* (Figures 5D and 5E, and data not shown). The Sir2-H364Y protein was also associated with DNA fragments corresponding to silent chromatin domains but with a lower efficiency than wild-type Sir2. Compared to wild-type Sir2, the efficiency of coprecipitation of DNA fragments with the Sir2-H364Y protein was reduced about 2.5-fold for rDNA fragments (Figure 5D) and about 7-fold for telomeric DNA fragments (Figure 5E). Because silencing is completely abolished in H364Y mutant strains but the Sir2-H364Y protein is still associated with rDNA and telomeric chromatin, we conclude that the enzymatic activity of Sir2 is required for a post-initiation step in assembly of silent chromatin.

Discussion

The experiments reported here demonstrate that the yeast Sir2 protein has an enzymatic activity that uses NAD^+ as a donor to covalently modify protein substrates. In addition to modifying itself, Sir2 can modify the core histones in vitro, suggesting that histones may be among the in vivo targets of Sir2. The identity of the donor compound (NAD^+) and the recognition of a modified form of Sir2, which requires enzymatically active Sir2 for its generation, by an anti-ADP-ribose antibody suggest that the relevant enzymatic activity is ADP-ribosylation. We further show that the loss of this enzymatic activity correlates with the loss of silencing at the silent mating–type loci, telomeric DNA regions, and the rDNA repeats. These results provide an example of a chromatin-associated enzymatic activity that is absolutely required for transcriptional repression.

ADP-ribosylation involves the transfer of an ADP-ribose moiety from NAD^+ to acceptor amino acids in protein substrates (Ueda and Hayaishi, 1985). The most prevalent form of this modification is mono-ADP-ribosylation, which involves the transfer of a single ADP-ribose moiety to amino acid side chains, typically to form an N-glycosidic bond with either arginine or asparagine and in certain cases with a modified amino acid side chain, diphthamide. The best characterized mono-ADP-ribosylation enzymes are bacterial toxins, such as diphtheria and cholera toxins, which interfere with translation and regulation of cAMP levels, respectively. A second class of enzymes in this family are poly(ADP)ribosylation enzymes (PARPs), which have only been identified in multicellular eukaryotes (Ueda and Hayaishi, 1985). PARPs usually catalyze the addition of poly(ADP)ribose chains via an O-glycosidic bond to either a glutamic acid side chain or the polypeptide carboxy terminus. Three lines of evidence suggest that Sir2 is a mono-ADP-ribosyltransferase. First, a modified form of Sir2 could be recognized by an antibody that has much higher affinity for mono-ADP-ribose than for poly(ADP)-ribose chains (Meyer and Hilz, 1986). Second, modification of proteins by Sir2 causes only a slight shift in their mobility in SDS-polyacrylamide gels, even when high concentrations of NAD^+ are included in the reaction. This is in contrast to dramatic shifts observed in the mobilities of proteins that are poly(ADP)ribosylated (Ueda and Hayaishi, 1985; Smith et al., 1998). Third, all known poly(ADP)ribose polymerases are inhibited by 3-aminobenzamide (Ueda and Hayaishi, 1985), which has no effect on the enzymatic activity of Sir2 (D. M., unpublished data).

If Sir2 uses NAD^+ as a cofactor to modify histones or other proteins in a step that is required for silencing, as the results presented here suggest, then defects in NAD^+ biosynthesis resulting in changes in the intracellular levels of NAD^+ may be expected to affect the efficiency of silencing in yeast. Indeed, Smith et al. have discovered that deletion of NPT1, encoding an enzyme that catalyses the formation of nicotinic acid mononucleotide, a precursor in NAD^+ biosynthesis, disrupts silencing in yeast (J. Boeke, personal communication). These results suggest that the efficiency of silencing is extremely sensitive to a reduction in the intracellular pools of NAD^+.

Sir2-like proteins have no obvious sequence similarity to any of the known ADP-ribosyltransferases and appear to define a distinct class of enzymes. All proteins in this class contain a conserved 250 amino acid region, which corresponds to the C-terminal domain of yeast Sir2 (Brachmann et al., 1995) (Figure 6). The enzymatic activity reported here and observed by Frye (1999) lies within this domain of Sir2. Interestingly, a 39 amino acid region within the core domain, centered around the conserved GAGxSxxxG sequence (Figure 6), is weakly similar to a 39 amino acid region of bacterial 6-phosphogluconate dehydrogenases (33% identical, 56% similar), which use $NADP^+$ (and with much lower efficiency, NAD^+) as a cofactor to catalyze the conversion of 6-phosphogluconate to ribulose 5-phosphate (Bisercic et al., 1991). The NAD^+-binding site in dehydrogenases is composed of two similar domains that bind to the nicotinamide and adenine moieties in NAD^+. The similarity between Sir2 and these dehydrogenases appears to be limited to the adenine-binding half of the NAD^+-binding domain (Phillips et al., 1998). The histidine that is required for the enzymatic activity of Sir2 is conserved in all Sir2-like proteins and is usually followed by glycine and serine or threonine (Figure 6). We note that the active sites

Table 1. List of Yeast Strains Used in This Study

Strain	Genotype	Source
Y1 (JRY2334)	*MAT***a** *ade2-1 can1-100 his3-11 leu2-3.112 trp1 ura3-1 GAL*	J. Rine
Y3 (JRY3433)	Y1, *sir2::HIS3*	J. Rine
Y10 (BJ5459)	*MAT***a** *ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*	E. Jones
Y235 (UCC1001)	*MAT***a** *ura3-52 lys2-801 ade2-1010 trp1Δ his3-Δ200 leu2-Δ1 TEL adh4::URA3*	D. Gottschling
Y1195	Y235, pRS315	This study
Y1197	Y235, pSIR2-LEU2	This study
Y1199	Y235, pH364Y-LEU2	This study
Y1240	Y235, pGST-LEU2	This study
Y1242	Y235, pGST-SIR2-LEU2	This study
Y1244	Y235, pGST-H364Y-LEU2	This study
Y1201	Y235, *sir2*Δ, pRS315	This study
Y1203	Y235, *sir2*Δ, pSIR2-LEU2	This study
Y1205	Y235, *sir2*Δ, pH364Y-LEU2	This study
Y1218	Y235, *sir2*Δ, pGST-LEU2	This study
Y1220	Y235, *sir2*Δ, pGST-SIR2-LEU2	This study
Y1222	Y235, *sir2*Δ, pGST-H364Y-LEU2	This study
Y236 (UCC1003)	*MAT***a** *ura3-52 lys2-801 ade2-1010 trp1Δ his3-Δ200 leu2-Δ1 adh4::URA3*	D. Gottschling
Y1236	Y236, pRS315	This study
Y480 (JS128)	*MAT***a** *his3Δ200 leu2Δ1 ura3-167 RDN1::Ty1-mURA3*	J. Smith and J. Boeke
Y1206	Y480, pRS315	This study
Y1208	Y480, pSIR2-LEU2	This study
Y1210	Y480, pH364Y-LEU2	This study
Y1224	Y480, pGST-LEU2	This study
Y1226	Y480, pGST-SIR2-LEU2	This study
Y1228	Y480, pGST-H364Y-LEU2	This study
Y1230	Y480, *sir2*Δ, pGST-LEU2	This study
Y1232	Y480, *sir2*Δ, pGST-SIR2-LEU2	This study
Y1234	Y480, *sir2*Δ, pGST-H364Y-LEU2	This study
Y1212	Y480, *sir2*Δ, pRS315	This study
Y1214	Y480, *sir2*Δ, pSIR2-LEU2	This study
Y1216	Y480, *sir2*Δ, pH364Y-LEU2	This study
Y478 (JS122)	*MAT***a** *his3Δ200 leu2Δ1 ura3-167 ???::TY1-mURA3*	J. Smith and J. Boeke
Y1238	Y478, pRS315	This study

of many poly- and mono-ADP-ribosylases contain an HGS/T sequence, where the histidine side chain hydrogen bonds with the 3′-hydroxyl group of the adenylate ribose in NAD^+ (Ruf et al., 1998; Kickhoefer et al., 1999). The histidine residue at position 364 of Sir2 may play a similar role in binding NAD^+. The significance of these limited sequence similarities, if any, will only become clear when the crystal structure of a Sir2-like protein is determined.

Previous studies have demonstrated that the Sir2 protein is a component of silent chromatin regions at the mating-type loci, telomeric DNA regions, and the rDNA repeats (Gotta et al., 1997; Strahl-Bolsinger et al., 1997). Sir2 also appears to be able to act more globally when it is overexpressed. Thus, overexpression of Sir2 from the yeast *GAL1* promoter causes a severe growth defect and reduced viability (Holmes et al., 1997). The basis for this toxicity is unknown, but it is accompanied by a general decrease in histone acetylation (Braunstein et al., 1993) and an increase in the rate of chromosome loss (Holmes et al., 1997), suggesting that Sir2 toxicity results from a global alteration in chromosome structure. One exciting possibility is that ADP-ribosylation of histones by Sir2 directly inhibits histone acetylation by preventing histone acetylases from gaining access to their recognition sites in histones and that this is the underlying cause of Sir2 toxicity. Consistent with this idea, overexpression of the enzymatically inactive Sir2 protein had little effect on growth (see Figure 4C).

What role might the enzymatic activity of Sir2 play in gene silencing? We have shown that the enzymatically inactive Sir2-H364Y protein can assemble into silencing complexes with Sir4 and Net1, respectively, and that the mutant protein is associated with the same DNA domains as wild-type Sir2. These results suggest that Sir2 modifies a chromatin component after its recruitment to DNA and that this modification is either directly or indirectly responsible for silencing. A first step in understanding the role of this modification is the identification of the true physiological substrates of Sir2. Among the candidate targets of Sir2 are the histones, which play a pivotal role in the assembly of silent chromatin domains (Kayne et al., 1988; Hecht et al., 1995, 1996; Bryk et al., 1997), and other proteins that are required for silencing. Sir2 might act on a common target that is involved in silencing at both mating-type/telomeric and rDNA regions, or it might modify different targets, depending on the locus to which it is recruited. The former possibility points to histones and Sir2 itself as the likely targets of Sir2, as both histones and Sir2 are involved in silencing at all of the silent loci. Although the activity of Sir2 under the reaction conditions employed in our experiments is rather promiscuous, histones and Sir2 itself were among the best substrates for its enzymatic activity in our in vitro labeling experiments. Furthermore, a fraction of overexpressed Sir2 protein purified from yeast is an ADP-ribosylated form that requires enzymatically active Sir2 for its generation. These

results identify Sir2 itself as one target of Sir2 activity, but the physiological consequences of this self-modification and whether Sir2 acts on histones in vivo remain to be determined. An exciting possibility is that the recruitment of Sir2 to sites that initiate silencing activates its enzymatic activity in a step that requires an interaction between Sir2 and histones and involves the auto-ADP-ribosylation of Sir2. Subsequently, Sir2 may modify histones or other silencing factors to promote gene silencing. Understanding the precise function of the enzymatic activity of Sir2 and its effect on chromatin structure should provide valuable lessons on how covalent modification of chromatin by mono-ADP-ribosylation can regulate gene expression.

Experimental Procedures

Strains, Plasmids, and Other Reagents

All the yeast strains used in this study are listed in Table 1. pGAL-GST was constructed by ligation of an XbaI/BamHI fragment containing the *GAL1* promoter driving GST from pRD56 (kind gift of Ray Deshaies, California Institute of Technology) into XbaI/BamHI-digested pRS315. The entire *SIR2* open reading frame was PCR amplified as an EcoRI fragment and ligated into the EcoRI site of pRD56 in frame with the C terminus of GST to generate pDM114a. A XbaI/XhoI fragment containing GAL1-GST-SIR2 was then ligated into XbaI/XhoI-digested pRS315 to generate pGAL1-GST-SIR2. The H364Y mutation was generated by overlap PCR as follows: SIR2 fragments were generated using the overlapping primers JT7 (5′ GTGCTATGGCTCTTTTGCTAC 3′) and JT8 (5′ GCCATAGCACTG CACCAGTTTATC 3′) and the outside primers JT9c (5′ GGCGAATTC GGAAGTGCAATATCTGTC 3′) and JT10 (5′ CGCGAATTCATCAC GATTAATCAGGACTTG 3′). Underlined letters indicate the position at which a point mutation was introduced. Separate PCR reactions were performed with primer pairs JT7/JT10 and JT8/JT9c (30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min). Portions of the resulting PCR products were then mixed and reamplified with primers JT9c and JT10. The final PCR product containing the appropriate point mutation was digested with StuI and BglII and used to replace a StuI/BglII fragment in pGAL/GST-Sir2 to generate pGAL-GST-H364Y. A BglII–StuI fragment encoding the C terminus of Sir2 and containing the H364Y mutation was then subcloned into the BglII–StuI sites of pSIR2-LEU2, which contains the entire SIR2 genomic region in the pRS315 backbone, to generate pH364Y/LEU2. All PCR-generated regions were entirely sequenced to ensure the absence of PCR-introduced errors. The entire SIR2 open reading frame was PCR amplified as an EcoRI fragment and subcloned into pGEX-1B to generate a GST-Sir2 fusion protein (pDM111). pGEX-1B is a derivative of pGEX-1 (Smith and Johnson, 1988) containing an insertion within its BamHI site that encodes thrombin and heart/muscle kinase recognition sites (D. M., unpublished data). pDM360 was constructed by subcloning the EcoRI–XhoI *SIR2/H364Y* fragment from pGAL1-GST-H364Y/LEU2 into pGEX-4T-1 (to make GST-Sir2-H364Y). GST fusion proteins constructed using pGEX-1B are 9 amino acids longer than GST fusion proteins constructed using pGEX-4T-1 (due to insertion of the heart muscle kinase site). In order to generate GST-Sir2 fusion proteins that were identical except for a single amino acid substitution at position 364 of Sir2, the BglII–XhoI fragment at the C terminus of *SIR2* in pDM360 (containing the H364Y mutation) was replaced with the corresponding wild-type *SIR2* BglII–XhoI fragment to generate pDM111a.

Purification of GST-Sir2 and GST-Sir2-H364Y

GST-Sir2 and GST-Sir2-H364Y were purified from DH5a cells transformed with pDM111a (or pDM111) and pDM360, respectively, as previously described (Moazed and Johnson, 1996). Identical yields were obtained for GST-Sir2 and GST-Sir2-H364Y. GST-SIR2 and GST-Sir2-H364Y proteins were also purified from *sir2*Δ yeast strains expressing each protein under the control of the *GAL1* promoter (Y3 transformed with pGAL-GST-Sir2 and pGAL-GST-H364Y, respectively) as previously described (Moazed et al., 1997).

Enzyme Activity Assays

Reactions were carried out in a volume of 10 μl containing 0.5 μg to 2 μg of each GST fusion protein, 0.1 μg to 5 μg of BSA, or 3 μg of calf thymus (Boehringer Mannheim) or *Tetrahymena* (generous gift of Nima Mossamaparast and David Allis) histones as indicated, and 3 μCi of ^{32}P-NAD$^+$ (1000 Ci/mmol, Amersham Pharmacia) in 50 mM Tris-HCl, pH 8.0 (at 22°C), 150 mM NaCl, 10 mM DTT. GST-Sir2 proteins were also active in reaction buffers containing 50 mM Glycine-KOH, pH 9.0, or 50 mM HEPES-KOH, pH 7.6 (Frye, 1999), instead of Tris-HCl. After incubation for 1 hr at 37°C, reactions were stopped by the addition of 90 μl ice-cold 22% trichloroacetic acid (TCA), incubated on ice for 15 min, and centrifuged at 13,000 g for 15 min. The pellets were resuspended in 20 μl 1.5× SDS sample buffer, heated to 100°C for 3 min, and 10 μl was loaded onto 8.5% or 15% SDS polyacrylamide gels. Ten micrograms of BSA was added to some reactions as a carrier before TCA precipitation. Where indicated, NADase (1 μl, 50 units/ml, Sigma) was added to the reactions at the same time as Sir2, and proteinase K (1 μl, 2 mg/ml, Boehringer Mannheim) was added at the end of the reaction with an additional incubation time of 30–60 min at 37°C. After electrophoresis, polyacrylamide gels were stained with Coomassie brilliant blue, destained, dried on Whatman paper, and exposed to Kodak XAR5 film for 3–12 hr at room temperature.

Silencing Assays

Telomeric and rDNA silencing assays were performed as previously described (Aparicio et al., 1991; Smith and Boeke, 1997). Cultures of the appropriate synthetic selective media were inoculated with overnight cultures of desired yeast strains and grown to log phase (A660 of about 1) at 30°C. Three microliters of 10-fold serial dilutions of each culture (in water) were spotted onto SD-LEU, SD-LEU-URA, and SD-LEU+5-FOA plates. For galactose induction dextrose in the medium was replaced with galactose. Plates were photographed after 2–3 days of growth at 30°C. Mating assays were performed as previously described (Sprague, 1991).

Immunoprecipitation Reactions and Western Detection

One liter cultures of yeast strains (Y1201, Y1203, and Y1205) were grown to mid-log phase in SD-LEU medium (A660 of 1.5, approximately 3 g/l). Cells were harvested by centrifugation, washed with ice-cold water, and flash frozen in liquid nitrogen. Cell lysis, immunoprecipitation, and transfer to PVDF membranes were performed essentially as described previously (Moazed et al., 1997). Membranes were blocked with 5% nonfat dry milk in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20 and probed with 1:5000 dilution of rabbit anti-Sir2 and anti-Sir4 affinity-purified antibodies (D. M., unpublished data), and 1:1000 dilution of a rabbit anti-Net1 antibody (J. H. and D. M., unpublished data). Secondary detection was performed using horseradish peroxidase coupled donkey anti-rabbit antibodies (Amersham) and the Renaissance enhanced chemiluminescence detection system (NEN).

ChIP experiments were carried out as previously described (Strahl-Bolsinger et al., 1997) using a monoclonal anti-Sir2 antibody. Primers and other details were as described in Straight et al. (1999).

For detection of ADP-ribosylated forms of Sir2, approximately 1 μg of GST-Sir2 fusion proteins purified from either yeast or *E. coli* were loaded onto 8.5% SDS polacrylamide gels, and after electrophoresis, blotted onto PVDF membranes. Several identical lanes were run on the same gel, the resulting blot was then cut into several longitudinal pieces, which were then probed separately with affinity-purified rabbit anti-ADP-ribose (1:10,000 dilution, Meyer and Hilz, 1986), anti-GST (1:5,000), or anti-Sir2 (1:5,000) antibodies. After incubation with secondary antibodies and detection by chemiluminescence (described above), blots were stained with Coomassie, and the Coomassie-stained bands and Western signals were aligned using stained molecular weight markers as standards.

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References

Aparicio, O.M., Billington, B.L., and Gottschling, D.E. (1991). Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. Cell *66*, 1279–1287.

Bisercic, M., Feutrier, J.Y., and Reeves, P.R. (1991). Nucleotide sequences of the gnd genes from nine natural isolates of Escherichia coli: evidence of intragenic recombination as a contributing factor in the evolution of the polymorphic gnd locus. J. Bacteriol. *173*, 3894–3900.

Brachmann, C.B., Sherman, J.M., Devine, S.E., Cameron, E.E., Pillus, L., and Boeke, J.D. (1995). The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. Genes Dev. *9*, 2888–2902.

Braunstein, M., Rose, A.B., Holmes, S.G., Allis, C.D., and Broach, J.R. (1993). Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev. *7*, 592–604.

Bryk, M., Banerjee, M., Murphy, M., Knudsen, K.E., Garfinkel, D.J., and Curcio, M.J. (1997). Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast. Genes Dev. *11*, 255–269.

Foss, M., McNally, F.J., Laurenson, P., and Rine, J. (1993). Origin recognition complex (ORC) in transcriptional silencing and DNA replication in S. cerevisiae. Science *262*, 1838–1844.

Fritze, C.E., Verschueren, K., Strich, R., and Easton Esposito, R. (1997). Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA. EMBO J. *16*, 6495–6509.

Frye, R.A. (1999). Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. Biochem. Biophys. Res. Commun. *260*, 273–279.

Golderer, G., and Grobner, P. (1991). ADP-ribosylation of core histones and their acetylated subspecies. Biochem. J. *277*, 607–610.

Gotta, M., Strahl-Bolsinger, S., Renauld, H., Laroche, T., Kennedy, B.K., Grunstein, M., and Gasser, S.M. (1997). Localization of Sir2p: the nucleolus as a compartment for silent information regulators. EMBO J. *16*, 3243–3255.

Gottschling, D.E., Aparicio, O.M., Billington, B.L., and Zakian, V.A. (1990). Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription. Cell *63*, 751–762.

Hecht, A., Laroche, T., Strahl-Bolsinger, S., Gasser, S.M., and Grunstein, M. (1995). Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. Cell *80*, 583–592.

Hecht, A., Stahl-Bolsinger, S., and Grunstein, M. (1996). Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. Nature *383*, 92–96.

Herskowitz, I. (1988). Life Cycle of the Budding Yeast *Saccharomyces cerevisiae*. Microbiol. Rev. *52*, 536–553.

Holmes, S.G., Rose, A.B., Steuerle, K., Saez, E., Sayegh, S., Lee, Y.M., and Broach, J.R. (1997). Hyperactivation of the silencing proteins, Sir2p and Sir3p, causes chromosome loss. Genetics *145*, 605–614.

Kayne, P.S., Kim, U.J., Han, M., Mullen, J.R., Yoshizaki, F., and Grunstein, M. (1988). Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell *55*, 27–39.

Kickhoefer, V.A., Siva, A.C., Kedersha, N.L., Inman, E.M., Ruland, C., Streuli, M., and Rome, L.H. (1999). The 193-kD vault protein, VPARP, is a novel Poly(ADP-ribose) polymerase. J. Cell Biol. *146*, 917–928.

Klar, A.J.S., Fogel, S., and MacLeod, K. (1979). *MAR1*—a regualtor of HMa and HMα loci in *Saccharomyces cerevisiae*. Genetics *93*, 37–50.

Kun, E., Chang, A.C., Sharma, M.L., Ferro, A.M., and Nitecki, D. (1976). Covalent modification of proteins by metabolites of NAD^+. Proc. Natl. Acad. Sci. USA *73*, 3131–3135.

Loo, S., and Rine, J. (1994). Silencers and domains of generalized repression. Science *264*, 1768–1771.

Lustig, A.J. (1998). Mechanisms of silencing in Saccharomyces cerevisiae. Curr. Opin. Genet. Dev. *8*, 233–239.

Meyer, T., and Hilz, H. (1986). Production of anti-(ADP-ribose) antibodies with the aid of a dinucleotide-pyrophosphatase-resistant hapten and their application for the detection of mono(ADP-ribosyl)ated polypeptides. Eur. J. Biochem. *155*, 157–165.

Moazed, D., and Johnson, A.D. (1996). A deubiquitinating enzyme interacts with SIR4 and regulates silencing in S. cerevisiae. Cell *86*, 667–677.

Moazed, D., Kistler, A., Axelrod, A., Rine, J., and Johnson, A.D. (1997). Silent information regulator protein complexes in Saccharomyces cerevisiae: a SIR2/SIR4 complex and evidence for a regulatory domain in SIR4 that inhibits its interaction with SIR3. Proc. Natl. Acad. Sci. USA *94*, 2186–2191.

Moretti, P., Freeman, K., Coodly, L., and Shore, D. (1994). Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. Genes Dev. *8*, 2257–2269.

Nasmyth, K.A., Tatchell, K., Hall, B.D., Astell, C., and Smith, M. (1981). A position effect in the control of transcription at yeast mating type loci. Nature *289*, 244–250.

Phillips, C., Dohnalek, J., Gover, S., Barrett, M.P., and Adams, M.J. (1998). A 2.8 A resolution structure of 6-phosphogluconate dehydrogenase from the protozoan parasite Trypanosoma brucei: comparison with the sheep enzyme accounts for differences in activity with coenzyme and substrate analogues. J. Mol. Biol. *282*, 667–681.

Rine, J., and Herskowitz, I. (1987). Four genes responsible for a position effect on expression from HML and HMR in Saccharomyces cerevisiae. Genetics *116*, 9–22.

Ruf, A., de Murcia, G., and Schulz, G.E. (1998). Inhibitor and NAD^+ binding to poly(ADP-ribose) polymerase as derived from crystal structures and homology modeling. Biochemistry *37*, 3893–3900.

Sherman, J.M., Stone, E.M., Freeman-Cook, L.L., Brachmann, C.B., Boeke, J.D., and Pillus, L. (1999). The conserved core of a human SIR2 homologue functions in yeast silencing. Mol. Biol. Cell *10*, 3045–3059.

Shore, D., and Nasmyth, K. (1987). Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. Cell *51*, 721–732.

Shou, W., Seol, J., Shevchenko, A., Baskerville, C., Moazed, D., Jiang, J., Chen, S., Charbonneau, H., Shenvchenko, A., and Deshaies, R.J. (1999). The termination of telophase is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from the nucleolar RENT complex. Cell *97*, 233–244.

Smith, D.B., and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene *67*, 31–40.

Smith, J.S., and Boeke, J.D. (1997). An unusual form of transcriptional silencing in yeast ribosomal DNA. Genes Dev. *11*, 241–254.

Smith, S., Giriat, I., Schmitt, A., and de Lange, T. (1998). Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. Science *282*, 1484–1487.

Soman, G., Haregewoin, A., Hom, R.C., and Finberg, R.W. (1991). Guanidine group specific ADP-ribosyltransferase in murine cells. Biochem. Biophys. Res. Commun. *176*, 301–308.

Sprague, G.F. (1991). Assay of yeast mating reaction. In Guide to Yeast Genetics and Molecular Biology, C. Guthrie and G.R. Fink, eds. (San Diego: Academic Press), pp. 77–93.

Strahl-Bolsinger, S., Hecht, A., Luo, K., and Grunstein, M. (1997). SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes Dev. *11*, 83–93.

Straight, A.F., Shou, W., Dowd, G.J., Turck, C.W., Deshaies, R.J., Johnson, A.D., and Moazed, D. (1999). Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. Cell *97*, 245–256.

Tsang, A.W., and Escalante-Semerena, J.C. (1998). CobB, a new member of the SIR2 family of eucaryotic regulatory proteins, is required to compensate for the lack of nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase activity in cobT mutants during cobalamin biosynthesis in Salmonella typhimurium LT2. J. Biol. Chem. *273*, 31788–31794.

Ueda, K., and Hayaishi, O. (1985). ADP-ribosylation. Annu. Rev. Biochem. *54*, 73–100.