Genes positioned close to telomeres in yeast are silenced by a heterochromatin-like structure containing Sir proteins. To investigate whether silencing also affects DNA repair, we studied removal of UV lesions by photolyase and nucleotide excision repair (NER) in strains containing the URA3 gene inserted 2 kilobases from a telomere. URA3 was transcriptionally active in sir3Δ mutants, partially silenced in SIR3 cells, or completely silenced by overexpression of SIR3 or deletion of RPD3. The active URA3 showed efficient repair by both pathways. Fast repair of the promoter and 3′ end by photolyase reflected a non-nucleosomal structure. Partial silencing had no remarkable effect on photolyase but reduced repair by NER, indicating differential accessibility for the two repair reactions. Complete silencing inhibits NER and photolyase in the coding region as well as in the promoter and the 3′-end. Conventional nuclease footprinting analyses revealed subtle changes in the promoter proximal nucleosome under partially silenced conditions but a pronounced reorganization of chromatin extending over the whole gene in silenced chromatin. Thus, both repair systems are sensitive to chromatin changes associated with silencing and provide direct evidence for a compact structure of heterochromatin.

Silencing refers to transcriptional inhibition characterized by the epigenetic formation of a repressive chromatin structure, frequently referred to as heterochromatin. In yeast Saccharomyces cerevisiae, silencing occurs at several genetic loci, including the cryptic mating-type loci (HML and HMR), the ribosomal DNA, and regions close to telomeres (1–4). Silencing of genes integrated in subtelomeric regions decreases with increasing distance from the telomere (telomere position effect) (5). In contrast to stable silencing at the HM loci, silencing of subtelomeric genes is variegated, resulting in stochastic patterns of repression of transcription (6–9).

Telomeric silencing depends on numerous proteins: three silent information regulators (Sir2, Sir3, and Sir4), histones, proteins required for chromatin assembly, proteins involved in telomere formation as well as enzymes that modify histones by deacetylation, ubiquitination, and methylation (5–7, 10–16). Silencing is enforced by the proximity to a pool of concentrated Sir proteins, clustered telomeres, and perinuclear localization (17–20). Perinuclear localization appears to be necessary, albeit not sufficient, for silencing (21). Current models propose a stepwise formation of telomeric heterochromatin (2, 4, 22). Rap1 binds to telomeric Rap1-binding sites and recruits Sir4. Sir4 recruits Sir3 and the NAD-dependent histone deacetylase Sir2. Sir2 deacetylates histone H4 at Lys-16, which allows Sir3 binding to nucleosomes. Sir3 recruits more Sir4 onto nucleosomes, and the process is repeated as the Sir complex spreads along chromatin away from the initiation site (23, 24). In addition, telomeric chromatin appears to be further stabilized by folding back of the telomere, which allows additional Rap1-Sir and Sir-Sir interactions (8, 22, 25). The extent of spreading of the Sir complex is regulated in part by the concentration of Sir3 (6) and may be counteracted in adjacent euchromatic regions by acetylation of histone H4 Lys-16 by histone acetyltransferase Sas2 (26, 27). Deletion of the histone deacetylase RPD3 as well as overexpression of SIR3 enhance telomeric silencing (6, 14).

It is widely believed that Sir-mediated silencing involves a compaction of the chromatin fiber into a more condensed form, which is refractory to the action of RNA polymerase and transcription factors. However structural information on heterochromatin is scarce and to some extent controversial. Immunoprecipitation experiments revealed hypoacetylated histones in silenced chromatin (8, 28, 29), which is consistent with the idea of a more compact chromatin structure. Nuclease digestion studies revealed arrays of positioned nucleosomes in HMR and HML that were dependent on SIR3 and the N-terminal tails of histone H4 (30, 31), suggesting that a special arrangement of nucleosomes might be required for silencing. With respect to telomeric silencing it was shown that deletion of SIR3 altered the chromatin structure in the promoter region of a retrotransposon at the left telomere of chromosome III (32). More information is available on the URA3 gene, which serves as a reporter for silencing in subtelomeric regions (5). Nuclease digestions revealed that a silenced URA3 gene positioned at telomere VII-L adopted a normal chromatin structure despite silencing (33). However, a unique site in the coding region of URA3 was largely resistant to Escherichia coli dam methylase expressed in yeast but accessible in a sir mutant (34). On the other hand, restriction sites in the coding region of URA3 were inaccessible even in sir mutants, whereas silencing-dependent inaccessibility was observed for sites in the promoter (8). In other constructs, silencing complexes targeted to the Hsp82 locus blocked a step downstream of polymerase recruitment (35). Taken together it is unclear to what extent heterochromatin restricts the accessibility to DNA and whether this affects the whole region or the promoter only. Here we address this topic with a DNA repair approach, which allows quantitative monitoring of the removal of UV lesions at individual sites and whole chromatin domains of living cells (36, 37).
Repair of Silenced Chromatin

DNA damage poses a serious risk to the integrity of cells, whereas improper repair can be mutagenic or lethal (38). Nucleotide excision repair (NER) and DNA repair by photolyase (photoreactivation) are the major pathways to remove the predominant UV-induced DNA lesions, cyclobutane pyrimidine dimers (CPDs), and (6-4) photoproducts (39). NER is divided in two subpathways. Global genome repair removes lesions from the non-transcribed part of the genome, whereas transcription-coupled repair removes lesions from the transcribed strand of genes transcribed by RNA polymerase II (RNAP2) (38–40). In yeast S. cerevisiae, global genome repair is modulated by nucleosomes (41–43) and depends on RAD7 and RAD16 (44–46). In transcription-coupled repair, RNAP2 is stalled at lesions on the transcribed strand. Thereby it acts in damage recognition, and leads to preferential repair of the transcribed strand (38).

Photoreactivation provides the clearest example on how chromatin exerts a repressive role on DNA damage recognition. Photolyase is inhibited by nucleosomes in vitro (53–55). In yeast, however, linker DNA and nuclease-sensitive regions such as promoters, 3′-ends of genes, and origins or replication are repaired in 15–30 min, whereas repair of nucleosomes requires 2 h (36, 56, 57). Repair of nucleosomes in vitro is attributed to dynamic properties of nucleosomes, which make DNA lesions accessible to the repair enzyme (58). In genes transcribed by RNAP1, −2, and −3 photoreactivation is slow in the transcribed strand, indicating that the stalled RNA polymerase inhibits the access of photolyase to the DNA lesion (36, 50, 59, 60). Thus, photolyase is a sensitive molecular tool to measure CPD accessibility in chromatin and the state of transcription in living yeast cells.

Because nucleosomes and transcription modulate NER and photoreactivation, it is conceivable that formation of heterochromatin inhibits damage recognition and leads to reduced repair. It was reported that the silenced HML was more slowly repaired with the transcribed strand (61, 62), photoreactivation that can be explained by transcription-coupled repair in the active MAT locus. Here, we investigate how telomeric silencing affects chromatin structure and DNA repair of a subtelomeric URA3 gene. We show that enhanced silencing by overexpression of the silencing proteins Sir3p or disruption of RPD3 reduces repair and generates chromatin rearrangements in the promoter and the coding region, thus providing direct in vivo evidence for a compact chromatin structure.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Yeast strains are listed in Table I. UCC506, UCC510, UCC5083 rpdl3, and the overexpression plasmid YEPSIR3 were kindly provided by D. Gottschling and K. Struhl. SIR3 was deleted by insertion of TRP1 using plasmid pKL12 (kindly provided by S. Gasser). RAD1 was deleted by insertion of KanMX (63). All rad1Δ strains exhibited a strong UV sensitivity (not shown).

UV Irradiation and Repair—UV irradiation and repair were done as described (36, 64). RRY6 and FTY132 were grown in SD minimal medium (2% dextrose, 0.67% yeast nitrogen base without amino acids) supplemented with the appropriate amino acids and uracil to prevent activation of URA3 and maintain the YEPSIR3 expression plasmid. All other strains were cultured in YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose). The cells were harvested at a density of ~3 × 10⁸ cells/ml at 30 °C, resuspended in SD minimal medium to yield about 4 × 10⁶ cells/ml, and rapidly irradiated with UV light using Sylvania G15T8 germicidal lamps (predominantly 254 nm) at a dose of 150 J/m² (measured by an UVX radiometer, UVP Inc., San Gabriel, CA). After irradiation, the medium was supplemented with the appropriate amino acids and uracil. For NER, the cells were incubated in the dark at room temperature. For photoreactivation, the cell suspensions were exposed to photoreactivating light (Sylvania Type F15 T8/BBL bulbs, peak emission at 375 nm) at ~1.5 milliwatts/cm² (measured by a UVX radiometer (UVP Inc., San Gabriel, CA) with a 365-nm photocell) for 3–120 min. The temperature was 22–26 °C. To avoid sedimentation, cells were repeatedly resuspended. After exposure, aliquots of 250 ml were collected and immediately chilled on ice. DNA was purified using Qiagen Genomic tips 500/G (Qiagen) and dissoluted in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Analysis of CPDs—CPDs were mapped by indirect end-labeling and quantified as described (36, 64). Genomic DNA was cut with the appropriate restriction enzymes (Fig. 1). Aliquots were incubated for 2 h at 37 °C with T4-endonuclease V (Epicerin) in 50 mM Tris, 5 mM EDTA, pH 7.5, or mock-treated with the same buffer. The DNA was electrophoresed on 1.5% agarose gels, blotted to Zeta GT nylon membranes, and hybridized with radioactively labeled strand-specific DNA probes. Strand-specific probes were generated by primer extension using small DNA fragments as templates (Fig. 1), strand-specific primers, α-32P]CTP, and T4 polymerase for 30 cycles. The membranes were analyzed and quantified using a PhosphorImager (Molecular Dynamics).

Analysis of Chromatin Structures by Micrococcal Nuclease (MNase) Digestion and Indirect End-labeling—The procedure was done as described (65). Cells of exponentially growing cultures were converted to spheroplasts using zymolase. Crude nuclei and genomic DNA were isolated and digested with different amounts of MNase. The DNA was purified, cut with the appropriate restriction enzyme (as for CPD mapping), fractionated by electrophoresis in 1% agarose gels, blotted to Zeta GT nylon membranes, and hybridized with radioactively labeled DNA probes. Probes were prepared by random priming using small double-stranded DNA fragments as templates (Fig. 1) and an oligolabeling kit (Amersham Biosciences).

Primers—The following oligonucleotides were used to generate DNA fragments as DNA templates for probes and as primers to generate strand-specific probes. The sequence is given in capital letters; additionally nucleotides added for subcloning are given in small letters: for UCC510 and derivatives, UCC510–5′ 5′ (5′-tgatggagctAAAATTTACCTCTCTGAGG-3′); for UCC506 and derivatives, UCC506–5′ 5′ (5′-tgatggagctCTTCTCACGATAAGATC-3′) and UCC506–5′ 5′ (5′-tgatggagctATCCAAATCGTGCGACGCGG-3′) for GAL10 mapping, GAL10–5′ 5′ (5′-cgccgAGTACATCGCTGATAGATAAGAG-3′) and GAL10–5′ 5′ (5′-cgccgAGTACATCGCTGATAGATAAGAG-3′) for GAL10–5′ 5′ (5′-cgccgAGTACATCGCTGATAGATAAGAG-3′) and GAL10–5′ 5′ (5′-cgccgAGTACATCGCTGATAGATAAGAG-3′) for GAL10–5′ 5′ (5′-cgccgAGTACATCGCTGATAGATAAGAG-3′) for GAL10–5′ 5′ (5′-cgccgAGTACATCGCTGATAGATAAGAG-3′).

RESULTS

We used a set of established yeast strains where transcription of URA3 is affected by a telomere position effect (Table I, Fig. 1). In UCC506 (URA3–2kb-T) and UCC510 (URA3–6kb-T), the URA3 gene is located at the right end of chromosome V with its promoter about 2 and 6.5 kb, respectively, from a telomere lacking the Y element. In UCC506, the URA3 gene is partially silenced, whereas it is more frequently transcribed in UCC510 (6). To measure DNA repair by photolyase independent of NER, the NER pathway was inactivated by deletion of RAD1. Analysis of the transcribed strand allows monitoring transcription-coupled repair and inhibition of photolyase by stalled RNAP2, thereby providing information on the transcriptional state of URA3. Analysis of the non-transcribed strand provides information on the nucleosomal state and chromatin accessibility (36, 41). Repair of URA3 was compared with the inactive GAL10, which serves as an internal chromatin standard. All experiments were done in glucose media, where GAL10 is repressed and packaged in an array of nucleosomes (66).

The photoreactivation results are presented in Figs. 2 and 3. Yeast cultures were grown in media containing uracil, irradiated with 150 J/m² to generate about 0.3 CPDs/kb, and exposed to photoreactivating light at room temperature for up to 120
Repair of Silenced Chromatin

TABLE I
Yeast strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| UCC506 | MATα ade2–101 his3–Δ200 leu2–Δ1 lys2–801 trp1–Δ1 ura3–52 URA3 (2kb from TEL-V-R) | De Rubertis et al. (6) |
| UCC506Δrpd3 | Same as UCC506, rpd3Δ::HIS3 | This study |
| RRY1 | Same as UCC506, rad1Δ::kanMX4 | This study |
| RRY2 | Same as UCC506, rad1Δ::kanMX4 rpd3Δ::HIS3 | This study |
| RRY6 | Same as UCC506, rad1Δ::kanMX4 YEpSIR3 (LEU2 SIR3) | This study |
| KMY7 | Same as UCC506, rad1Δ::kanMX4 sir3Δ::TRP1 | This study |
| KMY5 | Same as UCC506, sir3Δ::TRP1 | This study |
| FYI132 | Same as UCC506, YEpSIR3 (LEU2 SIR3) | This study |
| UCC510 | MATα ade2–101 his3–Δ200 leu2–Δ1 lys2–801 trp1–Δ1 ura3–52 URA3 (6kb from TEL-V-R) | De Rubertis et al. (6) |
| RRY3 | Same as UCC510, rad1Δ::kanMX4 | This study |

Fig. 1. Schematic illustration of chromosomal regions containing URA3 and GAL10. Indicated are the URA3 gene (arrow), the approximate distance of the promoter from the telomere (2kb-T, 6kb-T) (6), GAL10 in chromosome II, the restriction sites (ClaI, EcoRI, SalI) and the probes (black bar) used for indirect end-labeling.

min. The DNA was extracted and either mock-treated or cut with T4-endonuclease V at CPDs, and the cutting sites were displayed by indirect end-labeling from a restriction site using strand-specific probes (Fig. 1) (36). DNA of non-irradiated cells showed the intact restriction fragment (top band) irrespective of T4-endonuclease V treatment (Fig. 2, lanes 1 and 2). DNA of irradiated cells showed the intact restriction fragment in the mock-treated lanes (~T4-endonuclease V, lanes 3) but numerous bands and a weaker top band when cut at CPDs with T4-endonuclease V (lanes 4). The bands represent the yields and distribution of CPDs along the chromosomal DNA. Top and bottom strands (left and right gels) revealed different patterns demonstrating strand specificity. The patterns in the URA3 region were as those observed in URA3 of the minichromosome YRpTRURAP (36) and allowed us to unambiguously verify the position of URA3 in the subtelomeric region. Similarly, the GAL10 region displays the characteristic strand-specific CPD distribution (Fig. 2C) (59). The CPD patterns disappeared when cells were exposed to photoreactivating light (+PR), but remained unchanged when cells were kept in the dark (~PR, 120 min), demonstrating that repair was done by photolyase and that the NER was inactivated (lanes 6–16).

Photolyase Efficiently Repairs Subtelomeric URA3—In RRY1 (URA3-2kb-T) and RRY3 (URA3-6kb-T), repair was almost complete in 2 h, but there was substantial repair heterogeneity of fast and slowly repaired sites, reflecting structural heterogeneity in the chromatin substrate (Fig. 2).

Repair in the promoter region (5’-end (dot)) and the 3’-end of URA3 (arrowhead) was extremely fast and already complete in about 15 min as observed in the URA3 gene of a minichromosome where the promoter and 3’-end are known to be in an open conformation and not packaged in nucleosomes (36, 57, 67). Repair was similar in RRY1 and RRY3, demonstrating that the promoter and 3’-end maintained an open chromatin structure irrespective of their position close to the telomere.

Repair in the coding region (bars) was compared with repair of the inactive GAL10 gene (Fig. 2, graphs). Both strands of GAL10 were efficiently repaired, and there was no strand bias, which confirms that GAL10 was not transcribed. Unexpectedly, the non-transcribed strand of URA3 was repaired almost as rapidly as GAL10 in both strains. There was no inhibition of repair that would indicate a more compact chromatin structure of URA3 close to the telomere.

Transcription inhibits photoreactivation on the transcribed strand due to RNAP2 stalled at DNA lesions (36, 59). In RRY1 and RRY3, the transcribed strands were more slowly repaired than GAL10 and more slowly than the non-transcribed strand of URA3, indicating that URA3 was transcribed in both positions. Slow repair is obvious as a “repair footprint,” which extends over the whole coding region of URA3 (black bar). Repair of the transcribed strand was slightly faster in RRY1 than in RRY3, which is consistent with reduced levels of transcription when URA3 is 2 kb from the telomere. Thus, the repair data verify that URA3 was subjected to partial silencing (6) and that the state of reduced transcriptional activity was maintained during the repair period.

Enhanced Silencing by Overexpression of SIR3 or Disruption of RPD3 Inhibits Photoreactivation—To alter heterochromatinization and silencing we manipulated the expression of the silencing protein Sir3 by gene disruption and overexpression, which abolishes and enhances transcriptional silencing, respectively (6). When SIR3 was deleted in KMY7 (Fig. 3A), both strands of GAL10 were repaired at similar rates, indicating that the GAL10 gene remained inactive. The promoter and 3’-end of URA3 remained open, as seen by fast repair on both strands (dots). Repair of the transcribed strand was inhibited (repair footprint) and slower than repair in GAL10, consistent with transcriptional inhibition in URA3. The non-transcribed strand of URA3 was repaired as fast as GAL10. Therefore, removal of Sir3p did not lead to an enhanced accessibility of URA3 chromatin to photolyase.

However, when SIR3 was overexpressed in RRY6 using a multicopy plasmid YEpSIR3, photoreactivation of the non-transcribed strand of URA3 was significantly slower than repair of GAL10 (Fig. 3B). Even repair of the promoter region and the 3’-end was reduced (dots, arrowhead). In addition, the flanking regions toward the telomere appeared to be more slowly repaired than in wild type cells (RRY1) and in the sir3Δ mutant (KMY7). The transcribed strand (black bar) was re-
paired as fast as the non-transcribed strand (white bar). Because URA3 is strongly silenced when Sir3 is overexpressed (6), the reduced repair can be attributed to chromatin repression and not to stalled RNAP2. These results demonstrate that overexpression of Sir3 inhibits repair in the telomere region by formation of a compact chromatin structure. The repression is not confined to the promoter region but includes the transcribed region and the 3'-end of the URA3 gene.

The rpd3Δ deletion mutant, which enhances telomeric silencing (14), showed a very similar effect on photoreactivation as overexpression of SIR3 (Fig. 3C), namely reduced repair rates in the whole URA3 gene including 5'- and 3'-ends.

Silencing Affects NER—NER is a complex multienzyme, multistep pathway that requires more space than photolyase and, therefore, might be more sensitive to chromatin compaction. Fig. 4 summarizes CPD repair by NER and by both pathways together in UCC510 (URA3-6kb-T) and UCC506 (URA3-2kb-T).

NER was generally slower than photoreactivation alone (compare Figs. 2 and 4). NER and photoreactivation together most efficiently removed lesions from all regions, indicating that photolyase provides the major contribution to CPD repair under our conditions (Fig. 4). NER alone only slowly repaired the 5'- and 3'-end of URA3, although the promoter was "open" (rapidly repaired by photolyase). This demonstrates that DNA accessibility is not the cause for slow repair but the NER process per se.

In UCC510 (URA3-6kb-T), the transcribed strand of URA3 was repaired faster than GAL10 and faster than the non-transcribed strand of URA3. This strand bias is characteristic for transcription-coupled repair and confirms that URA3 was transcribed. In UCC506 (URA3-2kb-T), the transcribed strand of URA3 was only slightly faster repaired than the non-tran-
scribed strand and just as fast as GAL10, demonstrating that URA3 in UCC506 (URA3-2kb-T) was partially silenced.

Repair of the non-transcribed strand of URA3 was similar in both strains, indicating that there is no dramatic change in chromatin accessibility between those two loci. On the other hand, URA3 was more resistant to NER than the inactive GAL10, suggesting a reduced accessibility of chromatin to NER in the subtelomeric region. Photoreactivation alone (Fig. 2) and photoreactivation with NER (Fig. 4) showed only minor differences between repair of the non-transcribed strand of GAL10 and URA3. In contrast to photolyase, NER appears to be inhibited in subtelomeric chromatin.

To investigate how NER reacts on alterations in chromatin composition, repair experiments were done in strains that carry URA3 2 kb from the telomere and contain deletions of SIR3 (KMY5) or RPD3 (UCC506 rpd3Δ) and in a strain overexpressing Sir3p (FTY132) (Fig. 5). When SIR3 was deleted, the transcribed strand appeared more rapidly repaired by NER than the non-transcribed strand, which is consistent with transcription-coupled repair and suggests that silencing was relieved. In that case the non-transcribed strand of URA3 was as rapidly repaired as the GAL10 gene. Thus, the deletion of SIR3 leads to an enhanced accessibility of DNA to NER, consistent with the removal of a repressive structure. Again, this effect was less pronounced for PR alone (Fig. 3) and PR with NER (Fig. 5), suggesting that the NER process is more sensitive to structural changes induced by SIR3 than photoreactivation.

Overexpression of SIR3 and deletion of RPD3 resulted in reduced repair rates by NER and both pathways in the transcribed region, the promoter, and the 3’-end (Fig. 5, B and C). Thus, NER and photolyase have reduced access to the subtelomeric URA3 gene, and the chromatin structure appears to be less transparent for repair proteins. Moreover, both strands of URA3 were repaired with similar rates, demonstrating that the strand bias of transcription-coupled repair was lost and that the URA3 gene was silent. Although reduced repair of URA3 compared with GAL10 was most obvious in the rpd3Δ strain, the difference was smaller in FTY132, possibly as a consequence of using selective media required to maintain the YEpsir3 plasmid.

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**FIG. 3. Inhibition of photolyase by silencing.** DNA repair experiments were done as described in Fig. 2. A, KMY7 (URA3-2kb-T sir3Δ rad1Δ). B, RRY6 (URA3-2kb-T YEpsir3 rad1Δ). Less DNA was loaded in lane 16. C, RRY2 (URA3-2kb-T rpd3Δ rad1Δ). Graphs are from one experiment each and three gels (A), two gels (B), one gel (C). For comparison with RRY1 (URA3-2kb-T rad1Δ) see Fig. 2B. The GAL10 data were from the same experiments as the URA3 data.
Alterations in Chromatin Structures of URA3 by Silencing—To obtain complementary information on chromatin organization we did conventional chromatin analyses by MNase digestion (Fig. 6). In UCC510, where URA3 is not silenced, MNase footprinting revealed a chromatin structure that was very similar to that observed in minichromosomes (67) and in the normal locus of chromosome V (68) (Fig. 6A). The promoter (5’-end) and the 3’-end were nucleosome sensitive (cut in chromatin, not cut in free DNA). Six nucleosomes were positioned in the coding region (U1 to U6). Three nucleosomes were tightly packed (U3, U4, U5). The first nucleosome (U1) may occupy extreme positions (circles 1a, 1b) (68). The position U1a is characterized by cutting sites I and III and leaves the TATA box free (black bar, cutting site III). Position U1b, which covers the TATA box, is defined by cutting site II and cutting in the nucleosome-sensitive region.

When URA3 was placed closer to the telomere (2 kb), the chromatin structure changed slightly (Fig. 6B, UCC506, lanes 2 and 3). The bands I and II were of similar intensity, suggesting that some nucleosomes were shifted to position U1b, which covers the TATA box. This interpretation is consistent with the reduced transcriptional activity. Disruption of SIR3 had only a mild effect (lanes 4–6): U1 revealed a bias for position U1a (stronger band I), leaving the TATA box more accessible, thereby allowing enhanced transcription. The subtle differences observed in chromatin organization of those strains are consistent with partial silencing and the variegated phenotype, having some genes expressed, whereas others are silenced.

Changes in chromatin structure, however, were most pronounced in rpd3Δ strains (lanes 9–12). In those cases nucleosome footprints were missing in the coding region, and the cutting pattern resembled that of naked DNA. However, because the whole region was slowly repaired, we infer that nucleosomes were present but not positioned or that nucleosomes and linkers were covered with silencing proteins. Chromatin changes were also observed in the promoter proximal nucleosome. Although in naked DNA band II is weak and band III is strong, chromatin showed preferential cutting in band II and reduced cutting in band III, consistent with a bias for nucleosome position U1b covering the TATA-box. Moreover, those strains showed reduced nuclease accessibility in the 5’ region. The results are entirely consistent with reduced transcriptional activity and reduced DNA repair efficiency by photolyase in the coding and promoter region.

Overexpressing of SIR3 results in an intermediate chromatin structure (Fig. 6, lanes 7 and 8), with a preference for U1b, loss of nuclease sensitivity in the promoter, and a reduced cutting heterogeneity in the coding region. This is also consistent with the reduced repair rates in the coding region and the promoter (Fig. 5B).

**DISCUSSION**

Yeast telomeres reversibly repress transcription of genes positioned nearby by recruitment and spreading of a silencing complex (consisting of Sir2, Sir3, and Sir4 proteins) to nearby...
Despite the overwhelming biochemical and genetic evidence with respect to the distribution of silencing components and their interactions with histones, we remain quite ignorant with respect to (i) the ratio of each component per nucleosome, (ii) the effect of silencing components on nucleosome positions and stability, (iii) the overall effects of silencing on compaction and accessibility of DNA, and (iv) the effect of silencing on other DNA-dependent processes. Here, we show that two different DNA repair pathways, nucleotide excision repair and photoreactivation, are sensitive to alterations in chromatin structure associated with silencing. In addition, the UV repair approach provides direct in vivo evidence for a compact, less accessible chromatin structure, which affects not only the promoter region but also the whole URA3 gene.

A central problem is that silencing is stochastic and reversible. Only a fraction of the genes in a cell population is silenced, whereas others are active and might have a different chromatin structure (5, 34). Unfortunately, the different fractions cannot be separated physically for structural analyses. Thus, a convenient approach is to analyze the extreme conditions in the absence of silencing (sir3Δ) or with maximal silencing (overexpression of SIR3, rpd3Δ). Silencing might occur by chromatin-mediated occlusion of transcription factors from promoter regions (70). Indeed, the promoter of a telomere-linked URA3 gene was found to be largely inaccessible to a restriction enzyme, and the accessibility increased in a sir3Δ strain (8). Our MNase digestion results support those observations. They revealed subtle changes with respect to the promoter proximal nucleosome and the promoter region. Under fully silenced conditions, nucleosome position U1b, which covers the TATA box, was preferred, whereas in sir3Δ strains position U1a was prevalent, and an intermediate situation was observed when URA3 was partially silenced. In contrast to the in vitro nuclease digestion experiments, the photolyase results provide independent in vivo evidence for a silencing-dependent occlusion of the promoter. The promoter region was rapidly repaired in sir3Δ strains, whereas repair was inhibited under fully silenced conditions.

The chromatin immunoprecipitation data provided evidence for a spreading of the silencing complex and the corresponding histone modifications, suggesting that silencing might compromise the whole chromatin domain (8, 71). The structural data, however, are controversial. Original MNase digestion data of a silenced URA3 gene revealed a normal structure (33). Expression of E. coli dam methylase in yeast, on the other hand, showed that a methylation site in the coding region became accessible in sir mutants (8, 34), whereas other sites remained inaccessible to restriction enzymes in vitro (8). An explanation for the controversial result might come from the different location of the methylation and restriction sites. Although the methylation site was located at the edge of a nucleosome close to the linker DNA (assuming the normal chromatin structure.

**Fig. 5. Sir3 and Rpd3 affect NER and photoreactivation.** Representative gels of the top strand (left gels) and bottom strand (right gels) are shown. A, KMY5 (URA3–2kb-T sir3Δ). B, FTY132 (URA3–2kb-T YEpSIR3). C, UCC506 rpd3Δ (URA3–2kb-T). Conditions are as described in Fig. 4. The GAL10 data were from the same experiments as the URA3 data.
Fig. 6. Chromatin structures of subtelomeric URA3. A, UCC510 with URA3 positioned 6 kb from the telomere. B, comparison of strains with URA3 positioned 2 kb from the telomere. Protein-free genomic DNA (N) or chromatin (C) was digested with MNase, and the cutting sites were displayed by indirect end-labeling using restriction sites and probes as described in Fig. 1. Indicated are strains and relevant genotypes, promoter region (5′), 3′-end (3′), the TATA-box (black rectangle), nucleosome positions (circles), multiple positions (overlapping circles), nucleosomes in URA3 (U1–U6, circles 1–6); U1a and U1b are two proposed positions of U1 characterized by cutting sites of MNase (I, II, III). Lanes 2 and 3, 4 and 5, 7 and 8, 9 and 10, 11 and 12, 13 and 14 show two samples of the MNase time course.

(67, 68), the restriction sites were within nucleosomes. In contrast to those site-specific assays, the repair approach showed that silencing reduces accessibility of the whole region. Thus, silencing-dependent compaction includes the promoter as well as the coding region.

The details of the silencing structure remain obscure. Our MNase experiments did not resolve positioned nucleosomes in the coding region. A loss of nucleosome footprints could reflect destabilized nucleosomes. In that case, however, one would expect enhanced repair rather than inhibition of repair. Alternatively, silencing proteins or the establishment of silencing after replication might rearrange or randomize nucleosome positions. Again, this alone would not explain reduced repair. The observation of altered MNase cutting in the promoter proximal sites and the generally reduced accessibility of DNA to repair enzymes argues that rearrangement occurs combined with a substantial protection of nucleosomes and linker DNA by silencing proteins. At this level of resolution we did not recognize hot spots or cold spots of repair along the URA3 gene. We take this as an indication that silencing generates a rather homogeneous compaction of chromatin.

Yeast nucleosomes are relatively tightly packaged missing long linkers. This is in particular the case for the URA3 gene, which contains three tightly packed nucleosomes (Fig. 6) (67). Nucleosomes contain 147 bp of DNA wrapped around two pairs of four histone proteins, H2A, H2B, H3, and H4. The structured histone fold domain of the core histones contributes to the nucleosome formation, whereas less structured N-terminal domains extend beyond the nucleosome core (72, 73) and are accessible for post-translational modifications and interactions with structural or regulatory proteins, including silencing factors Sir3 and Sir4 (10). Because the first nucleosome core crystals made with recombinant Xenopus laevis histones revealed that the N-terminal tail of H4 contacts a negatively charged pocket in the neighboring nucleosome (72), it was proposed that in heterochromatin Sir3 replaces the internucleosomal interactions (69). More recent data on the yeast nucleosome core crystal, however, showed no such contacts. Instead, the H4 tail is poised to interact with the DNA of a neighboring nucleosome core (73). Moreover, a genetic study identified mutants in histone H3 and H4 that are crucial for transcriptional silencing and map on the top and bottom surface of nucleosomes (16). The mutants include Lys-79 of histone H3 that can be methylated by the yeast Dot1 protein (74–76). Both loss and overexpression of Dot1 result in loss of telomeric silencing (77). Thus, it appears possible that silencing is modulated by stabilizing face to face contacts between nucleosomes.

Because photolyase is strongly inhibited on the nucleosome surface in vitro (53–55), efficient repair observed in vivo is explained by dynamic properties of nucleosomes (36). Nucleosome mobility or transient nucleosome disruption may make DNA lesions accessible (37, 58). Although the accessibility of photolyase was compromised in silenced chromatin, we still observed a significant amount of repair, indicating that silenced chromatin may maintain dynamic properties.

In contrast to photoreactivation, NER was inhibited in URA3 2 and 6 kb from the telomere, and the inhibition was released in sir3Δ cells. Apparently, partially silenced chromatin is more resistant to NER. NER builds up a multisubunit complex for damage recognition (37, 40) and requires a substrate size of about 100 bp (78), which is much longer than the average linker DNA between nucleosome cores in yeast (about 15 bp) (79). Thus, it is likely that the damage recognition step or the subsequent formation of the repair complex will require nucleosome disruption or removal. This may occur either by intrinsic properties of nucleosomes (mobility, partial unfolding, and dissociation) or with the help of remodeling activities. Several in vitro experiments support a contribution of remodeling activities. It was shown that nucleosomes inhibit the human excision reaction (80, 81). An ATP-dependent chromatin assembly factor, however, facilitated repair of a (6-4) photoproduct in linker DNA but not in nucleosomes (80). On the other hand, two different ATP-dependent remodeling complexes (SWI/SNF and ISW2) were shown to remodel UV-damaged nucleosomes and facilitate the accessibility of CPDs as measured by photolyase. Although SWI/SNF generally enhanced the accessibility of nucleosomal DNA, ISW2 shifted the nucleosome position (55). In addition, SWI/SNF stimulated the human excision repair reaction on a nucleosome containing a bulky acetylaminofluorenuarine adduct (82). It seems possible, therefore, that silencing affects the intrinsic properties of nucleosomes as well as chromatin remodeling processes.

One concern during the experiments was that Sir3p might redistribute after UV-DNA damage formation, thereby leading to a derepression and facilitating DNA repair. It is known that Sir3p and the telomeric proteins yKu redistribute after double
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strand break formation. This redistribution, however, is very slow (several hours), and it was not observed as a consequence of UV irradiation (83, 84). Because photolyase is very efficient and repairs a significant fraction of lesions within a few minutes, a slow redistribution of silencing proteins is unlikely the cause for rapid repair.

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