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Remodeling of Yeast *CUP1* Chromatin Involves Activator-Dependent Repositioning of Nucleosomes over the Entire Gene and Flanking Sequences

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The yeast *CUP1* gene is activated by the copper-dependent binding of the transcriptional activator, Ace1p. An episome containing transcriptionally active or inactive *CUP1* was purified in its native chromatin structure from yeast cells. The amount of RNA polymerase II on *CUP1* in the purified episomes correlated with its transcriptional activity in vivo. Chromatin structures were examined by using the monomer extension technique to map translational positions of nucleosomes. The chromatin structure of an episome containing inactive *CUP1* isolated from *ace1*Δ cells is organized into clusters of overlapping nucleosome positions separated by linkers. Novel nucleosome positions that include the linkers are occupied in the presence of Ace1p. Repositioning was observed over the entire *CUP1* gene and its flanking regions, possibly over the entire episome. Mutation of the TATA boxes to prevent transcription did not prevent repositioning, implicating a chromatin remodeling activity recruited by Ace1p. These observations provide direct evidence in vivo for the nucleosome sliding mechanism proposed for remodeling complexes in vitro and indicate that remodeling is not restricted to the promoter but occurs over a chromatin domain including *CUP1* and its flanking sequences.

Regulation of gene expression is best understood in living cells, where access to promoters and other regulatory elements is generally restricted by chromatin structure. Mechanisms have evolved to render these accessible at the appropriate moment (17, 60), including (i) regulated posttranslational modifications of the core histones, particularly acetylation, which alter nucleosome structure; (ii) remodeling of specific regions of chromatin by multisubunit complexes which use ATP to disrupt, displace, or slide nucleosomes; (iii) regulated nucleosome positioning (48); and (iv) contributions of other chromatin proteins.

An ideal approach to studying these interactions would be to examine native chromatin structures in vitro by using the sophisticated techniques available for analysis of reconstituted chromatin and relate these to events in vivo. The main concerns are purity and the amounts of material available. The use of small episomes with high copy number facilitates the separation of large chromosomal fragments from the gene of interest, e.g., simian virus 40 minichromosomes (14). Budding yeast (*Saccharomyces cerevisiae*) offers a source of minichromosomes in the form of plasmids, with the advantage that a model gene can be chosen and studied in the context of its molecular genetics (13, 20, 45).

CUP1 encodes a copper metallothionein responsible for protecting yeast cells from the toxic effects of copper (7, 23). It was chosen as a model gene because its regulation is well understood and relatively simple, increasing the likelihood that its activity can be reconstituted in vitro. In the absence of toxic concentrations of copper, *CUP1* is not required for growth (18). It is strongly induced when copper ions enter the cell and bind to the N-terminal domain of the transcriptional activator Ace1p (also called Cup2p), which folds and binds specifically to upstream activating sequences (UASs) in the *CUP1* promoter (6, 16). Transcription of *CUP1* is activated via the C-terminal acidic activation domain (16). Thus, the signal transduction pathway is known in some detail. The only other transcription factor that influences *CUP1* expression directly is heat shock factor (33). Current models for transcriptional activation are highly complex, invoking requirements for many proteins (51). However, *CUP1* can be induced in vivo in the absence of many of the basal transcription factors: TATA-binding protein (TBP) has been detected at the *CUP1* promoter in vivo (25, 31), but induction is independent of TFIIA (32, 42), TFIIE (46), the Kin28 CTD kinase of TFIIH, and some components of the mediator, but not others (28, 29, 36). Furthermore, Ace1p activates transcription independently of most of the TAFs (37). Thus, *CUP1* appears to be an example of simplified regulation (27).

We are interested in the process by which a gene in its natural chromatin context is activated for transcription. Here we describe the native chromatin structures of the transcriptionally active and inactive forms of *CUP1* in a small episome purified from yeast cells. Activation of *CUP1* is accompanied by a gene-wide repositioning of nucleosomes, which requires the presence of Ace1p but is independent of transcription, providing evidence for an activator-dependent remodeling activity that moves nucleosomes on *CUP1* and its flanking sequences.

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MATERIALS AND METHODS

Construction of yeast strains and plasmids. The *CUP1* locus was deleted from BJ5459 (*MAT***a** *cir*$^+$ *ura3-52 trp1 lys2-801 leu2*Δ*1 his3*Δ*200 pep4::HIS3 prb1*Δ*1.6R*

can1 GAL) (21) (Yeast Genetic Stock Center, Berkeley, Calif.) by transformation with the 4.3-kb *Bst*1107I-*Swa*I fragment from pΔCup3, with *LEU2* flanked by *CUP1* locus flanking sequences. pΔCup3: The 828-bp *Hin*dIII fragment from cosmid ATCC 71209 containing sequence flanking the *CUP1* locus was inserted at the *Hin*dIII site of pNEB193 (New England Biolabs) to obtain pΔCup1A. The 1.8-kb *Dra*I fragment from cosmid ATCC 70887 containing sequence on the other side of *CUP1* was inserted at the *Sma*I site in pΔCup1A to obtain pΔCup2A. *LEU2* as a 2.0-kb *Bst*YI-*Sal*I fragment from pOF4 (30) (gift of J. Thorner) was inserted into pΔCup2A *Bam*HI/*Sal*I to give pΔCup3. This strain was cured of the 2μm circle plasmid to obtain YDCcup1Δ2 as described previously (3). *TRP1 ARS1* as a 1,453-bp *Hin*dIII fragment from pTB-B9 (54) (gift of A. Dean) was inserted at the *Hin*dIII site of pGEM13zf(+) (Promega), oriented such that *ARS1* is closest to the *Not*I site in the vector, to give pGEM-TRP1ARS1. *CUP1* was obtained either as a 1,998-bp *Kpn*I fragment containing the entire *CUP1* repeat or as a 925-bp *Kpn*I-*Nsi*I fragment containing just *CUP1* from YEp(CUP1)2A (ATCC 53233) and inserted into pUC19 cut with *Kpn*I only or *Kpn*I and *Pst*I to give pCP1A (with *CUP1* closest to the *Xba*I site in the vector) and pCP2, respectively. A 1,060-bp *Sph*I-*Pvu*II *CUP1* fragment from pCP2 was inserted into pSP72 (Promega) *Sph*I/*Pvu*II to give pSP72-CUP1. A 1,015-bp *Eco*RI *CUP1* fragment from pSP72-CUP1 was inserted at the *Eco*RI site in pGEM-TRP1 ARS1 to obtain pGEM-TAC(+), such that *CUP1* is transcribed in the opposite direction to *TRP1*. pGEM-TAC(−) was obtained by cutting pGEM-TAC(+) with *Hin*dIII and religating to obtain the opposite orientation. TAC DNA as a 2,468-bp linear *Hin*dIII fragment from pGEM-TAC was circularized with ligase and used to transform YDCcup1Δ2. pGEM-TAC with both TATA boxes mutated was constructed by PCR-based mutagenesis: the proximal TATA box, TTATAA, was converted to CCCGGG; the distal TATA box, TATAAA, was converted to GGGCCC. An *ace1*Δ strain with TAC was constructed as follows. A 1,492-bp *Sma*I-*Hin*dIII *ACE1* fragment from pRI-3 (gift of S. Hu and D. Hamer) (16) was inserted into pUC19 *Sma*I/*Hin*dIII to give pUC-ACE1. A *Bst*Z17I-*Msc*I fragment containing the *ACE1* open reading frame (ORF) was replaced with *URA3* to give pUC-ACE1ΔURA3. YDCcup1Δ2::TAC was transformed with the *Xba*I-*Hin*dIII fragment from pUC-ACE1ΔURA3. The replacement of *ACE1* with *URA3* was confirmed by Southern blotting. The copy number of TAC was determined by phosphorimager quantitation of the ratio of linearized TAC to chromosomal *Bgl*II fragment containing *TRP1* in Southern blots of *Bgl*II digests of genomic DNA, using the 238-bp *Hin*dIII-*Bgl*II fragment from TAC as a probe.

Purification of minichromosomes. The protocol is based on our previous method (3) with alterations resulting in increased yield, reflecting a systematic analysis of losses of TAC at each stage by quantitative Southern blot. Cells were grown at 30°C in synthetic complete medium lacking tryptophan to late log phase in flasks or in a fermenter and stored at −80°C. Cells (2.6 g) were thawed in 50 ml of spheroplasting medium (SM) (6.7 g of yeast nitrogen base with ammonium sulfate and without copper sulfate (Bio101) per liter, 2% D-glucose, 0.74 g of CSM-trp (Bio101) per liter, 1 M D-sorbitol, 50 mM Tris-HCl [pH 8.0]) with 20 mM 2-mercaptoethanol (2-ME) and warmed to 30°C for 15 min, with swirling. Lytic enzyme (120 mg) (Sigma L-4025; 1,000 U/mg) was dissolved in 3 ml of SM and added to the cells. Spheroplasting was followed by diluting aliquots of cells into 1% sodium dodecyl sulfate (SDS) and measuring A_{600}. When the A_{600} reached 5% of the starting value, in about 20 min, spheroplasts were collected (7,500 rpm, 5 min, Sorvall SS34 rotor, 4°C), washed twice with 25 ml of SM (no 2-ME) and resuspended in 50 ml of prewarmed SM with or without 5 mM $CuSO_4$. Spheroplasts were incubated at 30°C for 30 min at 225 rpm, collected as described above, and washed with 25 ml of cold 1 M D-sorbitol, 50 mM Tris-HCl (pH 8.0). Spheroplasts were lysed by vigorous resuspension with a pipette in 40 ml 18% (wt/vol) Ficoll 400, 40 mM potassium phosphate, 1 mM $MgCl_2$ (pH 6.5 [adjusted with phosphoric acid]), with 5 mM 2-ME, 0.1 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride], 5 μg of leupeptin per ml, and 15 μg of pepstatin A per ml. Two step gradients were used: 20 ml of lysate layered over 15 ml of 7% (wt/vol) Ficoll–20% (vol/vol) glycerol–40 mM potassium phosphate–1 mM $MgCl_2$ (pH 6.5), with additions as described above, and spun (14,000 rpm, 30 min, SS34 rotor, 4°C). Each nuclear pellet was resuspended in 3 ml of 50 mM Tris-HCl (pH 8.0)–5 mM Na-EDTA with additions as described above. Forty microliters of RNase (Qiagen; DNase free; 100 mg/ml) was added to each resuspension, left for 30 min on ice, and spun (10,000 rpm, 5 min; SS34; 4°C). The cloudy supernatants, containing the minichromosomes, were applied to a 700 μl of 30% sucrose cushion in TAE (pH 7.9) (40 mM Tris, 2 mM Na-EDTA; adjusted to pH 7.9 with acetic acid) containing 10 μg of bovine serum albumin (BSA) (Calbiochem; nuclease and protease free) per ml and additions as described above, in SW60 tubes, and spun (60,000 rpm, 2.5 h, SW60 Ti rotor, 4°C). The 30% cushions were pooled, syringe filtered to remove particles (0.45 μm pore diameter, low protein binding), divided between two prewashed Centricon 500 filtration units (Amicon), concentrated (6,000 rpm, SS34 rotor, 4°C) until the volume was 100 μl, and washed twice with wash buffer (WB), which was made up of TAE (pH 7.9) containing BSA and the additions described above. A sample was removed for analysis. (SDS was added to 1% and KOAc was added to 1 M, DNA was extracted with phenol-chloroform [1:1] and then chloroform and precipitated with ethanol in the presence of 20 μg of glycogen.) Minichromosomes were frozen on dry ice and stored at −20°C. For electroelution from agarose gels, a 60-ml 0.7% agarose gel (6 cm wide, 10 cm long; SeaKem GTG agarose for nucleic acids >1 kb; FMC) in TAE (pH 7.9) with a central 4.4-cm well (1.5 mm wide) flanked by marker wells was cooled to 4°C in a Bio-Rad Mini-Sub Cell with TAE (pH 7.9) as a running buffer. Marker wells were loaded with sample buffer containing xylene cyanol and bromophenol blue. Minichromosomes were adjusted to 10% sucrose (without dyes) and electrophoresed at 40 V for 1.3 h at 4°C with buffer recirculation. A gel slice defined by the midpoints of the two dye bands in the marker lanes was excised and placed in SpectraPor 7 dialysis tubing (flat width of 24 mm, molecular weight cutoff of 8,000), which had been soaked in TAE (pH 7.9), 0.01% NP-40, 10 μg of BSA per ml at 4°C. The same buffer (2.5 ml) was added, and the tubing was secured with dialysis clips. Electroelution was at 40 V, 1.5 h, 4°C with buffer recirculation. The current direction was reversed for 30 s. The eluate was syringe filtered as described above to remove pieces of agarose, protease inhibitors and 2-ME were added, and the eluate was concentrated to about 100 μl in a prewashed Centricon 30 for 40 min as described above. The preparation was washed twice with 1 ml of WB for 30 min, to a final volume of about 100 μl.

Preparation of RNA and Northern blot analysis. After induction, spheroplasts from 40 ml of culture were collected and resuspended in 400 μl of 10 mM Tris-HCl (pH 7.5)–10 mM EDTA–0.5% SDS, mixed with 400 μl of phenol and incubated at 65°C for 30 min with occasional, brief vortexing. The aqueous phase was extracted again with phenol and then chloroform. RNA was precipitated with ethanol after adding 0.3 M NaOAc (pH 5.3), resuspended in 400 μl of 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1% 2-ME, 1% SDS, 10% glycerol, and stored at −80°C. Equal amounts (0.25 μg) of RNA were mixed with 27 mM MOPS (morpholinepropanesulfonic acid), 6.9 mM NaOAc, 1.4 mM EDTA, 25% formaldehyde, 69% formamide, and 0.01% bromophenol blue to 16.7 μl and incubated for 15 min at 65°C. NH_4OAc (3.3 μl, 0.5 M) was added. Samples were electrophoresed at 5 V/cm for 12 h in a 1% agarose gel containing 17% formaldehyde, 20 mM MOPS, 5 mM NaOAc, and 1 mM EDTA. The gel was blotted and hybridized overnight at 68°C with the 600-bp *Bsa*BI-*Pac*I *CUP1* fragment from pGEM-TAC as a probe.

Topoisomer analysis. Purified TAC-DNA was loaded in a 1.2% (wt/vol) agarose gel (15 by 10 cm) in the presence or absence of chloroquine diphosphate and electrophoresed at 45 V for 4.6 h with buffer recirculation (9). Gels were blotted and probed with a *Hin*dIII digest of pGEM-TAC labeled by random priming. Linking number standards were prepared as described previously (9) using pSP72. For analysis of TAC directly from cells, DNA was isolated by rapid extraction of induced and uninduced spheroplasts with 1% SDS and purified as described above.

Monomer extension. To prepare core particles, 25 to 40 ng of TAC minichromosomes were incubated in 40 μl of 10 mM Tris-HCl (pH 8.0)–35 mM NaCl–3 mM $CaCl_2$, with 2 to 4 U of micrococcal nuclease (MNase) (Worthington) for 2 min at 30°C, and EDTA was added to 5 mM. Core particle DNA was extracted, purified from 3% agarose gels, and end labeled with T4 kinase. Core DNA was denatured with alkali, annealed with excess template, and extended with Klenow enzyme, in the presence or absence of a restriction enzyme (61). With pGEM-TAC(+) as template, *Bam*HI, *Sap*I, *Xcm*I, *Bsu*36I, and *Dra*III were used. With pGEM-TAC(−), *Sap*I, *Bam*HI, *Xcm*I, *Bgl*II, and *Bst*BI were used. Products were analyzed in 6% denaturing polyacrylamide gels.

Primer extension. Analysis was performed as described previously (56). Nuclei were prepared from 500 ml of cells as described above and resuspended in 4 ml of 10 mM HEPES-Na (pH 7.5), 0.5 mM $MgCl_2$, and 0.05 mM $CaCl_2$ with protease inhibitors and 2-ME as described above. MNase (Worthington) was added to 650-μl aliquots of nuclei to 0 to 80 U/ml and incubated for 10 min at 37°C. EDTA was added to 10 mM, SDS was added to 1%, and DNA was purified as described above and dissolved in 80 μl of Tris-EDTA (TE) buffer containing RNase. For a free DNA control, pCP1A was digested with MNase to various degrees. MNase-digested DNA (5 μl) was mixed with primer (end labeled with T4 kinase and [γ-^{32}P]ATP) at 10 nM with 1.6 U of Vent polymerase in buffer supplied by the manufacturer (NEB). The primers used were CUP1A (5′-CTT CACCACCCTTTATTTCAGGCTG-3′) and CUP1B (5′-CGAAATCTGGGGA TTCTATACAGAG-3′). Multiple rounds of extension were performed as follows. DNA was denatured for 5 min at 95°C; followed by 20 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min; followed by 72°C for 5 min. DNA was purified and analyzed in 6% sequencing gels.

Restriction enzyme accessibility. TAC minichromosomes (4 ng) were mixed with an appropriate plasmid (4 ng) as an internal control (either pGEM-TAC, pBR322, or pGEM-TRP1ARS1) and a *Bst*EII digest of λ DNA at 1 ng/μl in a mixture of 100 μl of 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mg of BSA per ml, 5 mM 2-ME, and 0.1 mM AEBSF and incubated at 30°C. A zero-time sample (18 μl) was removed, and then 2 to 20 U of restriction enzyme was added. Aliquots were removed at various times and quenched with an equal volume of 2% SDS–20 mM Na-EDTA. DNA was purified as described above. If the restriction site was not unique in TAC, the DNA was digested with *Hin*dIII. DNA was resolved in 0.8% agarose gels, and Southern blots were probed either with pGEM-TAC or, if a second digestion had been performed, with the 238-bp *Hin*dIII-*Bgl*II fragment from pGEM-TAC. Rates of digestion were determined with a phosphorimager.

Transcription run-on analysis. Electroeluted minichromosomes (15 to 50 ng) were incubated in a mixture of 90 μl of 10 mM Tris-HCl (pH 8.0), 0.1 M NH_4OAc; 0.6 mM (each) ATP, GTP, and CTP; 5 mM $MgCl_2$; 2.5 mM $MnCl_2$; 1 U of RNase Prime Inhibitor (Eppendorf) per μl; and 45 μCi of [α-^{32}P]UTP at 3,000 Ci/mmol in the presence or absence of 20 μg of α-amanitin per ml for 20 min at 30°C. Na-EDTA was added to 10 mM. Free label was removed with a Sephadex G-50 spin column, and purified RNA was denatured for use as a probe of a Southern blot.

RESULTS

Purification of the *TRP1 ARS1 CUP1* (TAC) minichromosome. *CUP1* is present in multiple copies per haploid genome, and strains with higher copy numbers are more resistant to copper (23). The genes are tandemly reiterated with a 2-kb repeat unit containing *CUP1* and another gene of unknown function (URF). The haploid strain BJ5459 was chosen for these studies because it carries protease mutations, which should reduce proteolysis of chromatin during isolation. It is resistant to 1 mM copper and contains about eight copies of *CUP1* at a single locus (not shown). These were deleted to obtain a *cup1*Δ strain, which was then cured of the 2μm circle plasmid (an endogenous yeast plasmid present at high copy number in most laboratory strains) as described previously (3). This strain was transformed with *TRP1 ARS1 CUP1* (TAC), a 2,468-bp yeast plasmid based on *TRP1 ARS1* (62) (Fig. 1A). To demonstrate that *CUP1* in TAC is fully functional, growth was measured in the presence of 1 mM copper (Fig. 1B). The parental strain (BJ5459) grew well in the presence of copper, with a doubling time about 20% longer. The *cup1*Δ strain grew at the same rate as BJ5459 in the absence of copper, but was inviable in the presence of copper. The introduction of TAC restored growth in 1 mM copper, indicating that *CUP1* in TAC is fully functional. The copy number of TAC was measured by Southern blot analysis at an average of 10 per cell (it is likely that asymmetric segregation of *ARS* plasmids at cell division (45) will result in cells with significantly more or less than 10 copies of TAC). The correlation between the copy number of *CUP1* and the degree of resistance to copper (23) suggests that most of the TAC episomes are likely to be active in the presence of 1 mM copper.

A method for the purification of the TAC minichromosome as chromatin was developed. In the first step, cells were resuspended in medium with sorbitol as an osmotic stabilizer and digested with lytic enzyme to obtain spheroplasts, which were then treated with 5 mM copper(II) sulfate to maximize induction of *CUP1*. The induction of *CUP1* under these conditions was followed by Northern blot analysis (Fig. 1C). *CUP1* was strongly induced in both the TAC-containing strain (about sixfold in 2 mM copper) and the parental strain with chromosomal *CUP1* (about ninefold). However, this level of induction was significantly less than we were able to measure in intact cells (about 30-fold; not shown), and there was significant expression of *CUP1* even in the absence of copper. Why *CUP1* is partially induced in spheroplasts is unclear, but it is possible that it is part of a stress response to spheroplasting (1). To obtain a strain containing TAC with inactive *CUP1*, the gene for its transcriptional activator, Ace1p, was deleted. This strain expressed *CUP1* at very low levels (not shown).

Native TAC chromatin was isolated from copper-induced, uninduced, and *ace1*Δ cells. Low-ionic-strength buffers were used to prevent nucleosome sliding and to retain proteins. Purity was assessed by analysis of extracted nucleic acids (Fig. 2A): TAC chromatin preparations also contained small amounts of mitochondrial nucleoids, genomic chromatin, and digested ribosomes. TAC DNA was mostly supercoiled, with only a little nicked circle, indicating that TAC chromatin was not damaged by nucleases. This represents a high degree of purity (yields were 40 to 60%, corresponding to about 1 μg of plasmid DNA per liter of cells). This preparation was used for analysis of chromatin structure (see below), but for transcription experiments, TAC was further purified by electroelution from agarose gels (Fig. 2A). Minichromosomes were analyzed in an agarose gel, with or without treatment with SDS to remove the proteins (Fig. 2B). In the absence of SDS, TAC DNA migrated more slowly, as chromatin, with no free TAC DNA present, confirming that the minichromosomes remained substantially intact after purification.

Topological analysis of TAC minichromosomes in vitro and in vivo. Topological analysis can be used to count nucleosomes on closed circular DNA, using the fact that a nucleosome protects one negative supercoil from relaxation by topoisomerase (49). DNA was extracted from TAC minichromosomes, and topoisomers were resolved in a chloroquine gel (Fig. 3). By comparison with a set of standards of defined linking number, the centers of the linking number distributions were determined (12). The topologies of purified TAC minichromosomal DNA from uninduced and copper-induced cells were very similar, with 12.0 ± 0.2 (n = 2) and 11.8 ± 0.8 (n = 3) negative supercoils, respectively. TAC purified from *ace1*Δ cells (not shown) contained 11.0 ± 0.9 (n = 3) negative supercoils, about one less than TAC from induced and uninduced cells. However, whether this difference is significant is unclear, because the standard errors overlap. The topologies of TAC purified from uninduced, induced, and *ace1*Δ cells were compared with those of TAC in vivo by rapid extraction of DNA from spheroplasts: the values were 12.0 ± 0.7 (n = 2), 12.5 ± 0.4 (n = 9), and 12.2 ± 0.6 (n = 3) supercoils, respectively. The values for induced TAC and TAC from *ace1*Δ cells are slightly higher than those for the purified minichromosomes (although the standard errors overlap). It was also noted that the topoisomer distributions of purified TAC were broader than those of TAC in vivo. An important point here is that the linker DNA in purified TAC chromatin is completely relaxed (it copurifies with topoisomerase activity) (data not shown), but in vivo, TAC minichromosomes might not be completely relaxed, depending on the balance between supercoiling and relaxing activities. In conclusion, topological analysis is consistent with the presence of 10 to 13 nucleosomes in TAC minichromosomes, both in vitro and in vivo. This estimate is sensitive to the

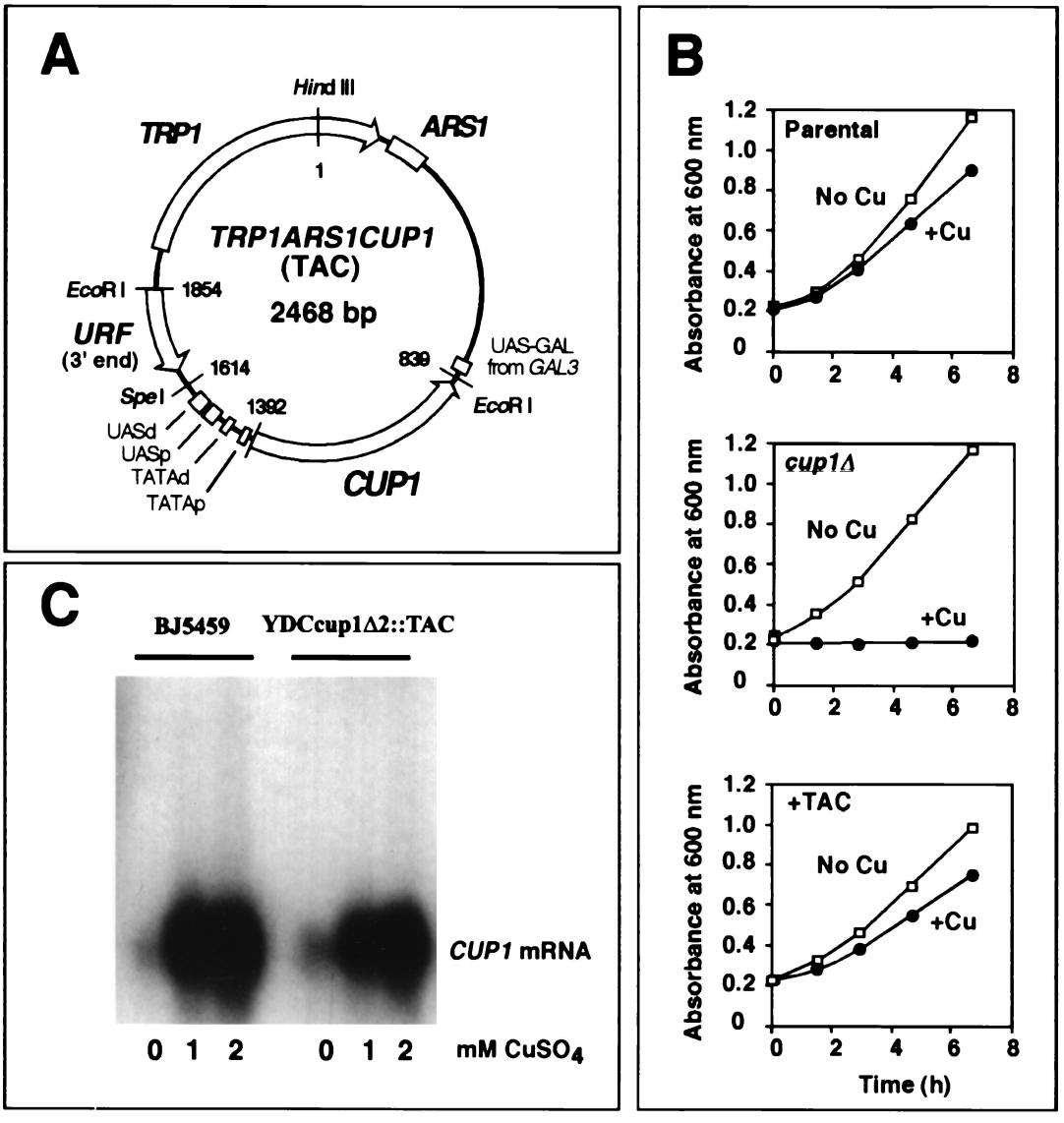

FIG. 1. The *TRP1 ARS1 CUP1* (TAC) episome protects yeast cells from the toxic effects of excess copper ions. (A) Map of the TAC episome. TAC is based on *TRP1 ARS1*, a yeast genomic *Eco*RI fragment (1,453 bp) capable of autonomous replication. *TRP1* encodes an enzyme required for the biosynthesis of tryptophan and is used as a selection marker. *ARS1* is the origin of replication. There is a UAS_{GAL} from *GAL3* within *TRP1 ARS1. CUP1* was inserted at the *Eco*RI site in *TRP1 ARS1*. The *CUP1* promoter contains two UASs: proximal (−106 to −142 [UAS_p]) and distal (−146 to −220 [UAS_d]), both of which contain binding sites for Ace1 and HSF. There are two good consensus TATA boxes, at −77 ($TATA_d$) and −33 ($TATA_p$) relative to the transcription start site. The insert also includes the 3′ untranslated region belonging to the URF neighboring *CUP1*. (B) TAC protects cells from copper toxicity. The growth of yeast cells was monitored by A_{600}. Cells from overnight cultures grown in the absence of copper were inoculated into medium with or without 1 mM copper(II) sulfate to an initial optical density of about 0.2. The parental strain, BJ5459 (doubling times were 160 min without copper and 185 min with copper), and the *cup1*Δ strain, YDCcup1Δ2 (170 min without copper), were grown in synthetic complete medium. YDCcup1Δ2::TAC was grown in synthetic complete medium lacking tryptophan (185 min without copper and 225 min with copper). (C) Induction of *CUP1* in spheroplasts. *CUP1* mRNA (about 600 nucleotides) was detected by Northern blot hybridization with a *CUP1* probe. Spheroplasts were incubated in SM with copper(II) sulfate added as indicated. RNA was prepared from spheroplasts after 30 min at 30°C with shaking at 225 rpm in an incubator.

possible contributions of RNA polymerase II (Pol II), remodeling complexes, and other factors to DNA supercoiling.

RNA Pol II is present on highly purified TAC minichromosomes. Transcriptionally active chromatin should contain RNA Pol II. Pol II forms stable elongation complexes which stall when nucleotides are removed. Nucleotides (including labeled UTP) were added to electroeluted minichromosomes to allow RNA polymerases to elongate nascent transcripts. To locate the sequences transcribed by Pol II, the labeled RNA was used as probe of a Southern blot of a gel with various restriction digests of pGEM-TAC (Fig. 4A). The digests divided TAC into separate *CUP1*, *TRP1*, and *ARS1* fragments (lane 2). In lane 3, the *CUP1* fragment was cut to give promoter and transcribed (ORF) fragments. The labeled RNA gave a strong signal with the TAC band and did not hybridize at all with the pGEM vector band, indicating that hybridization was specific

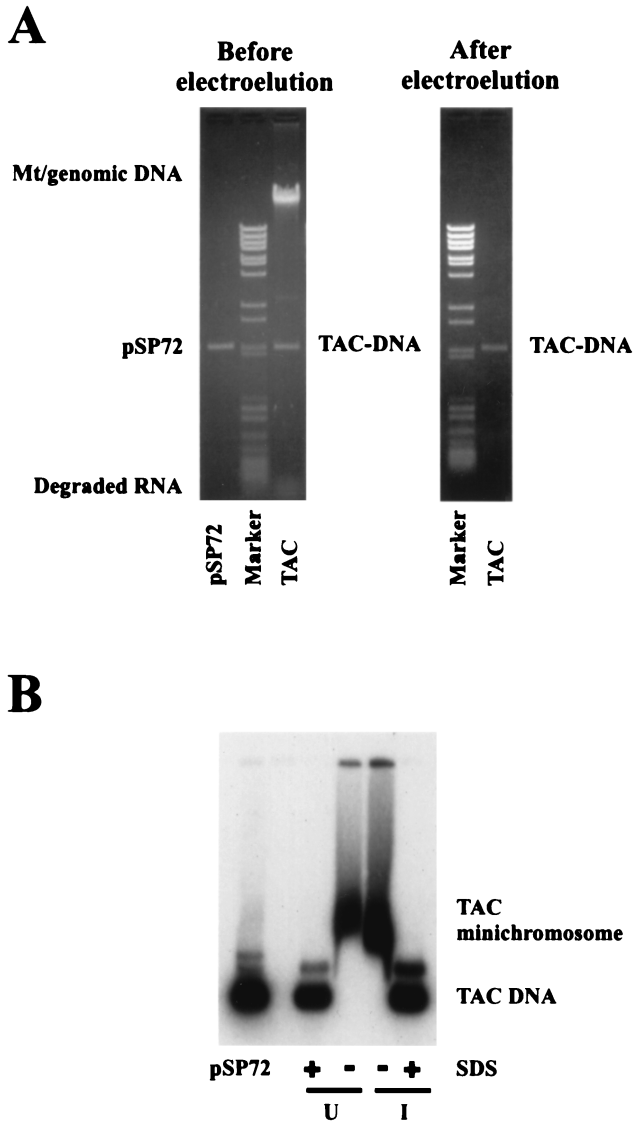

FIG. 2. Purification of the *TRP1 ARS1 CUP1* (TAC) episome. (A) Nucleic acids extracted from a typical preparation of TAC minichromosomes, before and after electroelution, analyzed in a 0.8% agarose gel stained with ethidium bromide. Supercoiled pSP72 (a plasmid of similar size to TAC; 2,472 bp) was used as a marker. Marker, a mixture of λ DNA *Bst*EII and pBR322 *Msp*I digests. (B) Analysis of TAC chromatin in an agarose gel. Electroeluted uninduced (U) and induced (I) TAC minichromosomes were incubated briefly with or without 1% SDS before loading in a 0.8% agarose gel. The gel was blotted and probed with radiolabeled pGEM-TAC.

(Fig. 4B). RNA from copper-induced minichromosomes gave a strong signal with the *CUP1* fragment, which was sensitive to α-amanitin (a specific inhibitor of Pol II, at low concentrations), indicating that Pol II is still present on *CUP1* after purification. Transcription was not completely inhibited by α-amanitin, probably because this drug inhibits the translocation step in RNA synthesis (58) and would allow the addition of a single nucleotide to the nascent transcript before blocking synthesis; if this nucleotide is UTP, the transcript would be end labeled. The *ARS1* fragment contains the 3′ end of *TRP1*, perhaps accounting for the signal on this fragment, but there might be some readthrough transcription into *ARS1* from *CUP1*. All transcripts hybridizing to *CUP1* were derived from the ORF, because the promoter fragment did not hybridize at all. This also shows that Pol II must have terminated transcription before reaching the *CUP1* promoter region. *TRP1* is transcriptionally active in all TAC preparations and was used to normalize the *CUP1* signals: induced TAC synthesized twice as much *CUP1* RNA as uninduced TAC and 5 times more than TAC from *ace1*Δ cells. These differences were less than expected given that *CUP1* is induced sixfold in spheroplasts (Fig. 1C), but could be accounted for if there were substantial readthrough from *CUP1* into *TRP1* in vitro.

In conclusion, TAC minichromosomes can be isolated substantially intact from yeast cells, and retain amounts of RNA Pol II that correlate with the transcriptional activity of *CUP1* in vivo.

The induced *CUP1* promoter is more accessible to restriction enzymes. Restriction enzymes were used to probe the accessibility of *CUP1* promoter DNA in copper-induced and uninduced TAC minichromosomes. Restriction enzymes cleave nucleosomal DNA at a very slow rate relative to naked DNA. In these experiments, minichromosomes were mixed with a plasmid as internal control and the kinetics of digestion of TAC chromatin and plasmid DNA were compared. This approach gives a quantitative estimate of the degree of protection of a particular restriction site (Fig. 5). This protection is likely to be predominantly due to nucleosomes, but other bound complexes might also contribute.

There is a *Mun*I site between the putative TATA boxes in the *CUP1* promoter. *Mun*I cleaved about 15% of uninduced TAC, reaching a plateau, indicating that 85% of the promoters are inaccessible (Fig. 5B). In contrast, about 35% of induced TAC was accessible. The other *Mun*I site in TAC is located just inside the transcribed region of *TRP1* and is strongly and equally protected in uninduced and induced TAC (about 10%

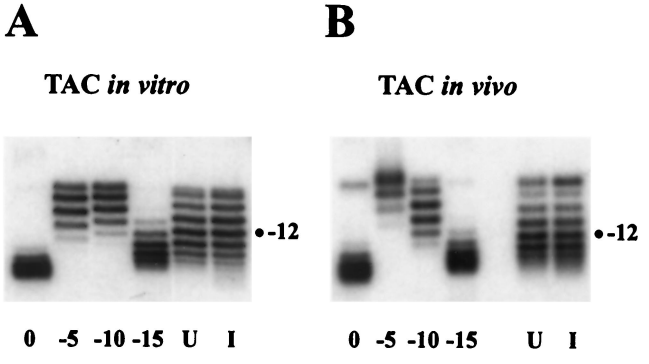

FIG. 3. Topological analysis of copper-induced and uninduced TAC minichromosomes in vitro and in vivo. (A) Determination of the linking numbers of uninduced and copper-induced TAC minichromosomes in vitro. DNA extracted from preparations of induced (I) and uninduced (U) TAC was electrophoresed in an agarose gel containing 10 μg of chloroquine per ml. Linking number standards with an average of 0 (relaxed), 5, 10, and 15 negative supercoils were prepared by using pSP72. Southern blots probed with pGEM-TAC are shown. (B) Determination of the linking numbers of uninduced and copper-induced TAC minichromosomes in vivo. DNA extracted directly from induced and uninduced spheroplasts was analyzed as in panel A, except that the gel contained 7.5 μg of chloroquine per ml.

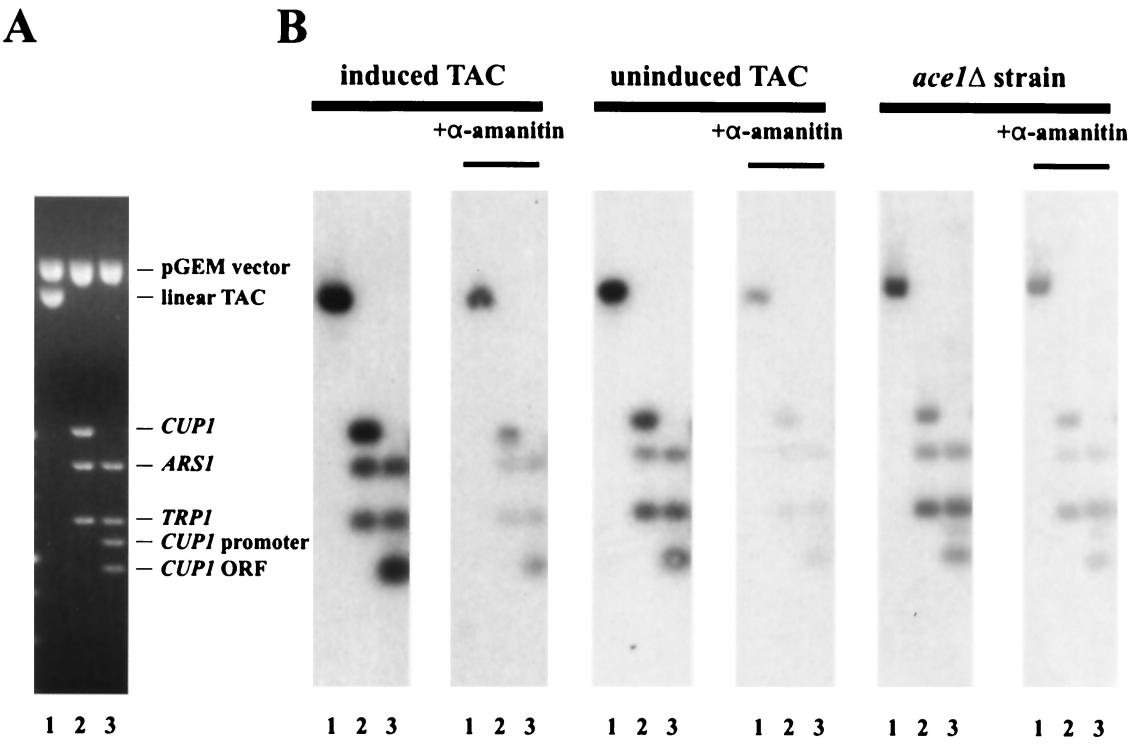

FIG. 4. Presence of RNA Pol II on *CUP1* in purified TAC minichromosomes. Electroeluted TAC minichromosomes were incubated with nucleoside triphosphates (including radiolabeled UTP) for synthesis of run-on transcripts in the presence or absence of α-amanitin. (A) Typical gel used for Southern blots (stained with ethidium). pGEM-TAC was digested with *Hin*dIII only (lane 1); *Hin*dIII and *Eco*RI (lane 2); or *Hin*dIII, *Eco*RI, and *Pac*I (lane 3). (B) Hybridization with RNA synthesized in the presence or absence of α-amanitin by electroeluted TAC minichromosomes isolated from induced, uninduced, or *ace1*Δ cells.

cleavage). Similarly, the *Msp*A1I site in the proximal UAS was accessible in 25% of uninduced TAC, whereas about 50% of induced TAC was accessible (Fig. 5C). In contrast, the *Hae*II site in the 3′ URF region was 20% accessible in both induced and uninduced TAC, indicating that induction had no effect on its accessibility. In summary, induced TAC episomes contained more accessible *CUP1* promoters than uninduced TAC, indicating that activation of *CUP1* coincides with increased exposure of the promoter, presumably to facilitate formation of the transcription complex.

Chromatin structure of the TAC minichromosome. The chromatin structures of TAC from induced and uninduced cells were examined initially by the indirect end-labeling method (54) to determine whether *CUP1* is present in a highly ordered chromatin structure. However, a cleavage pattern consistent with a more complex chromatin structure was observed (not shown), and nucleosome positions could not be determined. Therefore, the monomer extension method (61) for identifying nucleosome positions was used, which was developed to resolve arrays of overlapping positions in complex reconstituted chromatin structures, without ambiguity (10, 11). This method requires purified chromatin; it is unlikely to be effective with nuclei because of the large excess of nucleosomes from the rest of the genome.

Isolated minichromosomes were completely digested to nucleosome core particles by using MNase. DNA was extracted, and core particle DNA (140 to 160 bp) was purified from a gel and end labeled with T4 polynucleotide kinase. Labeled core DNA was then used as primer for extension by Klenow fragment with single-stranded pGEM-TAC DNA as template. The replicated DNA was digested with different restriction enzymes to resolve different regions of the TAC minichromosome. The lengths of the resulting DNA fragments were determined accurately in sequencing gels: each band defines the distance from the border of a nucleosome to the chosen restriction site. A control for sequence-dependent termination by Klenow enzyme involves omission of the restriction enzyme. The borders of each nucleosome were located precisely, with one using the positive strand as template and the other using the negative strand. The data from one template strand define all of the "upstream" nucleosome borders unambiguously and are sufficient to define the chromatin structure. The other strand should give the same nucleosome positions, this time defined by the "downstream" borders. The degree to which the two sets of positioning data are consistent can be assessed by calculating the average distance between the nucleosome borders, which should be close to 147 bp, the size of the core particle.

Because the monomer extension technique is not yet widely used, it is worthwhile discussing some technical points. A slight underdigestion of chromatin by MNase results in core particles that are not completely trimmed to 147 bp. Consequently, bands within about 20 bp of one another are likely to represent different degrees of trimming of the same positioned nucleosome. In the analysis, clusters of bands within 20 bp were counted as the same nucleosome. If core particles are overdi-

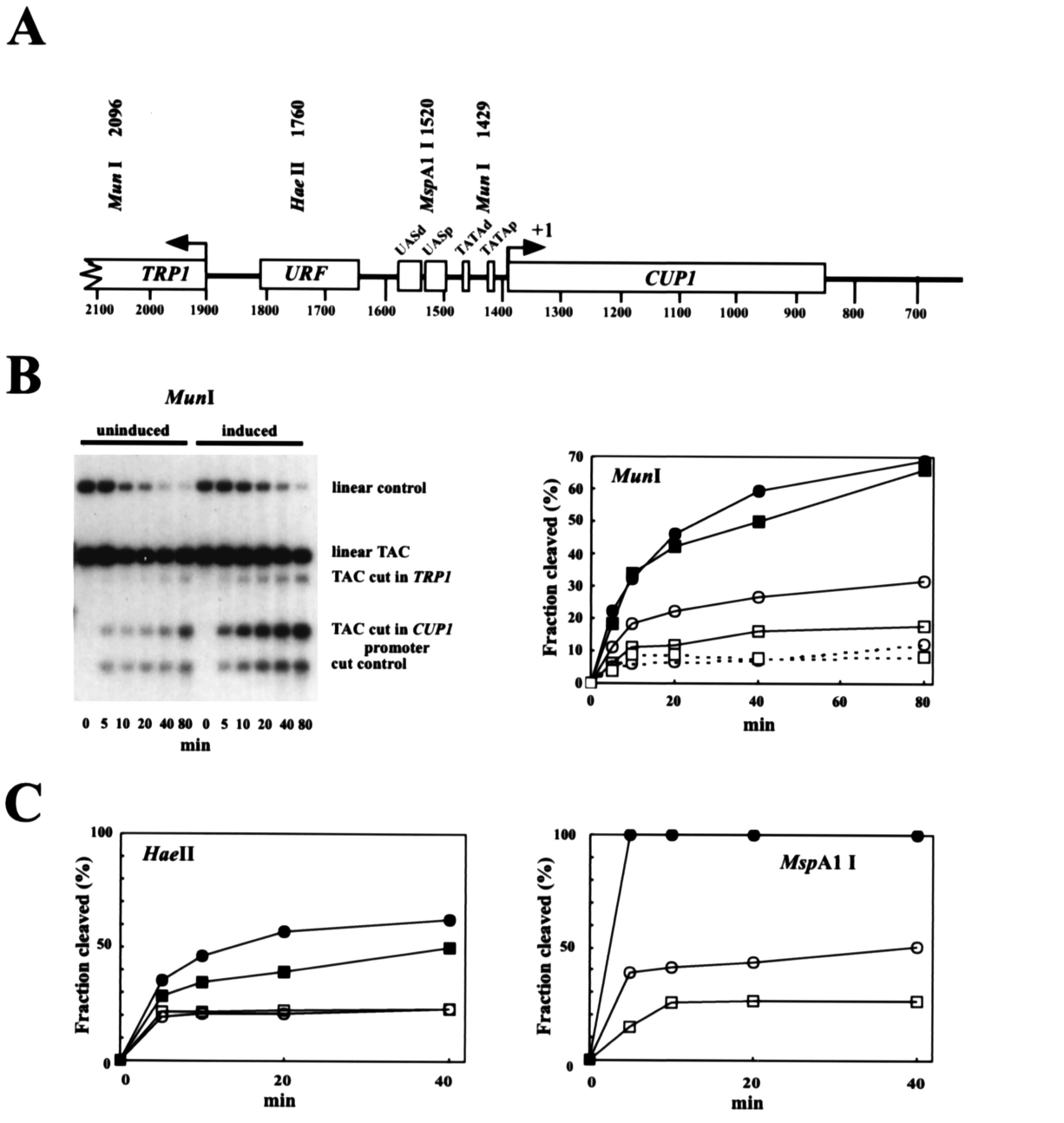

FIG. 5. Accessibility of restriction sites in the *CUP1* promoter in uninduced and induced TAC minichromosomes. (A) Map of relevant restriction sites in TAC. (B) Accessibility of the *Mun*I site in the *CUP1* promoter. TAC chromatin (not electroeluted) was mixed with plasmid DNA as an internal control and digested with *Mun*I for the times indicated. DNA was purified and digested with *Hin*dIII to linearize both TAC and the control plasmid (pGEM-TAC). A Southern blot was probed with the *Hin*dIII-*Bgl*II fragment from TAC (the *Bgl*II site is at 238). Data were quantitated by phosphorimager analysis and plotted for the uninduced control (■), uninduced TAC (□), induced control (●), and induced TAC (○). The dashed lines show the plots for the other *Mun*I site, in *TRP1*. (C) Accessibility of the *Hae*II and *Msp*A1I sites. Plots of data from phosphorimager analysis. These sites are both unique in TAC. Symbols are as in panel B.

gested by MNase, nicks begin to appear. Labeled core DNA was routinely checked in denaturing gels: the size range was typically 140 to 160 bp, with very little nicking. In any case, nicking would not affect the result, because kinase does not label nicks, and end-labeled nicked DNA strands liberated on denaturation of core DNA would give the correct result on extension. Proteins other than nucleosomes which might be bound to the minichromosome will not interfere with nucleosome mapping unless they protect 140 to 160 bp of DNA against extensive digestion by MNase (because the DNA is subsequently gel purified). The contributions of such proteins would appear as nucleosome-free gaps in the map (see below), but this might not be obvious unless there is close to 100% occupancy of their sites.

Initially, TAC episomes from copper-induced and uninduced cells were compared. A complex but highly reproducible

TABLE 1. Nucleosome positions on TAC minichromosomes[a]

Nucleosome	Position		Distance (bp)	*ace1*Δ[b]
	+ Border	− Border		
1	14 (9)	164 (12)	150	+
2	94 (19)	237 (13)	143	+
3	131 (1)	277 (8)	146	+
4	178 (11)	321 (8)	143	+
5	197 (6)	352 (8)	155	+
6	244 (7)	390 (15)	146	+
7	292 (18)	453 (26)	161	+
8	410 (7)	545 (3)	135	−
9	441 (10)	591 (12)	150	−
10	487 (12)	642 (15)	155	+
11	553 (20)	695 (19)	142	+
12	666 (12)	818 (10)	152	−
13	739 (22)	889 (13)	150	−
14	782 (14)	924 (17)	142	−
15	840 (11)	995 (10)	155	+
16	872 (9)	1028 (8)	156	+
17	923 (14)	1073 (8)	150	+
18	964 (14)	1120 (18)	156	+
19	1028 (15)	1188 (13)	160	+
20	1143 (14)	1291 (20)	148	−
21	1196 (17)	1347 (15)	151	−
22	1238 (7)	1394 (5)	156	+
23	1269 (10)	1429 (9)	160	+
24	1311 (13)	1464 (15)	153	+
25	1367 (14)	1516 (4)	149	−
26	1413 (13)	1552 (8)	139	−
27	1454 (5)	1595 (6)	141	−
28	1479 (10)	1651 (9)	172	+
29	1550 (5)	1690 (17)	140	+
30	1586 (10)	1735 (10)	149	+
31	1624 (6)	1770 (9)	146	+
32	1651 (14)	1810 (12)	159	+
33	1695 (10)	1866 (10)	171	−
34	1748 (16)	1893 (11)	145	−
35	1789 (6)	1939 (15)	150	−
36	1842 (18)	2005 (6)	163	−
37	1903 (17)	2052 (12)	149	+
38	1973 (22)	2106 (12)	133	+
39	2035 (2)	2179 (14)	144	−
40	2071 (10)	2227 (14)	156	−
41	2123 (15)	2268 (13)	145	+
42	2163 (2)	2311 (9)	148	+
43	2185 (7)	2346 (9)	161	+
44	2221 (9)	2373 (6)	152	+
45	2247 (11)	2411 (10)	164	+
46	2270 (1)			+
47	2300 (1)			+
48	2350[c]			+

[a] All band sizes were measured and converted to TAC coordinates, with +1 at the *Hin*dIII site, reading clockwise (see Fig. 1A). Data from five separate induced or uninduced minichromosome preparations are included, with analysis using several different restriction enzymes. The standard error is in parentheses and represents the error computed on the average coordinate for each set of bands attributed to a particular positioned nucleosome (due to different degrees of trimming) for the five preparations. The average core particle size is 151 ± 8 bp.

[b] *ace1*Δ in TAC from *ace1*Δ cells: +, position occupied; −, position unoccupied.

[c] This nucleosome border was measured only once, for the positive strand only.

band pattern was obtained with no qualitative differences between the induced and uninduced minichromosomes. Some data for induced TAC are shown in Fig. 6A (ind. lanes), and a summary of many data sets using different restriction enzymes to map different parts of TAC is shown in Fig. 6B. The pattern defined 48 different nucleosome positions on TAC (Table 1). The intensities of the bands indicate that some nucleosomes occur more frequently than others. Many of these positions are overlapping and therefore mutually exclusive. The first impression is that nucleosomes are positioned randomly in TAC, but this is not the case. A truly random distribution would yield 2,468 different positions and bands of equal intensity corresponding to every nucleotide position in the sequencing gel. Instead, 48 relatively strong positions were observed. It is emphasized that this complex pattern of nucleosome positions was highly reproducible; data were obtained from many independent preparations (Table 1). The standard errors were relatively small and reflect a combination of some measurements from the less accurate region at the top of each gel and small differences in the degree of trimming of core DNA by MNase. The average distance between nucleosome borders was 151 ± 8 bp, very close to the 147 bp expected, indicating that the data from the positive and negative strands are in agreement: they both describe the same chromatin structure.

Thus, induced and uninduced TAC minichromosomes have qualitatively very similar complex chromatin structures, perhaps representing all possible combinations of the 48 positions observed. They might differ quantitatively in their nucleosome distributions, but it is difficult to compare different monomer extension samples quantitatively, because a control band for normalization is not available. However, the increased accessibility of the restriction sites for *Mun*I and *Msp*A1I in the *CUP1* promoter observed on induction (Fig. 5) suggests that nucleosome positions over the promoter are less likely to be occupied after induction. There are multiple ways of arranging the nucleosomes in TAC with respect to one another. Most arrangements give a maximum of 12 or 13 nucleosomes in TAC, consistent with topological measurements in vivo and in vitro (Fig. 3). Thus, TAC chromatin is highly heterogeneous; each minichromosome is likely to have a slightly different chromatin structure from the next.

Ace1p-dependent nucleosome repositioning on *CUP1* and flanking regions. To determine the contribution of Ace1p to chromatin structure, TAC isolated from *ace1*Δ cells was analyzed (Fig. 6A). A simpler chromatin structure was observed in which only 32 of the 48 positions in induced TAC were prominent, i.e., a subset of the same positions (Table 1); the other 16 positions were rarely occupied. The nucleosomes observed can be divided into six clusters of overlapping positions (I to VI in Fig. 4B) separated by linkers of various lengths, some of which contain factor binding sites that could act as nucleosome phasing signals: the 18-bp linker between clusters IV and V contains the distal TATA box in the *CUP1* promoter; the very long (151 bp) linker between clusters II and III contains the UAS_{GAL}, and the 80-bp linker between clusters V and VI includes the region just upstream of the putative TATA box for *TRP1*, which might contain an activator binding site. The 41-bp linker between clusters I and II might contain a binding site for an unknown factor, since the function of the DNA between *ARS1* and *GAL3* remains to be elucidated. However, the 48-bp linker between clusters III and IV and the 22-bp linker between clusters VI and I are within the *CUP1* and *TRP1* ORFs, respectively, and so are unlikely to represent phasing signals. Nucleosome arrangements indicate a maximum of 11 nucleosomes in TAC from *ace1*Δ cells (1 or 2 less than TAC from Ace1p-containing cells).

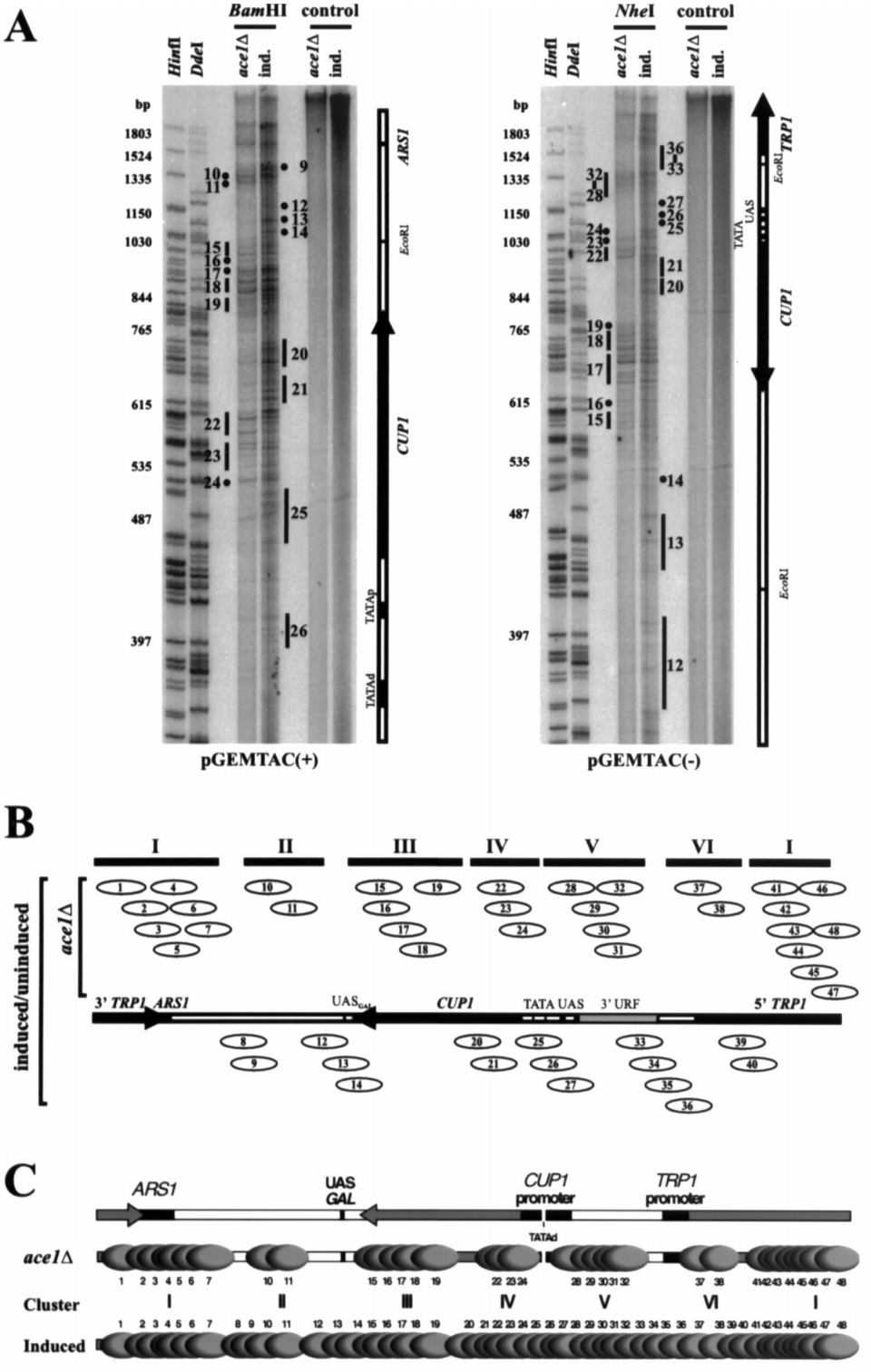

FIG. 6. Chromatin structure of purified TAC minichromosomes by monomer extension analysis. (A) Typical monomer extension analysis of positioned nucleosomes in TAC from copper-induced (ind.) and *ace1*Δ cells purified to the stage prior to electroelution. In the examples shown, translational positions were mapped from the *Bam*HI site (at 1833) on the positive strand of pGEM-TAC and the *Nhe*I site (at 423) on the negative strand. Controls had extension but no digestion with restriction enzyme. Nucleosome positions are indicated by numbered dots or bars (the latter indicating bands which were included within that nucleosome position, as discussed in the text). They are numbered from 1 to 48 (Table 1), beginning at the *Hin*dIII site in *TRP1* (Fig. 1A). The markers are *Hin*fI and *Dde*I digests of λ DNA labeled with T4 kinase; the sizes of some of the bands are indicated to the left. (B) Summary of nucleosome positions in TAC minichromosomes. Nucleosome positions are numbered in the order of their coordinates in TAC relative to the *Hin*dIII site in *TRP1* (=1). The *Eco*RI sites mark the boundaries of the *CUP1* insert. Positions observed in TAC from *ace1*Δ cells are shown above the map of TAC, and nonoverlapping position clusters I to VI are indicated. Cluster I includes positions 41 to 48 and 1 to 7. In TAC from uninduced and induced cells, all of the nucleosome positions shown were observed; the novel positions occupying linkers between the clusters are shown below the map of TAC. (C) Distribution of nucleosome positions in TAC. Gray ovals indicate positioned nucleosomes drawn to scale (numbered according to Table 1).

As discussed above, in the presence of Ace1p, more nucleosome positions were observed (Fig. 6; compare *ace1*Δ and induced lanes), including positions 25, 26, and 27 over the *CUP1* promoter and 20 and 21 in the *CUP1* ORF. Nucleosome repositioning also occurred over the sequences flanking *CUP1*, with positions 8, 9, 12, 13, and 14 downstream of the *CUP1* insert and positions 33 to 36 and 39 to 40 in the region upstream of *CUP1* appearing. These results are summarized in Fig. 6C, in which the nucleosome position clusters observed in *ace1*Δ cells are shown together with all of the 48 possible positions observed in Ace1p-containing cells. From these observations, it may be concluded that the binding of Ace1p coincides with the repositioning of nucleosomes on *CUP1* and its flanking regions from the clusters to the linkers.

TAC also has a complex chromatin structure in nuclei. A concern in all determinations of nucleosome positions is the possibility of nucleosome sliding. This seems unlikely to be occurring here; sliding requires elevated salt concentrations and temperature (>0.15 M at 37°C in the absence of histone H1) (50), which were avoided throughout purification. Furthermore, sliding would have had to occur to the same precise positions in all preparations. Although sliding seemed unlikely, we addressed the possibility that a single array of precisely positioned nucleosomes was present on *CUP1* in nuclei which was disrupted during the subsequent purification step, by analyzing the chromatin structure of TAC minichromosomes in nuclei. The monomer extension method is unlikely to be effective in nuclei, because there would be a very high background from contaminating core particles. Therefore, to examine the chromatin structure of TAC in nuclei, we used primer extension to map MNase-cut sites (56) with primers corresponding to both ends of the *CUP1* insert. Nuclei were prepared from uninduced and induced cells containing TAC and digested with increasing amounts of MNase. A typical nucleosomal ladder with a repeat length of about 160 bp was observed (Fig. 7A), as expected for yeast (55). Primer extension analysis of these samples (Fig. 7B) revealed a series of bands unique to chromatin, reflecting cleavage in the linkers, but they are not spaced by 147 bp. The pattern of protected regions of much less than 147 bp was consistent with the complex pattern of overlapping nucleosome positions revealed by monomer extension, and there was no evidence for a single array of positioned nucleosomes. There is almost no difference between induced and uninduced *CUP1*, although there are some subtle changes in the band pattern and degree of protection in the promoter region. We obtained similar maps for chromosomal *CUP1* in nuclei from BJ5459 cells (not shown). Thus, the primer extension map is consistent with the presence of multiple, overlapping positioned nucleosomes in TAC chromatin in nuclei. Furthermore, the fact that TAC minichromosomes from *ace1*Δ cells isolated in parallel gave a different chromatin structure also suggests that the isolation protocol preserves chromatin structure.

Nucleosome repositioning on *CUP1* is independent of transcription. Ace1p-dependent nucleosome repositioning might be due to transcription by RNA Pol II or to a chromatin remodeling complex recruited by Ace1p. The former seemed unlikely, because nucleosomes were repositioned in untranscribed regions as well as transcribed regions. To distinguish between the two, a yeast strain containing TAC with both TATA boxes in the *CUP1* promoter mutated to prevent transcript initiation was used (confirmed by mung bean nuclease mapping of transcripts) (data not shown). Nucleosome positions in these TAC minichromosomes were identical to those in the induced state, indicating that nucleosomes were repositioned in the presence of Ace1p, but in the absence of transcription (Fig. 8). Taken together, our observations constitute strong evidence for the recruitment by Ace1p of a nucleosome repositioning activity which acts over the entire *CUP1* gene and some flanking sequences.

DISCUSSION

CUP1 was chosen as a model for understanding gene activation in the context of chromatin structure, because its regulation is relatively simple and it has a strongly inducible promoter. An effective method for the purification of episomes was developed, and evidence that their chromatin structures remained substantially intact has been presented, including the retention of RNA Pol II in amounts correlating with transcriptional activity in vivo. The chromatin structures of purified TAC minichromosomes in various states of transcriptional activity were determined by the monomer extension method. A relatively ordered chromatin structure is observed in the absence of the transcriptional activator, Ace1p. In its presence, the clusters of nucleosome positions were disrupted, because nucleosomes were repositioned over linkers. Nucleosome repositioning requires Ace1p, but is independent of transcription, because it occurred even when the TATA boxes in the *CUP1* promoter were mutated.

Translationally positioned nucleosomes in TAC. TAC minichromosomes isolated from cells containing Ace1p are heterogeneous in chromatin structure: 48 differently positioned nucleosomes were identified. Overlapping nucleosome positions were observed over the entire plasmid. These can be occupied in many different combinations to give totals of 11 to 13 nucleosomes, in agreement with the topological analysis. The complexity of the chromatin structure of induced TAC minichromosomes is about what would be expected from in vitro reconstitution experiments. For example, on two 358-bp fragments containing a 5S RNA gene, 6 or 12 positions were observed (41) indicating "position densities" of about 1 per 30 or 60 bp, respectively, and for a 359-bp fragment containing the *Drosophila hsp70* promoter, 5 positions were observed (1 per 72 bp) (19). For TAC (2,468 bp), the value is 1 per 51 bp. In fact, the translational positions mapped in native induced TAC chromatin are the same as those formed by reconstitution of nucleosomes from purified components (C.-H. Shen and D. J. Clark, unpublished data). Therefore, DNA sequence determines the possible positions in TAC, but events on the plasmid determine which positions are occupied and when.

Chromatin structure of the TAC minichromosome. In the absence of Ace1p, the chromatin structure of *CUP1* and its flanking sequences is relatively ordered, with clusters of alternative overlapping nucleosome positions separated by linkers that are rarely occupied by nucleosomes. This may represent a relatively undisturbed chromatin structure laid down during nucleosome assembly coupled to DNA replication, which might be determined in part by factors acting as nucleosome phasing signals (15) bound at the *TRP1* and *CUP1* promoters,

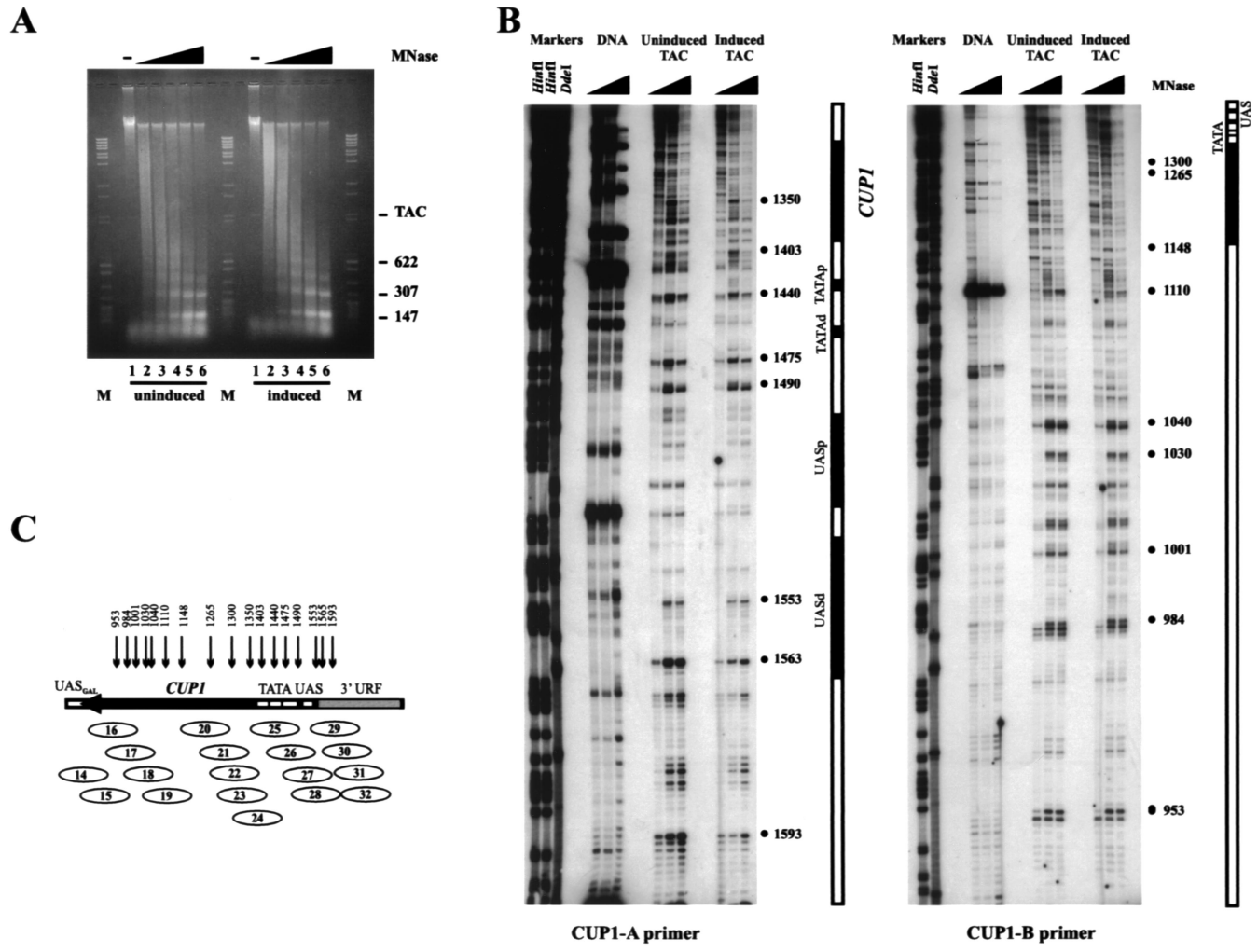

FIG. 7. Chromatin structure of *CUP1* in TAC minichromosomes in nuclei by primer extension analysis. (A) Analysis of DNA from nuclei from copper-induced and uninduced YDCcup1Δ2::TAC digested with MNase in a 1% agarose gel stained with ethidium bromide. Markers were a mixture of λ DNA digested with *Bst*EII and pBR322 digested with *Msp*I (some bands are labeled). (TAC is faintly visible in the undigested control lanes and is mostly nicked under the conditions used for incubation of the nuclei.) The band at the bottom in all lanes is residual RNA. (B) Primer extension mapping of copper-induced and uninduced *CUP1* in TAC minichromosomes in nuclei. Samples shown in panel A, lanes 1, 4, and 6, were used. DNA, pCP1A digested with MNase. Major bands are indicated with dots and coordinates in TAC. Markers (*Dde*I and *Hin*fI) are as in Fig. 6A. (C) Comparison of primer extension data for TAC in nuclei with the nucleosome map obtained for purified TAC by monomer extension (Fig. 6). The arrows indicate the major MNase-cut sites mapped by primer extension; these sites can be fit to linkers between positioned nucleosomes mapped by monomer extension.

at *ARS1* (the origin recognition complex), at the UAS_{GAL} and perhaps at other sites in TAC. All of these sites except *ARS1* are at least partly in the linker in *ace1*Δ cells. In the case of *ARS1*, a MNase-hypersensitive site was observed by indirect end labeling (not shown), previously reported by others (54), indicating that *ARS1* is accessible in a significant fraction of TAC minichromosomes (presumably those with arrays including nucleosomes 1 and 4 or 6 or 7, placing *ARS1* in the linker). In TAC from cells containing Ace1p, the presence of nucleosomes on a fraction of each of these binding sites implies that remodeling might lead to some displacement of these factors.

TRP1 was used as a selection marker for TAC and was therefore in its transcriptionally active state under all conditions examined. The activity of *TRP1* was insufficient to disturb the chromatin structure of *CUP1* in *ace1*Δ cells, although it might have had minor effects. The *TRP1* promoter in *TRP1 ARS1* is truncated and might be missing important regulatory elements which reduce its ability to recruit remodeling complexes as well as its transcriptional activity. Remodeling of *CUP1* does have effects on *TRP1*: in the presence of Ace1p, nucleosomes occupy positions 39 and 40 at the 5′ end of *TRP1* and positions 8, 9, and 12 to 14 near *ARS1*. The chromatin structure of the *TRP1 ARS1* minichromosome has been studied in detail by using indirect end labeling (54): three strongly positioned nucleosomes were identified next to *ARS1*. However, insertion of DNA at the *Eco*RI site disrupted the positioning of these nucleosomes (44). This is also where *CUP1* was inserted and probably accounts for the less ordered structure of the *ARS1* region in TAC. Another relevant factor is the much higher copy number of *TRP1 ARS1* (100 to 200 per cell) (62) relative to TAC and other *TRP1 ARS1* plasmids with inserts (20, 53): most *TRP1* genes in *TRP1 ARS1*-containing

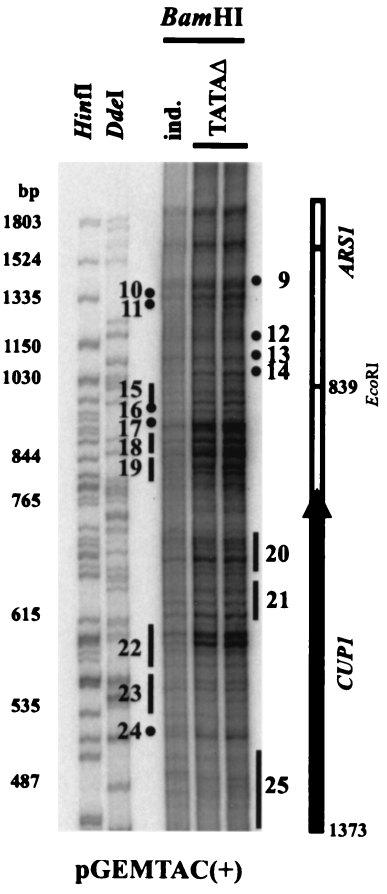

FIG. 8. Chromatin structure of TAC minichromosomes containing TATA box mutations in the *CUP1* promoter. Monomer extension analysis to compare nucleosome positions in copper-induced and TATA-mutant TAC minichromosomes. Two independently prepared TATA mutant samples are shown. Labeling is as in the legend to Fig. 6.

cells might be inactive and unremodeled, with more ordered structures.

How does Ace1p target the remodeling complex to *CUP1?* In the absence of Ace1p, the chromatin structure of the *CUP1* promoter is such that the distal TATA box is placed in the linker between two clusters of overlapping positions, but the UASs (coordinates 1510 to 1612) may be completely open (positions 30 to 32), partly covered (position 29), or completely contained within a nucleosome (position 28). For induction, Ace1p must bind to its site in order to target the remodeling complex. It is not known whether Ace1p, like the thyroid hormone receptor (59), can recognize its binding site in a nucleosome, or whether, like many transcriptional activators (2), it has greatly reduced affinity for its site when in a nucleosome. If the latter is the case, the presence of multiple binding sites for Ace1p (two in each UAS) offers a potential solution: for positions 29 to 32, at least one site is present in the linker and available for Ace1p to bind. In the case of position 28, in which all the sites are covered, the weakened binding of several Ace1p molecules might be sufficient to disrupt the nucleosome (2). In this model, Ace1p should be able to access at least one binding site independently of which nucleosome positions 28 to 32 happen to be occupied. An alternative, "concerted probing," model postulates the formation of a complex between copper-activated Ace1p and the remodeler, which then "probes" each nucleosome in turn until Ace1p recognizes its binding site.

It is instructive to compare *CUP1* with *PHO5*, for which the relationship between chromatin structure and gene expression in yeast has been most thoroughly studied (reviewed in reference 52). Induction of *PHO5* correlates with the disruption of an ordered array of four positioned nucleosomes on the *PHO5* promoter and requires the presence of a binding site for the Pho4p activator in the linker between the central pair of nucleosomes. *CUP1* has a much less ordered chromatin structure at the promoter, but there are four binding sites for Ace1p, which, as discussed above, could facilitate binding of Ace1p. The large increase in accessibility to nucleases at the *PHO5* promoter indicates that nucleosome disruption is likely to involve dramatic conformational changes or displacement of the four nucleosomes rather than just repositioning. For *CUP1*, relatively modest increases in accessibility to restriction enzymes were observed on induction (not shown). As for *CUP1*, chromatin remodeling at *PHO5* is dependent on the presence of activator, but transcription is not required. Whether remodeling of *PHO5* chromatin is confined to the promoter or whether, like *CUP1*, it involves the rest of the gene and flanking sequences is unclear.

Mechanism of remodeling of *CUP1* and its flanking sequences. It is not known which of the chromatin remodeling activities identified in yeast is involved in *CUP1* regulation. Nucleosome repositioning could be the direct result of recruitment by Ace1p of the SWI-SNF complex, RSC (8), or one of the I-SWI-like complexes. Alternatively, it might be the indirect consequence of a targeted histone modification, such as acetylation. Experiments addressing these possibilities are in progress. Current models for the mechanism of chromatin remodeling have been reviewed recently (22, 24, 38, 43). In the "activator model" (43), gene-specific activators recruit a remodeling complex directly to the promoter, which then alters local chromatin structure to facilitate transcription (39, 40). In vitro, remodeling complexes catalyze nucleosome sliding (19, 26, 57) and/or nucleosome transfer (35). Remodeled nucleosomes also have an altered conformation and can form dimer-like particles (4, 34, 47).

Much of the evidence for the mechanism of remodeling is based on biochemical data in vitro. We have provided direct support for the activator model in vivo by isolating and examining the structures of native chromatin: we have shown that remodeling of *CUP1* is dependent on its transcriptional activator, Ace1p, that remodeling involves the repositioning of nucleosomes, and that, perhaps surprisingly, remodeling is not confined to the *CUP1* promoter, but includes the entire gene and unrelated flanking regions also. While there is much evidence that gene activation is correlated with disruption of a relatively ordered chromatin structure, the structural nature of this disruption has not been elucidated. Our observations suggest that a major part of this structural transition is the dynamic redistribution of nucleosomes. Repositioned nucleosomes protected 147 bp of DNA from digestion by MNase, and so, by this criterion, are not conformationally altered. However, remodeled nucleosomes might be relatively short-lived

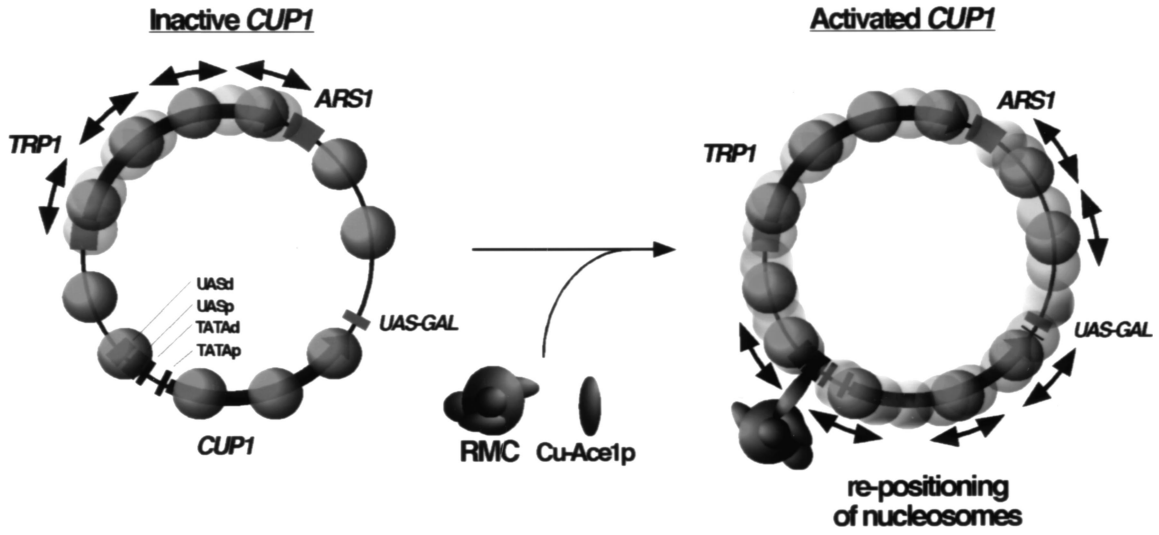

FIG. 9. A fluid chromatin model for the remodeling of the *CUP1* gene in TAC. A chromatin structure representative of TAC minichromosomes containing inactive *CUP1* (*ace1*Δ) is shown. *TRP1* is transcriptionally active and is shown as undergoing remodeling (double-headed arrows indicate nucleosome repositioning). The first step in activation of *CUP1* for transcription is the binding of copper-activated Ace1p to one of its binding sites in the UASs. How Cu-Ace1p might gain access to its sites is discussed in the text. Cu-Ace1p recruits a remodeling complex (RMC) to the *CUP1* promoter, which catalyzes nucleosome repositioning on *CUP1* and its flanking sequences. The continual free movement of nucleosomes between positions results in a fluid chromatin structure, rendering the underlying DNA transparent and facilitating the formation of a transcription complex at the *CUP1* promoter and the passage of RNA Pol II (see text for a discussion of possible mechanisms).

intermediates in vivo. Furthermore, remodeled nucleosomes might not protect 147 bp of DNA from MNase digestion (they might be relatively unstable or, if a dimer-like particle is formed, they might protect a larger piece of DNA which would not be present in core DNA preparations).

The remodeling activity recruited to *CUP1* by Ace1p is apparently capable of reorganizing a domain of chromatin structure defined by the limits of nucleosome repositioning observed. This extends from positions 8 and 9 near *ARS1* to positions 39 and 40 at the 5′ end of *TRP1*. These are outside the *CUP1* insert and indicate that the remodeling activity influences nucleosome positions over nearly 2 kb of DNA and perhaps over the entire TAC plasmid. The fact that remodeling is not confined to the promoter suggests that the remodeling complex recruited by Ace1p somehow reorganizes a domain of chromatin structure rather than working only on promoter nucleosomes. How it might achieve this is a matter for speculation, but the looping and tracking models suggested for enhancer action (5) are obvious candidates. Remodeling activity might create a "fluid" chromatin structure (24). Thus, the heterogeneity observed in TAC minichromosomes is likely to reflect a highly dynamic chromatin structure, in which facile nucleosome movement between observed translational positions is catalyzed by the remodeling complex recruited by Ace1p (Fig. 9). The fact that these positions overlap might be important in the mechanism of nucleosome transfer. Facile nucleosome movement should facilitate events such as the formation of a transcription complex at the *CUP1* promoter and the passage of RNA Pol II.

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REFERENCES

1. **Adams, C. C., and D. S. Gross.** 1991. The yeast heat shock response is induced by conversion of cells to spheroplasts and by potent transcriptional inhibitors. J. Bacteriol. **173:**7429–7435.
2. **Adams, C. C., and J. L. Workman.** 1993. Nucleosome displacement in transcription. Cell **72:**305–308.
3. **Alfieri, J. A., and D. J. Clark.** 1999. Isolation of minichromosomes from yeast cells. Methods Enzymol. **304:**35–49.
4. **Bazett-Jones, D. P., J. Côté, C. C. Landel, C. L. Peterson, and J. L. Workman.** 1999. The SWI/SNF complex creates loop domains in DNA and polynucleosome arrays and can disrupt DNA-histone contacts within these domains. Mol. Cell. Biol. **19:**1470–1478.
5. **Blackwood, E. M., and J. T. Kadonaga.** 1998. Going the distance: a current view of enhancer action. Science **281:**60–63.
6. **Buchman, C., P. Skroch, J. Welch, S. Fogel, and M. Karin.** 1989. The *CUP2* gene product, regulator of yeast metallothionein expression, is a copper-activated DNA-binding protein. Mol. Cell. Biol. **9:**4091–4095.
7. **Butt, T. R., E. J. Sternberg, J. A. Gorman, P. Clark, D. Hamer, M. Rosenberg, and S. T. Crooke.** 1984. Copper metallothionein of yeast, structure of the gene, and regulation of expression. Proc. Natl. Acad. Sci. USA **81:**3332–3336.
8. **Cairns, B. R., Y. Lorch, Y. Li, M. Zhang, L. Lacomis, H. Erdjument-Bromage, P. Tempst, J. Du, B. Laurent, and R. D. Kornberg.** 1996. RSC, an essential, abundant chromatin-remodeling complex. Cell **87:**1249–1260.
9. **Clark, D. J.** 1998. Counting nucleosome cores on circular DNA using topoisomerase I, p. 139–152. *In* H. Gould (ed.), Chromatin: a practical approach. Oxford University Press, Oxford, United Kingdom.
10. **Davey, C., S. Pennings, and J. Allan.** 1997. CpG methylation remodels chromatin structure *in vitro*. J. Mol. Biol. **267:**276–288.
11. **Davey, C., S. Pennings, G. Meerseman, T. J. Wess, and J. Allan.** 1995. Periodicity of strong nucleosome positioning sites around the chicken adult β-globin gene may encode regularly spaced chromatin. Proc. Natl. Acad. Sci. USA **92:**11210–11214.
12. **Depew, R. E., and J. C. Wang.** 1975. Conformational fluctuations of DNA helix. Proc. Natl. Acad. Sci. USA **72:**4275–4279.
13. **Ducker, C. E., and R. T. Simpson.** 2000. The organized chromatin domain of the repressed yeast a cell-specific gene *STE6* contains two molecules of the corepressor Tup1p per nucleosome. EMBO J. **19:**400–409.
14. **Eadara, J. K., and L. C. Lutter.** 1993. RNA polymerase locations in the simian virus 40 transcription complex. J. Biol. Chem. **268:**22020–22027.
15. **Fedor, M. J., N. F. Lue, and R. D. Kornberg.** 1988. Statistical positioning of

nucleosomes by specific protein-binding to an upstream activating sequence in yeast. J. Mol. Biol. **204:**109–127.
16. **Fürst, P., S. Hu, R. Hackett, and D. Hamer.** 1988. Copper activates metallothionein gene transcription by altering the conformation of a specific DNA binding protein. Cell **55:**705–717.
17. **Grunstein, M.** 1997. Histone acetylation in chromatin structure and transcription. Nature **389:**349–352.
18. **Hamer, D. H., D. J. Thiele, and J. E. Lemontt.** 1985. Function and autoregulation of yeast copperthionein. Science **228:**685–690.
19. **Hamiche, A., R. Sandaltzopoulos, D. A. Gdula, and C. Wu.** 1999. ATP-dependent histone octamer sliding mediated by the chromatin remodeling complex NURF. Cell **97:**833–842.
20. **Haswell, E. S., and E. K. O'Shea.** 1999. An in vitro system recapitulates chromatin remodeling at the *PHO5* promoter. Mol. Cell. Biol. **19:**2817–2827.
21. **Jones, E. J.** 1991. Tackling the protease problem in *Saccharomyces cerevisiae*. Methods Enzymol. **194:**428–453.
22. **Kadonaga, J. T.** 1998. Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. Cell **92:**307–313.
23. **Karin, M., R. Najarian, A. Haslinger, A. P. Valenzuela, J. Welch, and S. Fogel.** 1984. Primary structure and transcription of an amplified genetic locus: the *CUP1* locus of yeast. Proc. Natl. Acad. Sci. USA **81:**337–341.
24. **Kingston, R. E., and G. J. Narlikar.** 1999. ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. Genes Dev. **13:**2339–2352.
25. **Kuras, L., and K. Struhl.** 1999. Binding of TBP to promoters *in vivo* is stimulated by activators and requires Pol II holoenzyme. Nature **399:**609–613.
26. **Längst, G., E. J. Bonte, D. F. V. Corona, and P. B. Becker.** 1999. Nucleosome movement by CHRAC and ISWI without disruption or trans-displacement of the histone octamer. Cell **97:**843–852.
27. **Leblanc, B. P., C. J. Benham, and D. J. Clark.** 2000. An initiation element in the yeast *CUP1* promoter is recognized by RNA polymerase II in the absence of TATA-box binding protein if the DNA is negatively supercoiled. Proc. Natl. Acad. Sci. USA **97:**10745–10750.
28. **Lee, D., and J. T. Lis.** 1998. Transcriptional activation independent of TFIIH kinase and the RNA polymerase II mediator *in vivo*. Nature **393:**389–392.
29. **Lee, D., S. Kim, and J. T. Lis.** 1999. Different upstream transcriptional activators have distinct coactivator requirements. Genes Dev. **13:**2934–2939.
30. **Levin, D. E., O. F. Fields, R. Kunisawa, J. M. Bishop, and J. Thorner.** 1990. A candidate protein kinase C gene, *PKC1*, is required for the *S. cerevisiae* cell cycle. Cell **62:**213–224.
31. **Li, X.-Y., A. Virbasius, X. Zhu, and M. R. Green.** 1999. Enhancement of TBP binding by activators and general transcription factors. Nature **399:**605–609.
32. **Liu, Q., S. E. Gabriel, K. L. Roinick, R. D. Ward, and K. M. Arndt.** 1999. Analysis of TFIIA function in vivo: evidence for a role in TATA-binding protein recruitment and gene-specific activation. Mol. Cell. Biol. **19:**8673–8685.
33. **Liu, X., and D. J. Thiele.** 1996. Oxidative stress induces heat shock factor phosphorylation and HSF-dependent activation of yeast metallothionein gene transcription. Genes Dev. **10:**592–603.
34. **Lorch, Y., B. R. Cairns, M. Zhang, and R. D. Kornberg.** 1998. Activated RSC-nucleosome complex and persistently altered form of the nucleosome. Cell **94:**29–34.
35. **Lorch, Y., M. Zhang, and R. D. Kornberg.** 1999. Histone octamer transfer by a chromatin remodeling complex. Cell **96:**389–392.
36. **McNeil, J. B., H. Agah, and D. Bentley.** 1998. Activated transcription independent of the RNA polymerase II holoenzyme in budding yeast. Genes Dev. **12:**2510–2521.
37. **Moqtaderi, Z., M. Keaveney, and K. Struhl.** 1998. The histone H3-like TAF is broadly required for transcription in yeast. Mol. Cell **2:**675–682.
38. **Muchardt, C., and M. Yaniv.** 1999. ATP-dependent chromatin remodelling: SWI/SNF and Co. are on the job. J. Mol. Biol. **293:**187–198.
39. **Natarajan, K., B. M. Jackson, H. Zhou, F. Winston, and A. G. Hinnebusch.** 1999. Transcriptional activation by Gcn4p involves independent interactions with the SWI/SNF complex and the SRB/mediator. Mol. Cell **4:**657–664.
40. **Neely, K. E., A. H. Hassan, A. E. Wallberg, D. J. Steger, B. R. Cairns, A. P. H. Wright, and J. L. Workman.** 1999. Activation domain-mediated targeting of the SWI/SNF complex to promoters stimulates transcription from nucleosome arrays. Mol. Cell **4:**649–655.
41. **O'Donohue, M. F., I. Duband-Goulet, A. Hamiche, and A. Prunell.** 1994. Octamer displacement and redistribution in transcription of single nucleosomes. Nucleic Acids Res. **22:**937–945.
42. **Ozer, J., L. E. Lezina, J. Ewing, S. Audi, and P. E. Lieberman.** 1998. Association of transcription factor IIA with TATA binding protein is required for transcriptional activation of a subset of promoters and cell cycle progression in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **18:**2559–2570.
43. **Peterson, C. L., and J. L. Workman.** 2000. Promoter targeting and chromatin remodeling by the SWI/SNF complex. Curr. Opin. Genet. Dev. **10:**187–192.
44. **Roth, S. Y., A. Dean, and R. T. Simpson.** 1990. Yeast α2 repressor positions nucleosomes in TRP1/ARS1 chromatin. Mol. Cell. Biol. **10:**2247–2260.
45. **Roth, S. Y., and R. T. Simpson.** 1991. Yeast minichromosomes. Methods Cell Biol. **35:**289–314.
46. **Sakurai, H., and T. Fukasawa.** 1999. Activator-specific requirement for the general transcription factor IIE in yeast. Biochem. Biophys. Res. Commun. **261:**734–739.
47. **Schnitzler, G., S. Sif, and R. E. Kingston.** 1998. Human SWI/SNF interconverts a nucleosome between its base state and a stable remodeled state. Cell **94:**17–27.
48. **Simpson, R. T.** 1991. Nucleosome positioning: occurrence, mechanisms and functional consequences. Prog. Nucleic Acids Res. Mol. Biol. **40:**143–184.
49. **Simpson, R. T., F. Thoma, and J. M. Brubaker.** 1985. Chromatin reconstituted from tandemly repeated cloned DNA fragments and core histones: a model system for study of higher order structure. Cell **42:**799–808.
50. **Spadafora, C., P. Oudet, and P. Chambon.** 1979. Rearrangement of chromatin structure induced by increasing ionic strength and temperature. Eur. J. Biochem. **100:**225–235.
51. **Struhl, K.** 1996. Chromatin structure and RNA polymerase II connection: implications for transcription. Cell **84:**179–182.
52. **Svaren, J., and W. Hörz.** 1997. Transcription factors vs nucleosomes: regulation of the *PHO5* promoter in yeast. Trends Biochem. Sci. **22:**93–97.
53. **Tanaka, S., D. Halter, M. Livingstone-Zatchej, B. Reszel, and F. Thoma.** 1994. Transcription through the yeast origin of replication ARS1 ends at the ABFI binding site and affects extrachromosomal maintenance of minichromosomes. Nucleic Acids Res. **22:**3904–3910.
54. **Thoma, F., L. W. Bergman, and R. T. Simpson.** 1984. Nuclease digestion of circular *TRP1ARS1* chromatin reveals positioned nucleosomes separated by nuclease-sensitive regions. J. Mol. Biol. **177:**715–733.
55. **Thomas, J. O., and V. Furber.** 1976. Yeast chromatin structure. FEBS Lett. **66:**274–280.
56. **Weiss, K., and R. T. Simpson.** 1997. Cell type-specific chromatin organization of the region that governs directionality of yeast mating type switching. EMBO J. **16:**4352–4360.
57. **Whitehouse, I., A. Flaus, B. R. Cairns, M. F. White, J. L. Workman, and T. Owen-Hughes.** 1999. Nucleosome mobilization catalysed by the yeast SWI/SNF complex. Nature **400:**784–787.
58. **Wieland, T., and H. Faulstich.** 1991. Fifty years of amanitin. Experientia **47:**1186–1193.
59. **Wolffe, A. P.** 1997. Sinful repression. Nature **387:**16–17.
60. **Wolffe, A. P.** 1999. Chromatin, 3rd. ed. Academic Press, London, United Kingdom.
61. **Yenidunya, A., C. Davey, D. J. Clark, G. Felsenfeld, and J. Allan.** 1994. Nucleosome positioning on chicken and human globin gene promoters *in vitro*. Novel mapping techniques. J. Mol. Biol. **237:**401–414.
62. **Zakian, V. A., and J. F. Scott.** 1982. Construction, replication, and chromatin structure of *TRP1* RI circle, a multiple-copy synthetic plasmid derived from *Saccharomyces cerevisiae* chromosomal DNA. Mol. Cell. Biol. **2:**221–232.