Correlation between chromatin association and transcriptional regulation for the Act3p/Arp4 nuclear actin-related protein of *Saccharomyces cerevisiae*

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ABSTRACT

Actin-related proteins (Arps), which share a basal structure with actin but have distinct functions, have been found in a wide variety of organisms. While their functions are not yet clear, some Arps are localized in the nucleus and are suggested to contribute to the regulation of transcription. An essential gene of *Saccharomyces cerevisiae*, Act3p/Arp4, encodes the first identified nuclear Arp, which has been shown to bind to core histones *in vitro*. Here we have analyzed the *in vivo* function of Act3p/Arp4 on the *his4-912δ* promoter. Chromatin immunoprecipitation assays show that Act3p/Arp4 is bound to the entire *his4-912δ* promoter region. Conditional *act3/arp4* mutations affect transcription from the *his4-912δ* promoter, where decreased Act3p/Arp4 binding and a change in nuclease sensitivity of chromatin were observed, showing the involvement of Act3p/Arp4 in the regulation of gene expression through the organization of chromatin structure. Taken together with the presence of Act3p/Arp4 in chromatin remodeling and histone acetyltransferase complexes, it is suggested that Act3p/Arp4 functions in transcriptional regulation to recruit chromatin remodeling and histone acetyltransferase complexes onto chromatin.

INTRODUCTION

Actin-related proteins (Arps), a group of protein families that exhibit moderate sequence similarity among each other and to conventional actin (i.e. muscle actin), have been found in a variety of eukaryotic organisms (1,2). According to the known three-dimensional structure of rabbit muscle actin and sequence comparisons, Arps and conventional actin compose the actin branch within a superfamily of proteins that possess ATPase activity, a superfamily that includes the 70 kDa heat shock cognate protein and hexokinase (3). However, the predicted molecular surfaces of these proteins are quite divergent and thus each protein is thought to be involved in unique functions specific for each family member.

Poch and Winsor (4) proposed a classification of the Arps in *Saccharomyces cerevisiae* into 10 subfamilies, in which the Arp subfamilies are numbered according to their similarity to conventional actin: Arp1 is most and Arp10 is least similar to actin. Arp subfamilies 1–3 of various organisms have been found and analyzed, showing that these Arps are localized in the cytoplasm and have distinct functions from that of actin isoforms and that each subfamily of Arp has a similar function across eukaryotic phyla (2). On the other hand, in *S.cerevisiae* the more divergent Arp subfamilies, Arp4–9, are localized in the nucleus (5–8), and also in humans two members of the Arp4 subfamily, hArpNα and hArpNβ/BAF53, are localized in the nucleus (9,10). While functional information on more distantly related Arps than the Arp subfamilies 1–3 is still limited, the information that is available suggests that most of these nuclear Arps function in the organization of chromatin structure.

Saccharomyces cerevisiae Act3p, a member of the Arp4 subfamily, was the first Arp that was demonstrated to be localized in the nucleus (5,6). *ACT3/ARP4* is an essential gene coding for a polypeptide of 489 amino acids with a calculated molecular mass of 54.8 kDa (11). Genetic studies suggested that Act3p/Arp4 is involved in transcriptional regulation of, at least, *HIS4* and *LYS2* and in activity of the *his4-912δ* promoter (6).

Saccharomyces cerevisiae contains a retrovirus-like transposable element, Ty1, which contains two long terminal repeats, called δ elements, with strong promoter activity. Insertion of Ty1 or a δ element into the 5′-region of genes often causes inactivation of the adjacent gene because of interference or competition between transcriptional signals in the δ element and the native gene promoter (12). Selections for extragenic suppressors of Ty1- or δ element-inactivated genes have identified numerous *SPT* (Suppressor of Ty) genes and many of them were shown to be involved in transcriptional regulation via

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effects on chromatin structure (6,12). For example, genes for histones (*SPT11* and *SPT12*) (13), components of the Spt–Ada–Gcn5 acetyltransferase (SAGA) complex (*SPT3*, *SPT7* and *SPT20*) (14) and TATA-binding factor (*SPT15*) (15) were identified as *SPT* genes. Interestingly, *act3-3* and *act3-4* mutations cause variegated suppression of the δ element-inactivated *HIS4* gene (the *his4-912δ* allele) in cells of an identical genetic background (6), suggesting an epigenetic effect of the mutated Act3p/Arp4 proteins on transcription. However, the molecular mechanisms by which Act3p/Arp4 regulates transcription are not well understood.

Although Act3p/Arp4 shows obvious similarity to conventional actin, it is a unique member of the actin family in terms of the presence of two insertions, I and II (11). Insertion II, consisting of 83 amino acids, is relatively abundant in charged amino acids and is predicted to form a loop-like structure protruding from the surface of the molecule. We previously reported that insertion II interacts with each of the core histones *in vitro* (16). Additionally, Act3p/Arp4 is present in the NuA4 histone acetyltransferase complex and the Ino80 chromatin remodeling complex purified from cell extracts (17,18).

Here, to investigate the molecular contribution of Act3p/Arp4 to transcriptional regulation *in vivo*, we have analyzed the association of Act3p/Arp4 with chromatin and the effect of mutations on transcriptional activity of the *his4-912δ* promoter.

MATERIALS AND METHODS

Yeast strains, media and general methods

Strain MZ3 (*MATa pep4-3 trp1 leu2-Δ1 ura3-Δ1*) was derived from 20B12 (19). Yeast strains DY2864 (*MATa his4-912δ-ADE2 his4-912δ lys2-128δ can1 trp1 ura3 ACT3*), DY4285 (*MATa his4-912δ -ADE2 lys2-128δ can1 leu2 trp1 ura3 act3-ts26*) and DY4519 (*MATa his4-912δ-ADE2 lys2-128δ can1 leu2 trp1 ura3 act3-ts12*) were used for analyses of chromatin structure. To isolate new temperature-sensitive alleles of *ACT3*, a plasmid shuffle was performed as described (16,20). DNA sequencing shows that the *act3-ts26* and *act3-ts12* alleles each have a single amino acid substitution of G187R and G455S, respectively. Protease inhibitors (0.5 μg/ml each antipain, chymostatin, elastatinal, leupeptin and pepstatin A) were added to the buffers described below.

Mobility shift assay of mononucleosomes

The entire coding sequence of Act3p/Arp4 was amplified with a primer set consisting of ACT3-forward and ACT3-reverse (Table 1) by PCR. To construct a plasmid for a glutathione *S*-transferase (GST)–Act3p/Arp4 fusion protein, by taking advantage of the primer-derived *Bam*HI and *Eco*RI sites of the amplified PCR products for Act3p/Arp4, the entire coding sequences were inserted into the vector pGEX-2TK (Pharmacia Biotech, Uppsala, Sweden). *Escherichia coli* (XL-1 blue) was transformed with the constructed plasmid or with a control pGEX-2TK and expression of the fusion protein was induced by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After further incubation at 30°C for 3 h, cells were harvested by centrifugation and the cell pellet was resuspended in 10 ml of phosphate-buffered saline (PBS) (140 mM NaCl,

Table 1. Primers used in this work

ACT3 forward : 5' ATAGGATCCATGTCCAATGCTGCTTTGCAA 3'
ACT3 reverse : 5' CGCGAATTCCTATCTAAACCTATCGTTAAG 3'
A-primer forward : 5' GTTAGAATGTCATCCCATAGG 3'
A-primer reverse : 5' GAGATGATATACTGGTAGTGC 3'
B-primer forward : 5' ATATCGCAAACCTCGAGAAGA 3'
B-primer reverse : 5' GAGATGGTCTATCTCTTTCTC 3'
C-primer forward : 5' AGACAGCAAGCCAAACAAAAG 3'
C-primer reverse : 5' AAAACGTGAGTCACTGTGCAT 3'
D-primer forward : 5' ATACGGTGTTAGAAGATGACG 3'
D-primer reverse : 5' ACTTTGGGTAACTGCTATACC 3'
CEN3 primer forward: 5' CAGCGCCAAACAATATGGAAA 3'
CEN3 primer reverse: 5' ATATGAGCAAAACTTCCACCA 3'
upstream probe forward: 5' CGGGATCCGAGAAGCCTATCTAGAATCA 3'
upstream probe reverse: 5' CCCAAGCTTAGTGCGCTAGTCAAGTTCTA 3'
downstream probe forward: 5' GGGGATCCTTGGGACGTATGATTGTTGA 3'
downstream probe reverse: 5' GCAAGCTTAGGAACACTTTGGGTAACTG 3'

2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) containing 1 mM DTT and 0.1 mg/ml lysozyme. Cells were lysed and the supernatant was collected. After addition of 500 μl of a slurry of 50% glutathione–Sepharose 4B (Pharmacia Biotech), the fusion protein was bound to the matrix at 4°C for 12 h with shaking. Subsequently the matrix was washed five times with PBS and the fusion protein was recovered in 500 μl of elution buffer (50 mM Tris–HCl, pH 9.5, 20 mM reduced glutathione).

Mononucleosomes were reconstituted *in vitro* with purified core histones of chicken (16) and a 180 bp DNA fragment recovered from *Bss*HII-digested pBluescript SK+ (Stratagene) according to Tatchell and van Holde (21). Mobility shift assays were performed by mixing the reconstituted mononucleosome and the purified GST fusion protein in 10 μl of binding buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 5 mM DTT, 5 mM PMSF, 4 mM $MgCl_2$, 5% glycerol) for 15 min at 37°C and then by electrophoresis on 1.5% agarose gels in 1× TAE (8 mM Tris, 1 mM sodium acetate, 0.4 mM EDTA, pH 8.0). The DNA fragment was detected by Southern blotting probed with the 180 bp fragment labeled with ^{32}P as described below.

Immunoprecipitation of the chromatin fraction containing Act3p/Arp4

Yeast cells were grown in YPD to a concentration of 1.5×10^7 cells/ml. One hundred and fifty milliliters of cells were crosslinked with 1% formaldehyde for 15 min at 26°C. Cells were harvested and suspended in 600 μl of buffer A (0.1 mM Tris–HCl, pH 9.5, 10 mM DTT) and incubated for 15 min at 30°C. Cells were precipitated and washed with buffer B (1.2 M

sorbitol, 20 mM HEPES, pH 7.6) and suspended in 1.5 ml of buffer B containing 0.4 mg Zymolyase 100T. Spheroplasts were washed with 20 mM PIPES, pH 6.8, 1 mM MgCl$_2$, 1.2 M sorbitol and then with PBS. Yeast chromatin was prepared from the spheroplasts by incubating in 10 mM HEPES, pH 7.0, 200 mM NaCl, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100, followed by a wash with 10 mM HEPES, pH 7.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA. The chromatin fraction was isolated following lysis in lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1% SDS and protease inhibitors), sonication of the suspension (10 times for 10 s, resulting fragment size <1 kb) and clarification by centrifugation.

For immunoprecipitation of chromatin containing Act3p/Arp4, 1 ml of clarified chromatin fraction was suspended in 9 ml of Ab-binding buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) and incubated with 100 ng anti-Act3p/Arp4 antibody (5) for 12 h at 4°C. Twenty microliters bed volume of protein A–Sepharose 4B beads (Amersham Pharmacia, Buckinghamshire, UK) were added and the incubation continued for 3 h. The immunoprecipitates were successively washed for 10 min each with Ab-binding buffer, Ab-binding buffer containing 500 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1% deoxycholate, 1% NP-40, 0.25 M LiCl and, finally, TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). Finally, the precipitates were processed for DNA purification (22) and purified DNAs were dissolved in 40 µl of TE.

PCR analysis of immunoprecipitated DNA

All reactions were carried out in 100 µl volume with final concentrations of 1× PCR buffer (Sigma, St Louis, MO), 0.2 mM dNTPs, 1 µM each primer for fragments I–IV, 10 µl of immunoprecipitated DNA fraction and 0.5 µl of *Taq* polymerase (Sigma). Identical cycling conditions were used for all primer sets (Table 1). These comprised: an initial denaturation of 96°C for 3 min; 30 cycles of 96°C for 40 s, 54°C for 1 min and 73°C for 1 min. The resulting PCR products were examined by agarose gel electrophoresis.

Indirect end-labeling of micrococcal nuclease-digested chromatin

Yeast nuclei were prepared as described in Weber *et al.* (5). Nuclei were isolated from 360 ml of a mid-log YPD culture (1.5 × 10^7 cells/ml) and suspended in ice-cold 20 mM Tris–HCl pH 7.4, 1 M sorbitol, 1 mM CaCl$_2$, 50 mM MgCl$_2$, 5 mM NaCl, 1 mM 2-mercaptoethanol, 0.5 mM spermidine, 30 mM sodium azide and protease inhibitors at a concentration of 100 µg DNA/ml. After preincubation at 37°C for 2 min, micrococcal nuclease (0, 1, 3, 10 and 30 U/ml) was added to 200 µl aliquots of the suspension and chromatin was digested at 37°C for 5 min. The reaction was stopped by adding 20 µl of 250 mM EDTA, 5% SDS, and DNAs were purified from the partially digested chromatin. The purified DNAs were then completely digested with *Bsa*HI. Samples of 1 µg/lane of the processed DNAs were electrophoresed on either 1.25 or 1.75% SeaPlaque GTG agarose gels (FMC BioProducts, Englewood, CO) and transferred to nylon membranes. For indirect end-labeling of the membrane bound DNAs, two DNA fragments consisting of –2116 to –1785 and +41 to +375 (from the start of the *ADE2* open reading frame) of the *his4-912δ–ADE2* gene were amplified with primer sets for the upstream probe and the downstream probe (Table 1), respectively, and labeled with [α-^{32}P]dCTP

(Amersham Pharmacia) by the primer extension method (23). Hybridization and washing were performed according to Church and Gilbert (24) and radioactivity of the labeled DNAs on the membrane was detected.

Restriction endonuclease digestion of chromatin

Nuclei isolated from a mid-log YPD culture (1.5 × 10^7 cells/ml) were suspended in digestion buffer (20 mM Tris–HCl, pH 7.5, 1 M sorbitol, 5 mM MgCl$_2$, 1 mM CaCl$_2$, 0.5 mM spermidine, 50 mM NaCl, 1 mM 2-mercaptoethanal) or digestion buffer containing 100 mM NaCl at a concentration of 70 ng/µl DNA. Restriction enzymes (10 U) were added to 200 µl aliquots of the suspension and chromatin was digested at 37°C for 1 h. The reaction was stopped by adding 20 µl of 250 mM EDTA, 5% SDS. The detection of a digested fragment containing the *his4-912δ* region was performed as described above. For the detection of a digested fragment containing the *CEN3* region, the 0.7 bp *Sal*I–*Kpn*I fragment from pOS31 that contains the *CEN3* DNA sequence (25) was used as probe.

RESULTS

The effect of non-lethal *act3/arp4* mutations on transcription

To investigate the involvement of Act3p/Arp4 in transcriptional regulation, the activity of the *his4-912δ* promoter was tested in two yeast strains possessing conditional *act3/arp4* mutations (ts26 and ts12) (16,17). ts26 and ts12 have single amino acid substitutions of G187R and G455S in the genomic *ACT3/ARP4* gene, respectively. The *his4-912δ–ADE2* allele contains the *his4-912δ* promoter upstream of the *ADE2* gene (Fig. 1B) (6). A wild-type (*ACT3/ARP4*) strain with the *his4-912δ–ADE2* allele does not grow on SC medium lacking adenine (Fig. 1A), due to the δ element inserted in the *HIS4* promoter. However, an *act3/arp4* mutation (ts26 and ts12) allows a strain with the *his4-912δ–ADE2* allele to grow on Sc-Ade medium despite the δ element inserted in the *HIS4* promoter (Fig. 1A). These results demonstrate that the *act3/arp4* mutations affect the activity of the δ element.

Association of Act3p/Arp4 with the *his4-912δ* chromatin region *in vivo*

We have previously reported that the insertion II region of Act3p/Arp4 binds to yeast and chicken core histones (16). The binding of recombinant Act3p/Arp4 was checked by mobility shift assay with mononucleosomes reconstituted with chicken core histones and a DNA fragment from a cloning vector, pUC119, according to Tatchell and van Holde (21) (Fig. 2). A complex was detected between recombinant Act3p/Arp4 and mononucleosomes and the amount of complex was dependent on the amount of Act3p/Arp4 added (Fig. 2, lanes 3 and 4). A complex was not detected between mononucleosomes and GST (Fig. 2, lane 2) or between naked DNA and Act3p/Arp4 (Fig. 2, lane 5). These results show that Act3p/Arp4 binds to nucleosomes *in vitro*. Recently it was shown that recombinant Act3p/Arp4 bound *in vitro* to nucleosomes reconstituted with core histones from HeLa cells and a 5S ribosomal DNA nucleosome-positioning sequence (17). This shows that binding of Act3p/Arp4 to nucleosomes does not depend on specific DNA sequences.
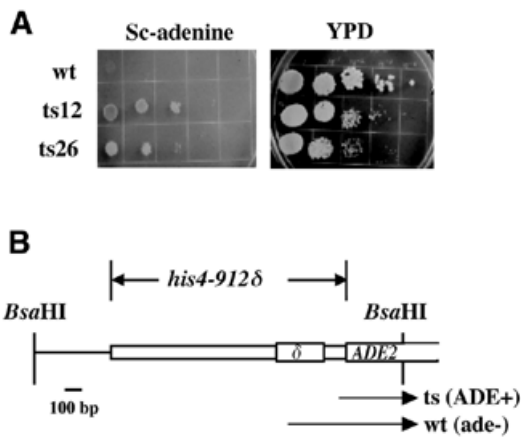
Figure 1. The effect of non-lethal *act3/arp4* mutations on transcriptional activity of the *his4-912δ* promoter. (**A**) One hundred microliter samples of YPD medium containing 2.0×10^8 cells/ml *ACT3/ARP4* wild-type (wt) or *act3/arp4* mutant (ts26 and ts12) strains were washed and suspended in 100 µl of sterile water. Ten-fold dilutions were prepared and 10 µl from each series was spotted onto Sc-Ade or YPD (complete) plates and incubated at 30°C. Incomplete suppression of *his4-912δ* was observed in ts12 and ts26 strains. (**B**) Structure of the allele used in this study. The *ADE2* promoter is replaced by the *his4-912δ* promoter where the 334 bp δ element is inserted 162 bp upstream from the original initiating ATG. Arrows show expected transcripts in the strains.
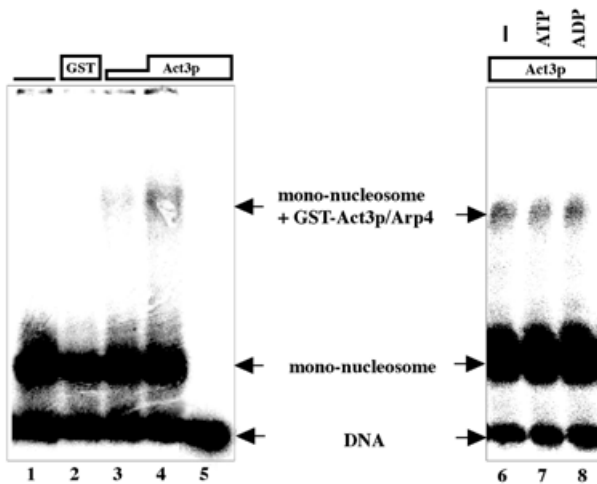

Figure 3. *In vivo* association of Act3p/Arp4 with the *his4-912δ* promoter region of wild-type and *act3/arp4* mutants. (**A**) Act3p/Arp4 was immuno-precipitated (IP/α-Act3p) from crosslinked chromatin from *ACT3/ARP4* wild-type (wt) and *act3/arp4* mutant (ts26 and ts12) strains. Rabbit immunoglobulin was used as a control (IP/Contr. IgG). DNA purified from the precipitated fractions and input bulk chromatin was analyzed by PCR using primer sets directed against four parts of the *his4-912δ* promoter region [I–IV in (A) and (B)] and the centromere region of chromosome III [*CEN3* in (B)]. (**B**) The resulting PCR products were resolved on an agarose gel.

Figure 2. *In vitro* association of Act3p/Arp4 with reconstituted mononucleosomes. Mobility shift assay of GST (lane 2) and recombinant Act3p/Arp4 (GST–Act3p/Arp4) (lane 3–5 and 6–8) was performed with reconstituted mononucleosomes (lanes 1–4 and 6–8) and naked DNA alone (lane 5). Half the amount of GST–Act3p/Arp4 was added in lane 3 as compared with lanes 4 and 5. In lanes 6–8, mobility shift assays with the same amount of GST–Act3p/Arp4 were performed in the absence (lane 6) or presence of 10 mM ATP (lane 7) and 10 mM ADP (lane 8). The positions of the complex consisting of mono-nucleosome + GST–Act3p/Arp4, mononucleosomes, and naked DNA are shown by arrows.

Actin binds ATP/ADP and nucleotide binding affects intramolecular associations, including actin filament formation. As Act3p/Arp4 can bind ATP (16), we suggested that the binding of ATP analogs could regulate binding of Act3p/Arp4 to the nucleosome. We tested this possibility using a mobility shift assay in the presence of ATP or ADP, which showed that
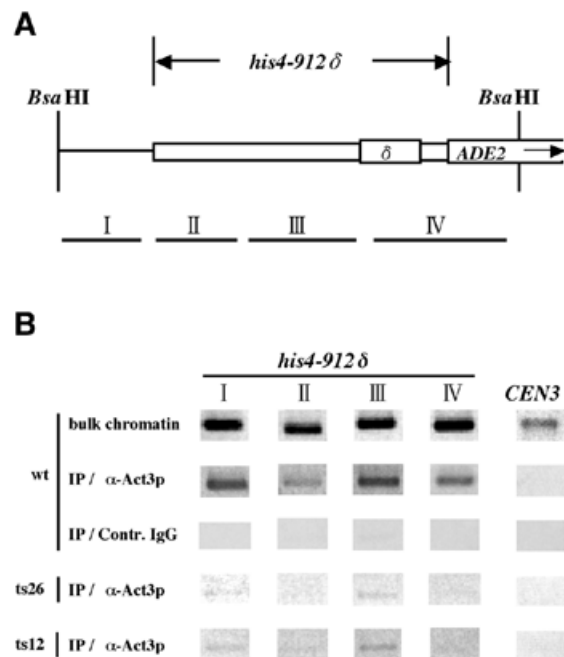
these analogs do not affect binding of Act3p/Arp4 to mono-nucleosomes (Fig. 2, lanes 6–8). On the other hand, it seems premature to suggest that a modification of Act3p/Arp4 regulates its binding to nucleosomes, because post-translational modification of Arps has not been reported in any organism.

To check the association of Act3p/Arp4 with the *his4-912δ* promoter region *in vivo*, we preformed chromatin immuno-precipitation analysis with a crosslinked and shared chromatin fraction. PCR amplification of precipitated DNA with primer sets directed against four parts of the *his4-912δ* promoter region (Fig. 3A) revealed that an anti-Act3p/Arp4 antibody (5,16), but not mouse immunoglobulin, precipitated chromatin containing each of the four segments of the *his4-912δ* promoter (I–IV in Fig. 3B) of the strain with wild-type *ACT3/ARP4* (wt). This shows that intact Act3p/Arp4 associates with chromatin of the *his4-912δ* promoter *in vivo*.

Regions I–IV of the *his4-912δ* promoter region were amplified from the precipitated bulk chromatin with equal efficiency. Taken together with the *in vitro* experiment with reconstituted nucleosomes containing different DNAs, this suggests that Act3p/Arp4 did not bind to the chromatin in a sequence-specific manner. However, the antibody against Act3p/Arp4 did not precipitate chromatin containing the *CEN3* sequence (Fig. 3B). Since the four conserved lysine residues in the H4 tail are required for *in vitro* binding with Act3p/Arp4 (17), acetylation of histones might contribute to this selectivity.
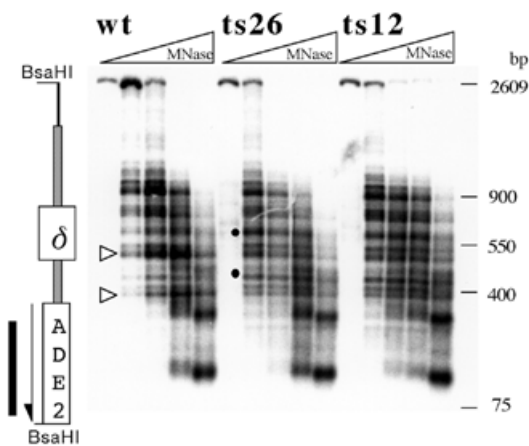
Figure 4. Chromatin structure around the *his4-912δ* promoter region of cells possessing an intact *ACT3/ARP4* gene and of *act3/arp4* mutants. MNase sensitivity of the *BsaHI–BsaHI* fragment of the *his4-912δ* promoter region in an *ACT3/ARP4* background (wt) and *act3/arp4* mutant (ts26 and ts12) strains was analyzed by the indirect end-labeling method using a probe located just downstream of the promoter (solid bar). Chromatin regions that are sensitive to MNase digestion were represented as bands. Positions of the regions correspond to the schematic drawing on the left of the panel. Arrows show the orientation of transcription, open triangles indicate MNase cleavage sites whose intensity was decreased in the mutants and dots show those whose intensity was enhanced in the mutants.

Decrease in the association of mutated Act3p/Arp4 with chromatin

We next determined the association of Act3p/Arp4 mutants with chromatin of the *his4-912δ* promoter. These Act3p/Arp4 mutations are outside the region (amino acids 296–399) used to prepare polyclonal antibody and thus the antibody will recognize both wild-type and mutant Act3p/Arp4 proteins with equal affinity. When the chromatin immunoprecipitation assay was performed with strains with a conditional *act3/arp4* mutation (ts26 and ts12) grown under permissive conditions, very little chromatin corresponding to regions I–IV was recovered with the anti-Act3p/Arp4 antibody, in contrast to the *ACT3/ARP4* strain (Fig. 3B). The chromatin immunoprecipitates from the mutants did not contain the *CEN3* region, as was seen for the *ACT3/ARP4* strain. This shows that Act3p/Arp4 mutants are defective in associating with the *his4-912δ* promoter region *in vivo*.

Micrococcal nuclease sensitivity of the *his4-912δ* promoter region

We next examined the effect of Act3p/Arp4 mutations on chromatin structure by analyzing the micrococcal nuclease (MNase) sensitivity of chromatin. Chromatin digested with MNase was analyzed by the indirect end-labeling method and regions that are sensitive to MNase were detected as bands. The experiment shown in Figure 4 examined the chromatin structure of the *his4-912δ* chromatin region in a strain possessing an intact *ACT3/ARP4* gene (wt) and conditional *act3/arp4* mutants (ts26 and ts12). Importantly, two of the major MNase cleavage sites observed in the *ACT3/ARP4* strain were less abundant in both of the *act3/arp4* mutants (Fig. 4, open triangles). Two other cleavage sites (Fig. 4, dots) were
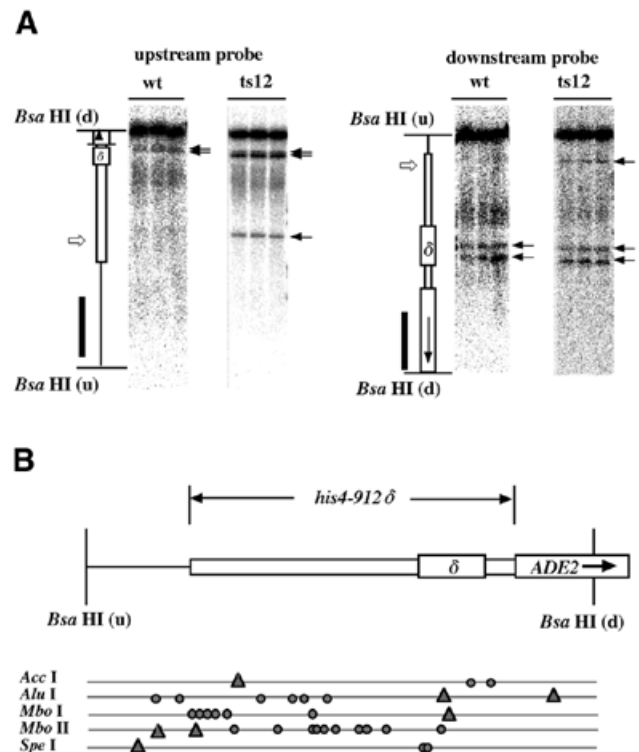

Figure 5. Chromatin structure around the *his4-912δ* promoter region of cells possessing an intact *ACT3/ARP4* gene and of of *act3/arp4* mutants detected by accessibility to a restriction enzyme. (**A**) *AccI* sensitivity of the *BsaHI–BsaHI* fragment surrounding the *his4-912δ* promoter region in an *ACT3/ARP4* background (wt) and an *act3/arp4* mutant (ts12) strain were analyzed by the indirect end-labeling method using a probe located far upstream (left) or just downstream (right) of the promoter (solid bars). Positions of the regions are shown on the schematic drawing on the left of each panel. Horizontal arrows indicate observed *AccI* cleavage sites and open arrows indicate *AccI* cleavage sites whose intensity was increased in the mutants. Three lanes in each panel correspond to cultures shifted to 26, 30 and 37°C (from left to right) before harvest. (**B**) The sites where the sensitivity to restriction enzymes was changed in the mutants (triangles) are shown under a map of the *his4-912δ* promoter region.

more pronounced in both of the *act3/arp4* mutants, demonstrating that mutations of Act3p/Arp4 caused a change in chromatin structure in the region examined.

Restriction enzyme sensitivity of the *his4-912δ* promoter region

To confirm the change in chromatin structure in the mutants, we digested chromatin in isolated nuclei with restriction endonucleases. In Figure 5A chromatin was digested with the restriction enzyme *AccI*, and *AccI* sensitivity was detected by the method of indirect end-labeling using a probe located far upstream (Fig. 5A, left) or downstream (Fig. 5A, right) of the promoter (solid bars). There are three *AccI* sites present in the *his4-912δ* promoter region, but the most upstream site was not digested in the *ACT3/ARP4* strain because of the chromatin structure surrounding the site (Fig. 5A, open arrows on the schematic map). However, the *AccI* site was digested in the *act3/arp4* mutants: ts12 (Fig. 5A) and ts26 (data not shown). This shows that the local chromatin structure surrounding the most upstream *AccI* site was changed in the *act3/arp4* mutants. Reproducible changes in nuclease sensitivity in the *his4-912δ* promoter region were seen with the five enzymes tested in the
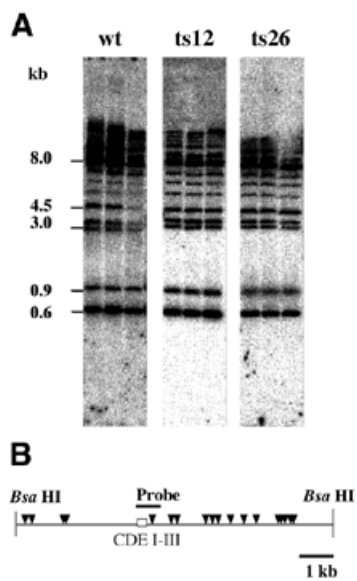
Figure 6. Chromatin structure around the *CEN3* centromere region of cells possessing an intact *ACT3/ARP4* gene and of *act3/arp4* mutants detected by accessibility to a restriction enzyme. (**A**) *Mbo*I sensitivity of the *Bsa*HI–*Bsa*HI fragment surrounding the *CEN3* region in an *ACT3/ARP4* background (wt) and *act3/arp4* mutant (ts26 and ts12) strains were analyzed by Southern blot analysis using a probe located in the CDE I–III region (solid bar). There is no reproducible difference between the wild-type and mutants. (**B**) A genetic map of the *Bsa*HI−*Bsa*HI fragment surrounding *CEN3*. The relative positions of the CDE I−III elements are shown by a box and *Mbo*I sites are indicated by triangles.

act3/arp4 mutants: the results are summarized in Figure 5B. Thus, changes in chromatin structure were detected in various areas in the *his4-912δ* promoter region, but were not limited to a restricted area.

Restriction enzyme sensitivity of a centromere region

Centromeric regions in *S.cerevisiae* have been shown to have distinctive chromatin structures, with positioned nucleosomes emanating from the CDEI, CDEII and CDEIII elements required for centromere function (26). We used the restriction endonuclease *Mbo*I to examine nuclease sensitivity of the *CEN3* region in isolated nuclei in the *ACT3/ARP4* strain and *act3/arp4* mutants (ts26 and ts12) (Fig. 6). No reproducible differences were noted, suggesting that *act3/arp4* mutations do not affect chromatin structure of centromere regions. This result is consistent with the chromatin immunoprecipitation results (Fig. 3), indicating that Act3p/Arp4 is not associated with the *CEN3* centromere region.

DISCUSSION

We used chromatin immunoprecipitation assays to characterize the association of Act3p/Arp4 with chromatin. We showed that Act3p/Arp4 associates with the *his4-912δ* promoter region and that *act3/arp4* mutations which alter expression from *his4-912δ* also result in decreased association of Act3p/Arp4 with the promoter. While the insertion II region of Act3p/Arp4 is responsible for binding to core histones *in vitro* (16), the mutants analyzed here contain amino acid substitutions not within insertion II but in the actin fold. The actin fold is a tertiary structure centered around an ATP/ADP-binding pocket

present in actin superfamily members, including Arps (3). In actin, the actin fold undergoes major conformational shifts in response to the 5′ phosphorylation-hydrolysis state of the adenine nucleotide and such shifts are thought to be crucial to the functions of the various actin superfamily members (3,27). Mutations within the actin fold might affect the stability of Act3p/Arp4 and/or cause the formation of irregular Act3p/Arp4 complexes and these changes could affect its association with chromatin.

In mutant cells, in which less of the mutated Act3p/Arp4 is found associated with the *his4-912δ* chromatin region, changes in chromatin structure of this region are seen, showing the involvement of Act3p/Arp4 in the organization and/or maintenance of chromatin structure. As Act3p/Arp4 interacts with histones through their N-terminal domains, which extend from the nucleosome core (17), it is unlikely that binding of Act3p/Arp4 alters the nucleosome core structure. In addition, while Act3p/Arp4 possesses a putative ATP/ADP-binding motif, this motif is not expected to take part in chromatin remodeling (28), and no sequence motif for any histone modification is found in Act3p/Arp4 (11). Therefore, it is unlikely that Act3p/Arp4 will modulate chromatin structure by itself.

It has been reported that Act3p/Arp4 is a stoichiometric component of the NuA4 histone acetyltransferase complex and Ino80 chromatin remodeling complex (17,18). Taken together with the requirement of Act3p/Arp4 for proper chromatin structure and promoter activity, we suggest that Act3p/Arp4 functions to recruit components of the complexes onto the chromatin for proper chromatin function. On the other hand, while a limited number of complexes involved in chromatin modulation have been characterized so far, actin family proteins, consisting of actin and actin-related proteins, are found in many of them (9,28–31). Thus it is possible that Act3p/Arp4 may recruit other chromatin modifying complexes, in addition to NuA4 and Ino80, to chromatin. Importantly, mutations of Act3p/Arp4 not only affect activity of the *his4-912δ* promoter, but also activity of some native promoters, including *HIS4* and *LYS2* (6). Act3p/Arp4 could be involved in the transcriptional regulation of a number of genes by recruiting specific complexes onto chromatin.

While Act3p/Arp4 does not seem to bind nucleosomes in a sequence-specific manner, there is evidence for selective recruitment of complexes involved in the modulation of chromatin structure. Chromatin immunoprecipitation experiments to globally ask what promoters have bound Esa1p, the catalytic subunit of the NuA4 histone acetyltransferase complex that also contains Act3p/Arp4, showed that NuA4 is specifically recruited to ribosomal protein promoters by the DNA-binding factor Rap1 or Abf1 (32). Moreover, depletion of Esa1p leads to a dramatic decrease in acetylation globally (32,33), showing that specific targeting of Esa1p occurs in a background of its global activity on chromatin. Since association of Act3p/Arp4 with chromatin *in vitro* and *in vivo* does not depend on specific nucleotide sequences, Act3p/Arp4 could be responsible for such global recruitment of chromatin remodeling and histone acetyltransferase complexes, including the NuA4 complex, rather than for sequence-specific targeting. The global activity of the complexes would serve to maintain a genome-wide balance between active and inactive states of chromatin. At the *his4-912δ* promoter in the *act3/arp4* strains, this balance was altered and activation of the δ element was changed.

On the two promoters, the promoter of the δ element and the native *HIS4* promoter responsible for functional Ade2p, the transcriptional effects and changes in chromatin structure caused by *act3/arp4* mutations appear to be contradictory. However, recent studies showed that chromatin remodeling is utilized not only for transcriptional activation, but also for transcriptional repression. A chromatin remodeling complex, hSWI/SNF, not only catalyzes the disruption of nucleosome structure, but also the reverse reaction *in vitro* (34), and a microarray analysis revealed that yeast SWI/SNF acts both as an activator and as a repressor of transcription *in vivo* (35). It is possible that chromatin remodeling activity is required globally for the maintenance of chromatin fluidity, and its contribution to transcriptional activation or repression depends on the balance between other co-activator or co-repressor activity (36). In the case of the *his4-912δ* promoter, chromatin remodeling activities would be required for a chromatin structure suitable for both activation of the promoter in the δ element and repression of the *HIS4* promoter.

While the association of Act3p/Arp4 with chromatin correlates with correct transcriptional regulation, it is unlikely that the nucleosome-binding property is shared by all nuclear Arps because this insertion II region is specific to Act3p/Arp4 and is not conserved in other Arps. On the other hand, as for Act3p/Arp4, most, if not all, nuclear Arps are expected to be components of chromatin remodeling complexes. Arp7 and Arp9 of *S.cerevisiae* are components of chromatin remodeling complexes SWI/SNF and RSC (28,29), and a putative human ortholog of Act3p/Arp4, hArpNβ/BAF53 (10), is contained in various protein complexes including the BAF chromatin remodeling and Tip60 histone acetyltransferase complexes (9,30,31). Therefore, the role of nuclear Arps could be more than just recruitment of the complexes and nuclear Arps might also have structural and regulatory functions in these chromatin remodeling complexes. Further analysis of other nuclear Arps with appropriate mutants would be helpful to address their role in transcriptional regulation through changes in chromatin structure.

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