Gal4-VP16 Directs ATP-Independent Chromatin Reorganization in a Yeast Chromatin Assembly System†

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ABSTRACT: Major insights into the regulation of chromatin organization have stemmed from biochemical studies using Gal4-VP16, a chimeric transcriptional activator in which the DNA binding domain of Gal4p is fused to the activation domain of viral protein VP16. Unexpectedly, given previous intensive efforts to understand how Gal4-VP16 functions in the context of chromatin, we have uncovered a new mode of chromatin reorganization that is dependent on Gal4-VP16. This reorganization is performed by an activity in a crude DEAE (CD) fraction from budding yeast which also supports ATP-dependent assembly of physiologically spaced nucleosome arrays. Biochemical analysis reveals that the activity tightly associates with chromatin and reorganizes nucleosome arrays by a mechanism which is insensitive to ATP depletion after nucleosome assembly. It generates a chromatin organization in which a nucleosome is stably positioned immediately adjacent to Gal4p binding sites in the template DNA. Individual deletion of genes previously implicated in chromatin assembly and remodeling, namely, the histone chaperones *NAP1*, *ASF1*, and *CAC1* and the *SNF2*-like DEAD/H ATPases *SNF2*, *ISW1*, *ISW2*, *CHD1*, *SWR1*, YFR038w, and *SPT20*, does not significantly perturb reorganization. Therefore, Gal4-VP16-directed chromatin reorganization in yeast can occur by an ATP-independent mechanism that does not require SAGA, SWI/SNF, Isw1, or Isw2 chromatin remodeling complexes.

Nuclear DNA is packaged into nucleosomes, which are fundamentally comprised of 146 bp of DNA wrapped around an octamer of core histone proteins. This packaging is necessary for productive execution of virtually all steps of nuclear DNA metabolism (*1*). In the context of transcription, DNA assembly into nucleosomes can be important for repression and present an obstacle to activation (*1*−*3*). Cells call on a variety of mechanisms to overcome the barrier to activation due to template organization into chromatin. One such mechanism, the remodeling of chromatin in the course of DNA binding by sequence-specific transactivators, has been particularly well studied. Chromatin assembly systems from higher eukaryotes and purified factors have been important tools in such studies (*4*−*9*). Most work has focused on remodeling which occurs when a DNA binding factor is added to a target template previously assembled into nucleosome arrays. A factor widely used as the starting point in this type of analysis has been Gal4-VP16, the fusion protein of the DNA binding domain of yeast Gal4p with the acidic activation domain of the herpes simplex virus protein VP16. The Gal4p DNA binding domain by itself binds to Gal4 elements incorporated into nucleosomes, but does not affect the positioning of the nucleosome. In the presence of remodeling factors and ATP, the nucleosomes are displaced upon binding of the Gal4p DNA binding domain (*6*).

Gal4-VP16-dependent remodeling requires accessory factors which include a subunit belonging to the *SNF2*-related family of DEAD/H DNA-dependent ATPases. Members of this family fall into three main classes (*10*). Those containing a bromo domain comprise the SWI2 group; the ISWIs contain a SANT domain, and CHD proteins contain one or more chromo domains. *SWI2* and ISWI group members have well-established roles in Gal4-VP16-dependent remodeling. No representative of the CHD group has been tested for activity in Gal4-VP16-dependent remodeling assays, although chromo domain protein 1 (Chd1p) of budding yeast is known to remodel nucleosomes in the absence of DNA binding factors (*11*).

The first characterization and molecular identification of transactivator-dependent chromatin remodeling factors in metazoans relied on the development of crude cell extracts which supported remodeling (*4*, *7*−*9*). Biochemical fractionation of these extracts eventually led to molecular cloning of their active constituents (*4*, *12*, *13*). In general, this unbiased approach is advantageous because the discovery process is independent of preconceptions about the molecular identity of the factors of interest. That is, new classes of remodeling factor can be discovered by this methodology.

Although the usefulness of crude systems as a starting point for studies of chromatin remodeling has been well documented, work in yeast has relied almost exclusively on the use of highly purified factors. A crude fraction of yeast nuclear extract has been used to study remodeling in the presence of the Pho2p and Pho4p transactivators but not other factors (*14*), and to our knowledge, chromatin remodeling that is dependent on Gal4-VP16 has never been demonstrated

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in a crude yeast system (*15*). Nonetheless, it is likely that crude yeast systems will be put to good use in studies of the regulation of chromatin remodeling. Assay of crude extracts from yeast knockout strains (*16*) could conceivably lead to identification of previously unknown remodeling factors, and such extracts could be the starting point for analysis of remodeling events involving multiple interacting factors.

We recently developed a simple yeast system which supports rapid replication-independent assembly of physiologically spaced nucleosomal arrays in vitro (*17*). The critical component of this system is a crude DEAE (CD)[1] fraction of extracts from nuclei or spheroplasts. Using this system, we obtained evidence of a role for chromo domain protein Chd1p in chromatin assembly (*17*). Having reconstituted an assembly activity reliant upon a member of the *SNF2*-related family of ATPases, we were encouraged to explore the ability of the system to support Gal4-VP16-dependent chromatin remodeling. We find that Gal4-VP16-dependent reorganization of chromatin structure is supported in the assembly system. Surprisingly, however, the activity in the CD fraction differs from previously described Gal4-VP16-dependent activities in several respects. The pattern of nucleosome reorganization it promotes has not been reported for other Gal4-VP16-dependent remodeling reactions. Chromatin reorganization can occur when ATP is depleted from the system prior to addition of a transactivator. Additionally, the reorganizing activity can tightly associate with chromatin in a manner that is independent of Gal4-VP16. A targeted screen of 10 genes previously implicated in chromatin assembly and/or remodeling suggests that none of several well-characterized remodeling factors is necessary, on its own, for the Gal4-VP16-dependent reaction supported by the CD fraction. Our results suggest that not all possible modes of Gal4-VP16-dependent chromatin reorganization by yeast factors have been identified in previous in vitro studies. Because of its distinctive properties, we refer to the reaction supported by the CD fraction as chromatin "reorganization" rather than remodeling.

EXPERIMENTAL PROCEDURES

The yeast CD fraction was prepared from protease-deficient strain DSY904 or from BY4741 and its null mutant derivatives. Strains, growth conditions, and methods of CD fraction preparation from spheroplasts and nuclei have been fully described elsewhere (*17*). Note that chromatin reorganization was supported in the CD fraction from spheroplasts or nuclei.

Chromatin Assembly Reactions. As described by Robinson and Schultz (*17*), the CD fraction and core histones were added to yR buffer with 2 mM $MgCl_2$ and incubated for 15 min at room temperature. The ATP regeneration mix and pGIE-0 plasmid DNA (*18*) that had been purified by two sequential CsCl gradients were added, and the reaction mixture was incubated at 30 °C for the times indicated in the figures. Where indicated, yeast histones were used at a concentration of 9 μg/mL and fly core histones were used at 7.5 μg/mL. Apyrase (Sigma, A6410) was added to a final concentration of 2 units/mL. *Drosophila* histones (*19*) and Gal4-VP16 (*20*) were purified as described previously. Where indicated, Gal4-VP16 was added to a final concentration of 200 nM.

Restriction Enzyme Access. Fifty microliter assembly reaction mixtures were mixed with 25 μL of RNG [10 mM Hepes (pH 7.5), 10 mM KCl, and 12 mM $MgCl_2$]. Aliquots (25 μL) of this mix were treated with either 10 units of *Xba*I, 5 units of *Bam*HI, or no restriction enzyme, and incubated at 37 °C for 30 min. Digestion was stopped with 20 mM EDTA; the samples were deproteinized, and the DNA precipitated. All DNA samples were then digested with *Bgl*II and RNase A.

Micrococcal Nuclease Digestion and Chromatin Reorganization Assays. These assays were performed as described in ref *21*. Micrococcal digestions were stopped with EDTA, the samples treated with RNase A, and the digestion products isolated. For both RE access and micrococcal nuclease assays, the DNA was resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The oligonucleotides used in the nucleosomal disruption assay were the proximal probe (5′-TCGGAGGACAGTACTCCGCTCGGAG) and the distal probe (5′-GGCGTATCACGAGGCCCTTTCGTCT). To map the 250 bp band, the assembly reaction and micrococcal nuclease digests were performed as described above. The DNA isolated from these reactions was left uncut, or digested with *Hin*DIII or *Xba*I, prior to Southern blot analysis. In addition to the proximal and distal probes, the 5′*Hin*DIII probe (5′-GTATTCTATAGTGTCACCTAAATCGTATGT) and MP37 probe (5′-GTGCAGAGCGAGTATATATAGGACTGGG) were used.

Topoisomerase II Assays. To assay topoisomerase II activity, the CD fraction was diluted with yR buffer and supplemented with the ATP regenerating system or with only $MgCl_2$. Apyrase was added at a concentration of 2 units/mL to a sample containing the ATP regenerating system, and all samples were incubated at 30 °C for 30 min. Kinetoplast DNA (kDNA; TopoGEN, Columbus, OH) was added to a final concentration of 0.5 μg/mL and the reaction allowed to proceed for 2 h at 30 °C. As a control, kDNA was decatenated for 30 min at 37 °C with purified topoisomerase II (Amersham Biosciences).

Purification of Assembled Chromatin. Assembled chromatin was isolated by spinning the assembly reaction mixtures through two Sepharose CL-4B spin columns (SizeSep 400, Amersham Biosciences) that had been pre-equilibrated with 40 mM KCl, 6 mM $MgCl_2$, and 0.5 mg/mL bovine serum albumin in yR buffer. Where indicated, Sarkosyl was added to a final concentration of 0.05% and the reaction mixtures were incubated at room temperature for 5 min before they were applied to the spin columns. To improve the purification efficiency of spin column chromatography, assembly reactions were set up in half the normal volume and therefore with twice the normal concentration of CD fraction, histones, and DNA. yR buffer and ATP regenerating mix were added to the eluate after spinning, bringing the final DNA concentration to 0.5 μg/mL.

RESULTS

Gal4-VP16-Dependent Chromatin Reorganizing Activity in a Crude Yeast System. All experiments used previously well-characterized reagents: the Gal4p DNA binding domain

[1] Abbreviations: CD, crude DEAE; RE, restriction enzyme; kDNA, kinetoplast DNA.

fused to the activation domain of VP16 (Gal4-VP16) and a DNA template containing five tandem copies of the Gal4p binding site (*18*, *20*). In anticipation of reproducing Gal4-VP16-dependent remodeling as observed in the *Drosophila* S-190 extract, the conditions used in our assays closely resembled those under which remodeling occurs in the fly system (same template, Southern blotting protocols and probes, and similar micrococcal nuclease digestion). Furthermore, *Drosophila* embryo extract and the Gal4-VP16 used in the current study fully reproduced the pattern of Gal4-VP16-dependent chromatin remodeling previously reported for the fly system (see below). Therefore, the reagents we have generated and the conditions we use are suitable for biochemical analysis of chromatin remodeling by Gal4-VP16.

Restriction enzyme (RE) accessibility has previously been used to monitor the ability of an extract or factor to remodel or disrupt a nucleosomal array at the DNA binding site of an added DNA binding factor. For example, increased sensitivity to restriction enzymes has been interpreted to indicate the remodeling activity of NURF directed by either GAGA factor (*8*) or Gal4-VP16 (*22*). Similarly, the ability of Isw1p to disrupt nucleosomes in the context of GAGA factor binding has been monitored by RE accessibility (*23*).

Chromatin reorganization in the CD fraction supplemented with yeast or fly core histones (the results were identical) was assayed by monitoring the accessibility of the assembled plasmid DNA to either *Bam*HI or *Xba*I, which cut 9 or 3 bp downstream of the Gal4p binding sites, respectively (Figure 1A). After incubation with the restriction enzyme, the DNA was purified and cut with *Bgl*II at a site 915 and 921 bp from the *Xba*I and *Bam*HI sites, respectively (Figure 1B). *Bgl*II generates a 3.3 kb linear molecule from all previously uncut plasmids (Figure 1C, bottom panel) and two DNA fragments if *Xba*I or *Bam*HI had previously cut the nucleosomal template (Figure 1C, top two panels). Naked DNA is efficiently digested by *Bam*HI and *Bgl*II, while *Xba*I cutting does not go to completion under the conditions that were used (Figure 1C, lanes 1 and 5). Particularly as judged by the diminished yield of the shortest *Bam*HI−*Xba*I fragment, assembly of the template into nucleosomes with either *Drosophila* or yeast histones protects the *Bam*HI and *Xba*I sites from enzyme access (Figure 1C; for *Drosophila* histones, compare lanes 1 and 2, and for yeast histones, compare lanes 5 and 6). Under standard reaction conditions using either *Drosophila* or yeast histones, addition of Gal4-VP16 either before or after assembly increased access of both *Xba*I and *Bam*HI to their respective sites in the chromatinized template (Figure 1B, lanes 2−4 for *Drosophila* and lanes 6−8 for yeast). This result suggests that the structure of chromatin assembled by the CD fraction is altered upon binding of Gal4-VP16 to the template DNA.

Southern blotting (with a probe that anneals to the Gal4 elements) after chromatin assembly and partial micrococcal digestion reveals a population of molecules in which nucleosomes are physiologically spaced over the Gal4 element. These molecules yield a nucleosomal ladder upon micrococcal nuclease digestion of control assembly reactions using either *Drosophila* or yeast histones (Figure 2; lanes 1 and 2 for *Drosophila* histones and lanes 13 and 14 for yeast histones). When Gal4-VP16 is added at the start of assembly reactions using either *Drosophila* or yeast histones, this ladder is substantially disrupted (Figure 2; compare lanes 1 and 2 with lanes 3 and 4 for *Drosophila* and compare lanes 13 and 14 with lanes 15 and 16 for yeast). Most strikingly, the monomer and dimer disappear and a band of approximately 250 bp appears. In addition, the trimer becomes smeary. The chromatin organization of the Gal4p binding site is similarly disrupted when Gal4-VP16 is added after assembly is essentially complete (Figure 2; lanes 5 and 6 for *Drosophila* and lanes 17 and 18 for yeast). This disruption does not occur at a site 1 kb from the Gal4 binding sites, so it is not simply due to global, nonspecific interference with de novo nucleosomal assembly (lanes 7−12 for *Drosophila* and lanes 19−24 for yeast).

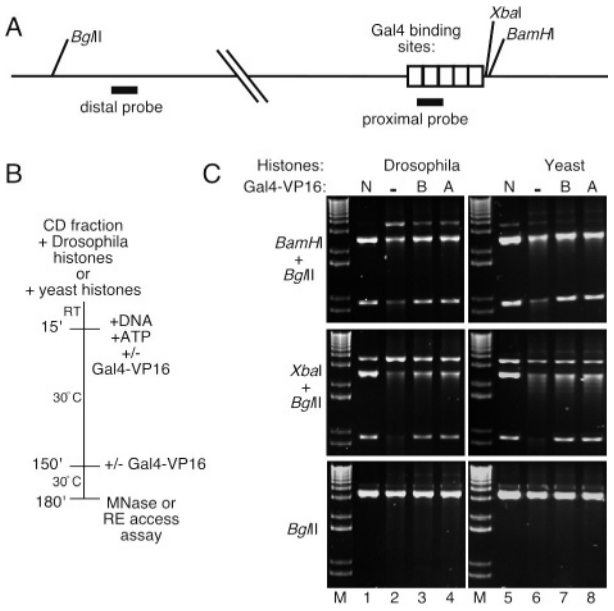

FIGURE 1: Analysis by restriction enzyme accessibility of a factor in the CD fraction that collaborates with Gal4-VP16 to reorganize chromatin. (A) Diagram of the plasmid used for assembly. *Xba*I and *Bam*HI sites are adjacent to the Gal4p binding sites; the *Bgl*II site is approximately 925 bp from *Xba*I and *Bam*HI sites. The proximal probe anneals at the Gal4p binding site; the distal probe anneals at a distance of approximately 800 bp. (B) Outline of the experiments shown in panel C and Figure 2. Nucleosomal templates were assembled with either *Drosophila* or yeast histones, and the reaction mixtures were incubated without Gal4-VP16 (−) or with addition of 200 nM Gal4-VP16 (+): M, 1 kb plus DNA ladder; MNase, micrococcal nuclease; RE, restriction enzyme; RT, room temperature. (C) Assay of restriction enzyme accessibility to the *Bam*HI and *Xba*I sites flanking the five-Gal4p binding site cassette. Gal4-VP16 was added at the same time as the DNA (B, before assembly) or 30 min before the end of the incubation (A, after assembly). Digestion products were purified, cut with *Bgl*II, resolved by agarose gel electrophoresis, and visualized by ethidium bromide staining. N denotes naked DNA.

The observed pattern of Gal4-VP16-dependent remodeling in the CD fraction was unexpected. Because in our hands *Drosophila* S-190 extract supported the pattern of remodeling previously reported for Gal4-VP16 in the fly system, it seemed unlikely that the results obtained using the yeast CD fraction were due to pervasive artifacts in our assays. This idea was confirmed in a side-by-side comparison of remodeling in the yeast CD fraction and *Drosophila* S-190 extract. Specifically, we tested if the micrococcal nuclease digestion pattern characteristic of chromatin reorganization by the CD fraction is also observed when it is replaced with fly extract (Figure 3A). Figure 3B shows that *Drosophila* S-190 extract supports general Gal4-VP16-dependent destabilization of

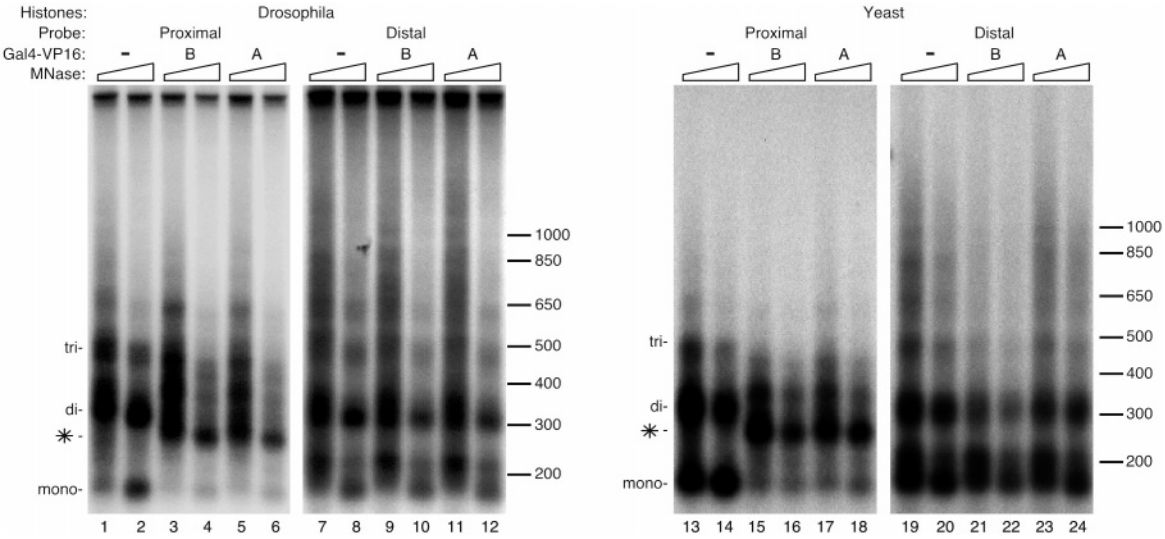

FIGURE 2: Analysis by partial micrococcal nuclease (MNase) digestion and Southern blotting of a factor in the CD fraction that collaborates with Gal4-VP16 to reorganize chromatin. As outlined in Figure 1B, assembly reaction mixtures using *Drosophila* (lanes 1−12) and yeast (lanes 13−24) core histones either were not supplemented with Gal4-VP16 (−) or received Gal4-VP16 at the start of assembly (B, before assembly) or 30 min from the end of the incubation (A, after assembly). Micrococcal nuclease-digested reaction products were purified and resolved by agarose gel electrophoresis. Blots were probed with an oligonucleotide that hybridizes at the Gal4p binding sites (Gal4 element proximal, lanes 1−6 and 13−18), stripped, and reprobed with an oligonucleotide that hybridizes 800 bp downstream from the Gal4 binding sites (Gal4 element distal, lanes 7−12 and 19−24). The migration of the mono-, di-, and trinucleosome length DNA fragments is indicated on the left border (mono-, di-, and tri-, respectively). The asterisk in the left panel indicates the product of approximately 250 bp resulting from chromatin reconfiguration. DNA size markers are as indicated at the right.

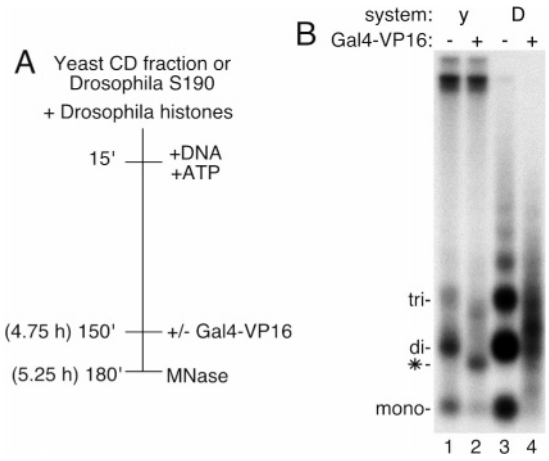

FIGURE 3: Comparing reorganization of nucleosomes assembled with the yeast CD fraction to remodeling in *Drosophila* S-190. (A) Outline of the experiment. The times in parentheses were used for assembly with the *Drosophila* S-190 extract. MNase denotes micrococcal nuclease. (B) Reactions were performed with the yeast CD fraction (y, lanes 1 and 2) and the *Drosophila* S-190 extract (D, lanes 3 and 4). Gal4-VP16 was added after 15 min. The products of micrococcal nuclease digestion were analyzed as described in the legend of Figure 2 using the Gal4 element proximal probe.

nucleosome arrays in the presence of ATP (lane 4), exactly as previously reported (*5*, *6*), but not generation of the distinct band between mono- and dinucleosomes that distinguishes chromatin reorganization in the yeast system (lane 2). We conclude that the novel reorganization of chromatin observed in yeast extract is not due to an unknown difference between our assay conditions and those reported in the literature.

The length of the fragment (~250 bp) which appears upon addition of Gal4-VP16 is consistent with protection of the five-Gal4 binding sites (95 bp), and the DNA of an immediately adjacent nucleosome (~160 bp) which could be located at either the 5′ or 3′ border of the Gal4 sites. Alternatively, the 250 bp fragment could arise because approximately 80 bp on either side of the five-Gal4 element is protected from nuclease digestion. It cannot be a digestion product of previously naked DNA that becomes resistant to digestion after addition of Gal4-VP16 because it does not simply materialize between the bands of a persistent and normal nucleosomal ladder. Rather, the appearance of the 250 bp fragment is accompanied by the disappearance of the previously well-defined monomer and dimer bands specifically over the Gal4p binding sites.

Mapping experiments were performed to determine the DNA composition of the 250 bp chromatin reorganization product (outlined in Figure 4A). After micrococcal nuclease digestion, DNA from assembly reactions was re-isolated and incubated with either *Hin*DIII, which cuts 20 bp upstream of the Gal4 element, or *Xba*I, which cuts 3 bp downstream (Figure 4B). Southern blot analysis with a probe that hybridizes to the Gal4 element shows that the 250 bp reorganization product produced in the presence of Gal4-VP16 persists when the re-isolated DNA is cut with either *Hin*DIII or *Xba*I (Figure 4C, middle panel). We conclude that micrococcal nuclease digestion of reorganized chromatin generates two populations of 250 bp fragments, one which includes only the *Hin*DIII site and one which includes only the *Xba*I site.

If 250 bp DNA fragments spanning the 5′ *Hin*DIII site do not also include DNA 3′ to the five-Gal4 binding sites, then Southern blotting analysis of *Hin*DIII-cut DNA with a probe that hybridizes 1 bp upstream of the *Hin*DIII site should yield only one fragment, of approximately 140 bp (*Hin*DIII cuts 20 bp upstream of the 5′ boundary of the five-Gal4 binding sites). Furthermore, digestion with *Xba*I should not affect the length of the product detected by a probe that hybridizes just upstream of the *Hin*DIII site. These predictions are both confirmed in the left panel of Figure 4C: *Xba*I digestion does not affect the recovery of the 250 bp product (lane 4),

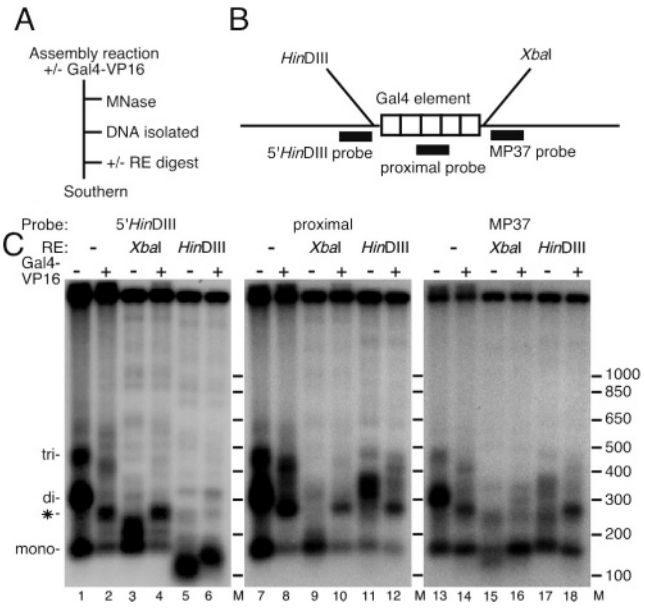

FIGURE 4: Mapping the 250 bp reorganization product. (A) Outline of the experiment shown in panel C. Chromatin was assembled with or without Gal4-VP16, and partially digested with micrococcal nuclease. The DNA was re-isolated, and aliquots were digested with restriction enzymes that flank the Gal4 binding sites and subjected to Southern analysis using probes that were upstream of (5′*Hin*DIII), within (proximal), or downstream of (MP37) the Gal4 element. (B) Diagram of the plasmid template showing the location of the restriction enzyme sites and the probes. (C) Southern blotting analysis of assembly products after micrococcal nuclease and RE digestion. Blots were probed with the proximal probe (middle panel), stripped and reprobed with the MP37 probe (right panel), and stripped and reprobed again with the 5′*Hin*DIII probe (left panel). The migration of the mono-, di-, and trinucleosome length DNA fragments is indicated on the left border (mono-, di-, and tri-, respectively). The asterisk in the left panel indicates the product of approximately 250 bp resulting from chromatin reconfiguration. DNA size markers are as denoted with dashes and the sizes indicated at the right. RE denotes the restriction enzyme.

whereas *Hin*DIII digestion yields a band of approximately 140 bp (lane 6).

Similarly, when probed with an oligonucleotide that hybridizes 10 bp downstream of the *Xba*I site (Figure 4C, right panel), the 250 bp reorganization product disappears upon digestion with *Xba*I (lane 16), but remains after *Hin*DIII digestion (lane 18). Comparison of the migration of the mononucleosome-sized fragment in lane 13 with the faster-migrating species in lane 16 suggests, as expected, that the *Xba*I digestion product is close to the length of DNA incorporated into a mononucleosome (compare lanes 13 and 16).

In summary, we conclude that the 250 bp band is composed of the Gal4 element and adjacent DNA which was incorporated into a nucleosome. Thus, the CD fraction supports Gal4-VP16-dependent chromatin reorganization, and this reorganization generates a chromatin structure not reported in previous studies of remodeling in the presence of Gal4-VP16.

Effect of ATP Depletion on Gal4-VP16-Dependent Chromatin Reorganization. In crude *Drosophila* embryo extracts, depletion of ATP by apyrase treatment after chromatin assembly but before Gal4-VP16 addition severely compromises Gal4-VP16-dependent remodeling. To determine if chromatin reorganization in the yeast system is similarly dependent on ATP, assembly reactions were set up using either *Drosophila* or yeast histones and Gal4-VP16 was added either at the start of assembly or after apyrase treatment (Figure 5A). Apyrase treatment was performed by a protocol that inhibits remodeling in the fly system (*6*, *24*) and completely inhibits ATP-dependent chromatin assembly by the CD fraction (*17*); this protocol is therefore expected to cripple other ATP-dependent reactions in the system. Chromatin reorganization was monitored by RE access and Southern blotting after micrococcal nuclease digestion (Figure 5B,C).

As expected, apyrase treatment following a 90 min assembly reaction in the presence of Gal4-VP16 did not prevent the increase in accessibility of DNA to *Xba*I and *Bam*HI which characterizes remodeling reactions in the presence of ATP (Figure 5B, *Bam*HI and *Xba*I panels; lanes 2 and 3 for *Drosophila* histones and lanes 6 and 7 for yeast histones). We next tested if, as in fly extracts, chromatin reorganization by Gal4-VP16 is inhibited when the transcription factor is added to a reaction mixture after assembly and ATP depletion. Surprisingly, this inhibition was not observed; Gal4-VP16 promoted similar access of both *Xba*I and *Bam*HI to DNA in the Gal4 element if Gal4-VP16 was added to reaction mixtures before assembly or after assembly and apyrase treatment (Figure 5B, compare lanes 3 and 4 for *Drosophila* histones and lanes 7 and 8 for yeast histones).

Micrococcal nuclease digestion and Southern blotting analysis were also used to monitor chromatin reorganization after ATP depletion. The experiment in Figure 5C examines reorganization in assembly reactions using either *Drosophila* or yeast histones supplemented with apyrase. The ~250 bp band was generated when the reaction mixtures with Gal4-VP16 added at the beginning of assembly were treated with apyrase for 75 min before probing with micrococcal nuclease (Figure 5C; compare lanes 3 and 4 to lanes 1 and 2 for *Drosophila* and lanes 15 and 16 to lanes 13 and 14 for yeast). In other words, as suggested by RE digestion analysis, chromatin reorganization can occur during the course of ATP-dependent assembly. Reorganization was also readily detected by micrococcal nuclease digestion analysis of reaction mixtures to which Gal4-VP16 was added after ATP depletion. That is, the nucleosomal ladder is smeared, and a prominent band appears between the monomer and dimer if, after assembly, the reaction mixture is treated with apyrase and then supplemented with Gal4-VP16 (Figure 5C; compare lanes 5 and 6 to lanes 1 and 2 for *Drosophila* and lanes 17 and 18 to lanes 13 and 14 for yeast). Interestingly, while the species source of histones has almost no effect on the generation of the 250 bp band, micrococcal nuclease digestion analysis does reveal some differences in the pattern of reorganization of chromatin comprised of *Drosophila* versus yeast histones. When *Drosophila* histones were used for assembly, bands shifted up from the standard size of the di- and trinucleosomes were prominent after micrococcal nuclease digestion of reaction mixtures in which ATP depletion preceded addition of Gal4-VP16 (Figure 5C; compare lanes 4 and 6). The same shifted bands are barely detectable when yeast histones were used in the assembly reactions (compare lanes 16 and 18). Under all conditions, however, the ladders revealed by the distal probe were essentially unchanged, demonstrating that binding of Gal4-VP16 after ATP depletion affects chromatin reorganization specifically at the Gal4p

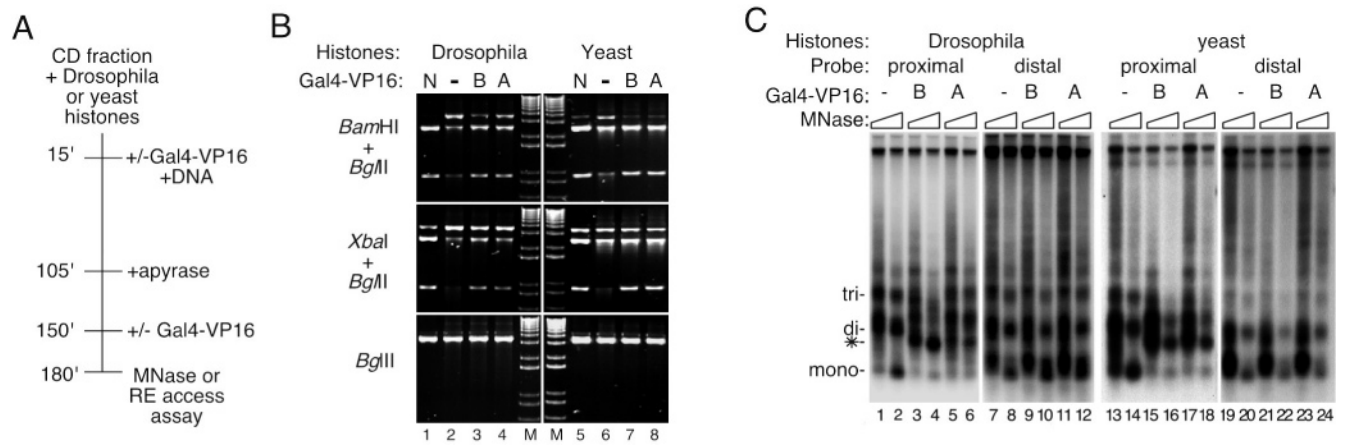

FIGURE 5: Chromatin reorganization mediated by Gal4-VP16 after depletion of ATP. (A) Outline of the experiment. Nucleosomal templates were assembled with either *Drosophila* or yeast histones, and the reaction mixtures were incubated without Gal4-VP16 (−) or with 200 nM Gal4-VP16 (+) at the beginning of the reaction or after apyrase treatment: MNase, micrococcal nuclease; RE, restriction enzyme. (B) Restriction enzyme accessibility assay of reactions to which Gal4-VP16 was added at the start of assembly (B, before assembly) or after apyrase was added (A, factor added after both assembly and apyrase treatment). Purified digestion products were cut with *Bgl*II, resolved by agarose gel electrophoresis, and visualized by ethidium bromide staining: N, naked DNA; M, 1 kb plus DNA ladder. (C) Micrococcal nuclease digestion assay. Digested reaction products were purified and resolved by agarose gel electrophoresis after reactions were performed using *Drosophila* (lanes 1−12) and yeast (lanes 13−24) core histones. Blots were probed with the Gal4 element proximal probe (lanes 1−6 and 13−18), stripped, and reprobed with the Gal4 element distal probe (lanes 7−12 and 19−24). The migration of the mono-, di-, and trinucleosome length DNA fragments is indicated on the left border (mono-, di-, and tri-, respectively). The asterisk in the left panel indicates the product of approximately 250 bp resulting from chromatin reconfiguration.

binding site rather than globally (Figure 5C; lanes 7−12 for *Drosophila* histones and lanes 19−24 for yeast histones). These results suggest that a fundamental step in Gal4-VP16-dependent chromatin reorganization in the yeast system is supported in the absence of ATP.

Transactivator-dependent nucleosome reorganization in the absence of ATP is contrary to the accepted dogma. We therefore considered the possibility that apyrase treatment might not be particularly effective at depleting ATP from the CD fraction. This seemed highly unlikely, given that assembly of correctly spaced nucleosome arrays in this system requires an ATP-dependent ATPase (Chd1p) and is inhibited by apyrase treatment according to a protocol which inhibits remodeling in crude extracts from fly embryos (*17*). Nonetheless, we performed a further functional test for apyrase depletion of ATP in the presence of the CD fraction. In this test, we monitored the DNA decatenation activity of DNA topoisomerase II as outlined in Figure 6A. DNA topoisomerase II is the only topoisomerase that can decatenate linked circles of double-stranded DNA, and this activity is dependent upon ATP (*25*). The standard substrate for these reactions is kinetoplast DNA (kDNA), which barely enters agarose gels because the catenated monomers of kDNA form a high-molecular weight complex (Figure 6B, faint band of stained material in well of lane 1). Decatenation activity in the CD fraction was assayed under the conditions used for chromatin assembly (the amount of apyrase added and the concentrations of CD fraction, starting ATP, and DNA were all the same as those used for assembly). After incubation with commercially available topoisomerase II, kDNA is decatenated into a DNA monomer that runs at roughly 3.5 kb (Figure 6B, lane 2). The CD fraction contains topoisomerase II activity that also decatenates the kDNA to yield a 3.5 kb product (Figure 6B, lane 3). No such product was formed, however, if the reaction mixture did not include an ATP regenerating system (lane 4). The same result was obtained when reaction mixtures containing the CD fraction

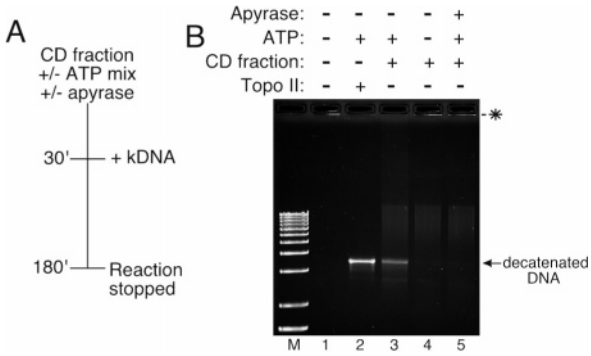

FIGURE 6: Apyrase effectively depletes the assembly reaction of ATP. (A) Outline of the experiment shown in panel B. The CD fraction was incubated with or with an ATP regenerating system, and with or without apyrase for 30 min at 30 °C. Kinetoplast DNA (kDNA) was added and the reaction mixture incubated for a further 2 h. (B) Ethidium bromide-stained agarose gel of the re-isolated DNA. The asterisk indicates catenated DNA which does not enter the gel: ATP, ATP regenerating system; Topo II, topoisomerase II.

and ATP regenerating system were treated with apyrase prior to addition of high-molecular weight kDNA (lane 5). The failure to detect the monomer product in lanes 4 and 5 was not due to DNA degradation, since high-molecular weight kDNA remains visible in the well in lanes 4 and 5. Collectively, our analysis of the effect of apyrase treatment on two distinct ATP-dependent activities, assembly of nucleosome arrays and kDNA decatenation by topoisomerae II, reveals that apyrase effectively depletes ATP in this system. We conclude that the yeast CD fraction contains activity which can reorganize chromatin in response to a DNA binding factor after ATP has been removed.

Is this activity due to an RNA? This seems unlikely because RNA on is own has not been reported to remodel chromatin. On the other hand, RNA can assemble histones onto DNA by an ATP-independent mechanism. We therefore tested whether ATP-independent chromatin reorganization

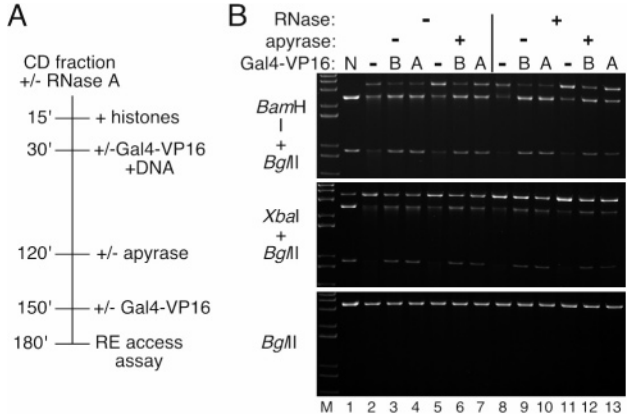

FIGURE 7: RNase A treatment does not affect restriction enzyme accessibility after ATP depletion by apyrase. (A) Outline of the experiment shown in panel B. The CD fraction was (+) or was not (−) preincubated with RNase A at 30 °C for 15 min. (B) Purified digestion products were cut with *Bgl*II, resolved by agarose gel electrophoresis, and visualized by ethidium bromide staining. Lane 1 shows digestion of naked DNA. M denotes the 1 kb plus DNA ladder.

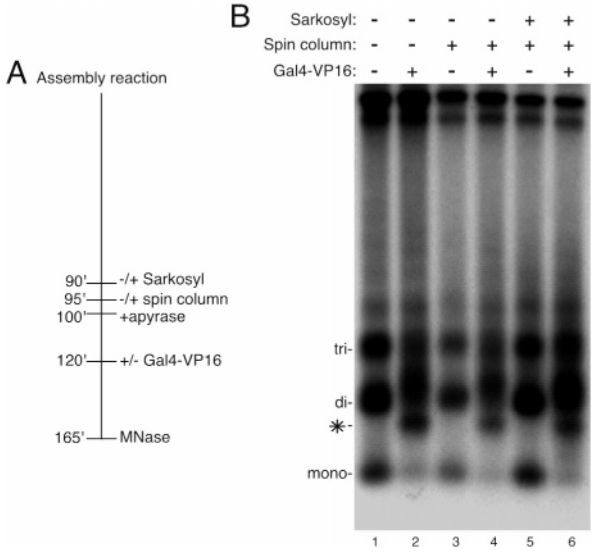

FIGURE 8: Chromatin reorganization is insensitive to Sarkosyl treatment. (A) Outline of the experiment shown in panel B. After 90 min, assembly reaction mixtures were incubated at room temperature for 5 min with or without 0.05% Sarkosyl, and then spun through two CL-4B spin columns. All samples were treated with apyrase, divided, and incubated with or without Gal4-VP16. Following micrococcal nuclease digestion, the samples were analyzed by Southern blotting. (B) Southern blot of the experiment (proximal probe). The migration of the mono-, di-, and trinucleosome length DNA fragments is indicated on the left border (mono-, di-, and tri-, respectively). The asterisk indicates the product of approximately 250 bp resulting from chromatin reconfiguration.

in the CD fraction was due to an RNA species by comparing the activity of the untreated and RNase-treated CD fraction [outlined in Figure 7A; the same protocol was previously used to show that RNA does not mediate assembly in this system (*17*)]. Chromatin reorganization was monitored using the RE accessibility assay. As demonstrated above, Gal4-VP16 directs reorganization events which lead to exposure of *Xba*I and *Bam*HI sites adjacent to the Gal4 binding elements, either in the presence (Figure 7B; compare lane 2 to lanes 3 and 4) or in the absence (Figure 7B; compare lane 5 to lanes 6 and 7) of ATP. An identical result is obtained using the extract pretreated with RNase (Figure 7B; compare lane 8 to lanes 9 and 10 and lane 11 to lanes 12 and 13). For example, in the control CD fraction, the yield of the fastest-migrating *Xba*I−*Bgl*II fragment is low when reaction mixtures do not include Gal4-VP16 because these sites are incorporated into nucleosomes (Figure 7B, lane 2). The same is true in the RNase A-treated CD fraction (lane 8). In the control CD fraction, the yield of this fragment increases in the presence of Gal4-VP16 because Gal4-VP16 directs reorganization which increases RE site accessibility (Figure 7B, lane 3). An essentially identical increase in the yield of the diagnostic *Xba*I−*Bgl*II fragment is obtained in the RNase A-treated CD fraction (lane 9). We conclude that ATP-independent chromatin reorganization was not due to an RNA species.

Stable Association of the Reorganizing Activity with Reconstituted Chromatin. Some ATP-dependent chromatin remodeling factors, for example, NURF and CHRAC, are able to physically associate with reconstituted chromatin (*26*). Chromatin devoid of these factors can be obtained by treating reconstitution reaction mixtures with Sarkosyl and then purifying the chromatin by size exclusion chromatography. We tested if this protocol also removes ATP-independent reorganizing activity from chromatin assembled in the CD fraction.

As outlined in Figure 8A, assembly reaction mixtures were either treated with Sarkosyl or left untreated, and then spun through a gel filtration column. Purified chromatin was tested for reorganizing activity in the presence or absence of Gal4-VP16. To exclude the possibility that this procedure unmasks an ATP-dependent activity in the samples, the protocol included apyrase treatment prior to transactivator addition. Southern blot analysis of micrococcal nuclease-digested chromatin revealed a Gal4-VP16-dependent 250 bp product in control reactions using a spin column eluate which had not been previously treated with Sarkosyl (Figure 8B; compare lanes 3 and 4). Therefore, ATP-independent reorganizing activity cofractionates with reconstituted chromatin. A 250 bp product is also readily detected in reactions performed with input material that had been treated with Sarkosyl and then purified by gel filtration chromatography (compare lanes 5 and 6). These results suggest that the reorganizing activity in the CD fraction can associate with reconstituted chromatin and that this association is not significantly disrupted by Sarkosyl treatment which can strip NURF, CHRAC, and other remodeling enzymes from chromatin.

Effect of Gene Deletion on Gal4-VP16-Dependent Reorganization: A Targeted Screen. We took advantage of the availability of the collection of viable haploid deletion strains to screen for mutants which yield a CD fraction with altered chromatin reorganizing activity. The CD fraction was assayed with and without apyrase treatment to confirm the ATP independence of the activity detected in mutant extracts. A subset of gene deletion mutants was selected for screening: representatives of three subfamilies of DNA-dependent ATPases in yeast (*ISW1*, *ISW2*, *CHD1*, *SNF2*, *SWR1*, and YFR038w), three histone chaperones (Asf1p, Cac1p, and Nap1p), and a critical noncatalytic subunit of the SAGA/ADA HAT complexes (Spt20p). As expected, the quality of the nucleosome ladder generated in the CD fraction in the absence of Gal4-VP16 depended upon the complement of endogenous yeast proteins ("−" lanes in Figure 9B,C). The

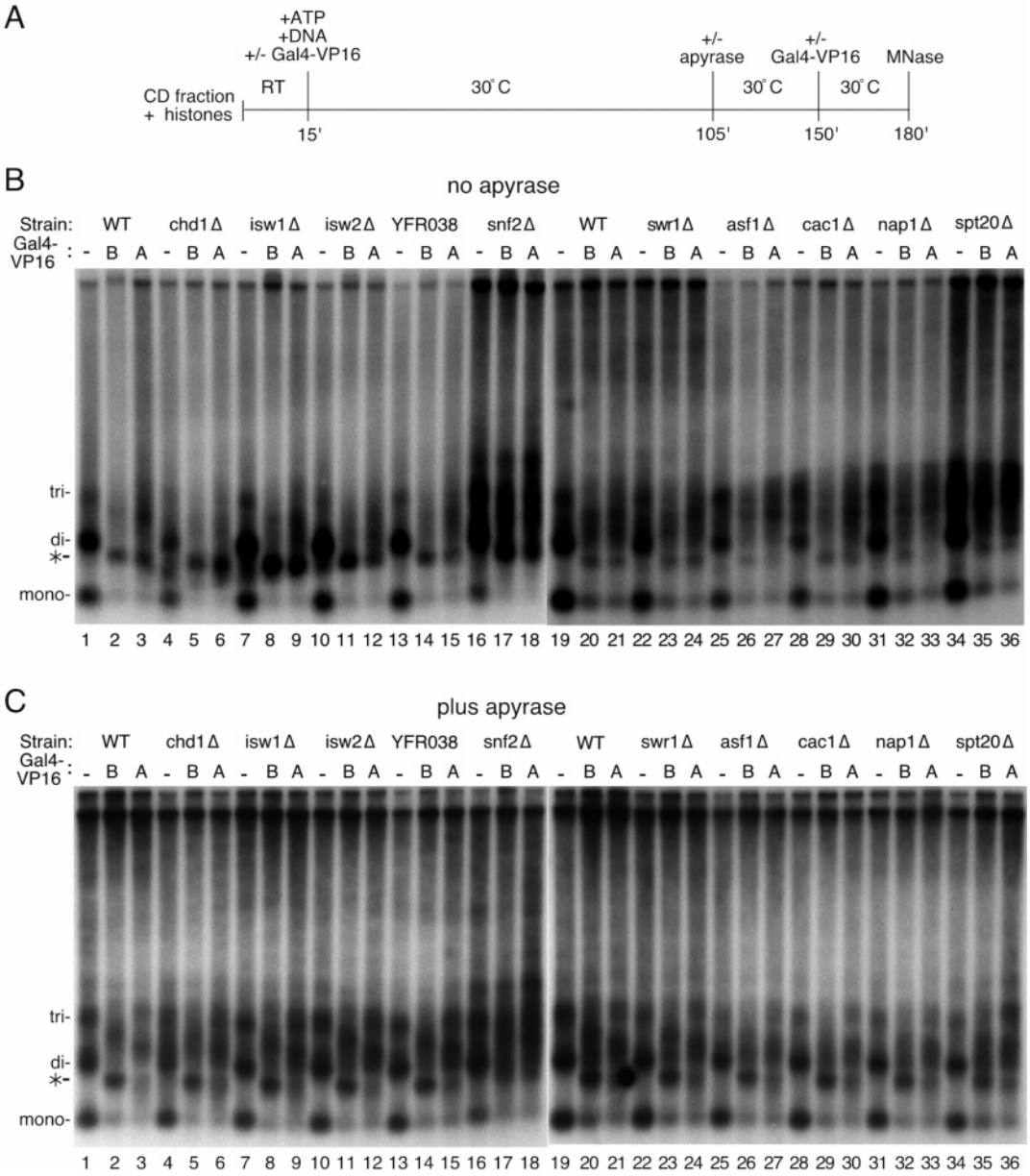

FIGURE 9: Screening of the CD fraction from 10 deletion mutants for chromatin reorganizing activity. (A) Outline of the experiment. RT denotes room temperature and MNase micrococcal nuclease. (B) Assembly reactions were performed under standard conditions with the CD fraction from the wild type (BY4741, WT; lanes 1−3 and 19−21) and the indicated deletion strains. Gal4-VP16 was added at the same time as the DNA (B, before assembly) or 30 min before the end of the incubation (A, after assembly). Reorganizing was analyzed by micrococcal nuclease digestion and Southern blotting using the Gal4 element proximal oligonucleotide probe shown in Figure 1A. The mono-, di-, and trinucleosome length DNA fragments are indicated (mono-, di-, and tri-, respectively) on the left border of the figure. The asterisk indicates the position of the product of approximately 250 bp resulting from reorganization. (C) Reactions were performed as described for panel A except that apyrase was added 45 min prior to adding Gal4-VP16. "B" indicates that Gal4-VP16 was added at the beginning of assembly (at 15 min, along with the addition of DNA), and "A" indicates its addition after assembly (at 150 min).

trinucleosome band, for example, is diminished in reactions that included *chd1*Δ extract because Chd1p contributes to correct spacing of nucleosomes during assembly; larger DNA fragments are detected after micrococcal nuclease digestion of chromatin assembled in *snf2*Δ extract because this chromatin is more resistant to digestion than wild-type chromatin (*17*). Nonetheless, all assayed null mutant fractions supported chromatin reorganization, yielding the novel band pattern detected by micrococcal nuclease digestion analysis and Southern blotting (Figure 9). In particular, whether Gal4-VP16 is added before or after assembly in the continuous presence of ATP, the monomer yield diminishes, the dimers typically disappear, and a 250 bp band of variable intensity becomes apparent (Figure 9B). Treatment with apyrase before addition of Gal4-VP16 did not substantially affect the pattern of reorganization in any mutant CD fraction (Figure 9C). We conclude that no single factor among those tested is exclusively required for Gal4-VP16-dependent chromatin reorganization in the yeast CD fraction.

DISCUSSION

Chromatin remodeling by yeast proteins has typically been studied using highly purified components, for example, nucleosome arrays reconstituted by salt gradient dialysis and remodeling factors obtained by multistep fractionation schemes. In higher eukaryotes, crude systems have also

served well for studies of chromatin remodeling, sometimes providing the machineries for both assembly of the chromatin substrate and its remodeling. Accordingly, we explored the possibility that a crude chromatin assembly extract from yeast might also support chromatin remodeling that is dependent upon an added transactivator. We indeed have observed robust Gal4-VP16-dependent reorganization of chromatin in the CD fraction. Therefore, in addition to assembly, chromatin reorganizing activity can be detected in a relatively crude chromatographic fraction of extract from whole yeast cells and nuclei.

Distinctive Properties of a New Chromatin Reorganizing Activity. These results are readily compared to those obtained in *Drosophila* because our analytical approach closely approximated that used to study Gal4-VP16-dependent remodeling in extracts of fly embryos (identical template, comparable micrococcal nuclease digestion, same Southern blotting protocols, and identical probes). This comparison reveals several distinctive properties of the Gal4-VP16-dependent activity detected in the yeast CD fraction. Most importantly, unlike conventional chromatin "remodeling", nucleosome reorganization in the CD fraction does not require ATP. While there is no precedent for Gal4-VP16-dependent chromatin remodeling in the absence of ATP, proteins that do promote nucleosome reorganization without ATP hydrolysis are now well-known. The FACT complex of higher eukaryotes, for example, is able to extract an H2A−H2B dimer from a nucleosome by an ATP-independent mechanism (*27*). Removal of the H2A−H2B dimer from nucleosomes can also occur by an ATP-independent mechanism if Nap1 is available as an acceptor (*28*). Note that because activity persists in the CD fraction from *nap1*Δ cells, it is unlikely that Nap1 is required for Gal4-VP16-dependent chromatin reorganization in yeast (Figure 9). Finally, type B high-mobility group (HMGB) proteins can also reorganize chromatin without the expenditure of ATP. Among known HMG proteins, Nhp6 of yeast is the best studied in this regard. Nhp6 binding to nucleosomes forms a complex which can recruit two other proteins, the Spt16 and Pob3 subunits of yeast FACT. Formation of the nucleosome−Nhp6−Spt16-Pob3 complex is associated with ATP-independent reorganization of the nucleosome (*29*). Travers (*30*) has argued that this reorganization might promote nucleosome mobility in vivo.

The second distinguishing feature of the chromatin reorganizing activity in the CD fraction is its capacity to generate a chromatin structure which has not been reported in previous studies of Gal4-VP16-dependent remodeling by factors in crude extracts of embryonic cells (*6*). Mapping studies suggest a chromatin reorganization process in which the array of nucleosomes proximal to the Gal4p sites is reconfigured in the CD fraction to stably locate a nucleosome immediately adjacent to these binding sites. In this arrangement, the DNA between the nucleosome and the Gal4p sites is evidently shielded from nuclease cutting by the continuous presence of a protein factor. Considering that many known remodeling enzymes can interact both with a DNA-bound transactivator and with histones (*31*), we speculate that the DNA between the Gal4 sites and the immediately adjacent nucleosome is protected from nuclease digestion by a chromatin reorganizing factor which simultaneously contacts the Gal4-VP16−DNA complex and the adjacent nucleosome. Alternatively, the chromatin reorganizing activity may not itself shield the DNA but promote interactions between other components of chromatin which protect DNA between the nucleosome and Gal4 binding sites from nuclease digestion.

The third distinguishing feature of the chromatin reorganizing activity in the CD fraction is its robust association with chromatin. That is, Sarkosyl treatment of chromatin followed by gel filtration using a resin with an exclusion limit of 20 MDa does not separate the chromatin from the ATP-independent chromatin reorganizing activity in the CD fraction (both are recovered in the void volume). The same treatment does separate *Drosophila* NURF and CHRAC from chromatin (*26*). The behavior of the chromatin reorganizing activity suggests that it binds to chromatin quite tightly, and functions in its reorganization only upon interaction with DNA-bound Gal4-VP16. Alternatively, the reorganizing activity may both be resistant to Sarkosyl disruption and have biophysical properties which preclude its entry into the pores of the resin. We do not favor the latter possibility because no remodeling factor larger than approximately 2 MDa has been described in the literature, and none of these factors is reported to exist in multimeric forms.

Considering how intensely yeast remodeling factors have been studied, it is surprising that the chromatin reorganizing activity characterized here has not been described previously in yeast. It may be that chromatin reorganization requires more than one factor, in which case previous studies of individual remodeling machines might not have suggested the existence of a reaction pathway which generates the Gal4-VP16-dependent chromatin state we report. Indeed, it is now clear that remodeling in vivo can involve collaboration of a remodeling machine with other factors. Reorganization of chromatin at the IFN-β promoter by human Swi/Snf is a two-step process in which TBP binding facilitated by Swi/Snf is followed by TBP-induced bending of promoter DNA that is required for nucleosome sliding (*32*). Alternatively, the outcome of reorganization in the CD fraction may correspond to an intermediate step in remodeling by a known factor that occurs rapidly in a highly purified system but slowly in a crude extract. It would not be surprising to find that a particular step in chromatin reorganization is sensitive to conditions prevailing in the CD fraction. The histone N-terminal tails, for example, are not required by yeast SWI/SNF when remodeling is performed under noncatalytic conditions (SWI/SNF is not recycled during remodeling) but are required under "multiple-round" conditions involving template switching of SWI/SNF (*33*, *34*). A remote possibility is that in our hands Gal4-VP16 alone is able to remodel chromatin. We will directly test this possibility in the unlikely event that none of the non-histone proteins detected in the chromatin fraction obtained by gel filtration chromatography (KMR and MCS, data not shown) is responsible for the reorganization described here.

Implications of Screen Results. Chromatin reorganizing activity was readily detected in the CD fraction prepared from all the null mutants that were tested. Therefore, none of the tested genes is uniquely required for ATP-independent chromatin reorganization in the CD fraction. Current efforts to identify the chromatin reorganizing activity are taking into consideration several possibilities raised by this finding. First, an untested gene might be necessary and sufficient for chromatin reorganization. Candidates yet to be tested include

DNA-dependent ATPases [for example, Ino80 (*35*)], chromatin assembly factors [for example, the Hir proteins (*36*)], subunits of FACT (*27*), and Cti6, a coregulator of *GAL1* in yeast (*37*). Second, Gal4-VP16-dependent chromatin reorganization might be performed by redundant pathways. Third, the activity might be performed by an essential gene such as Spt6, a H3/H4 chaperone required for maintenance of the normal chromatin organization of transcribing genes (*38*). Finally, an unknown gene might be necessary and sufficient for Gal4-VP16-dependent chromatin reorganization. In view of these various possibilities, both biochemical and genetic approaches are being used to further characterize the chromatin reorganizing activity in the CD fraction.

Little misregulation of chromatin reorganizing activity in the CD fraction was observed despite significant phenotypic variation between the strains that were examined. Three points are noteworthy in this respect. First, a comparable level of chromatin reorganizing activity was recovered from strains which differ substantially in their proliferation rate. For example, *spt20*Δ and *swr1*Δ cells have doubling times of 145 and 92 min, respectively, but yield extracts which reconfigure chromatin to a similar extent, as judged by generation of the 250 bp DNA product in the micrococcal nuclease digestion assay (Figure 9C; compare lanes 23 and 24 to lanes 35 and 36). Therefore, Gal4-VP16-dependent chromatin reorganizing activity in the CD fraction is not strongly regulated by proliferation signals. Second, pervasive misregulation of chromatin metabolism due to inactivation of histone chaperones or ATP-dependent chromatin remodeling enzymes is not associated with misexpression of chromatin reconfiguring activity in the CD fraction. For example, deletion of *ISW1* causes induction of 667 genes (*39*) but does not substantially affect recovery of chromatin reorganizing activity in the CD fraction. Deletion of *CAC1* and *ASF1* has readily evident effects on global chromatin assembly in vivo (*40*, *41*) but no significant effect on chromatin reorganizing activity. Finally, since *asf1*Δ cells have a substantial G2/M cell cycle progression defect and yield robust chromatin reconfiguring activity, it is unlikely that the latter is regulated by G2/M signaling cues. In summary, while no tested mutation conferred significant misregulation of chromatin reorganizing activity in the CD fraction, the results of the screen have served to focus ongoing studies aimed at defining the physiological context in which ATP-independent chromatin reorganizing activity is regulated in vivo.

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