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NuA4, an essential transcription adaptor/histone H4 acetyltransferase complex containing Esa1p and the ATM-related cofactor Tra1p

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Post-translational acetylation of histone H4 N-terminal tail in chromatin has been associated with several nuclear processes including transcription. We report the purification and characterization of a native multisubunit complex (NuA4) from yeast that acetylates nucleosomal histone H4. NuA4 has an apparent molecular mass of 1.3 MDa. All four conserved lysines of histone H4 can be acetylated by NuA4. We have identified the catalytic subunit of the complex as the product of *ESA1*, an essential gene required for cell cycle progression in yeast. Antibodies against Esa1p specifically immunoprecipitate NuA4 activity whereas the complex purified from a temperature-sensitive *esa1* mutant loses its acetyltransferase activity at the restrictive temperature. Additionally, we have identified another subunit of the complex as the product of *TRA1*, an ATM-related essential gene homologous to human TRRAP, an essential cofactor for c-Myc- and E2F-mediated oncogenic transformation. Finally, the ability of NuA4 to stimulate GAL4–VP16-driven transcription from chromatin templates *in vitro* is also lost in the temperature-sensitive *esa1* mutant. The function of the essential Esa1 protein as the HAT subunit of NuA4 and the presence of Tra1p, a putative transcription activator-interacting subunit, supports an essential link between nuclear H4 acetylation, transcriptional regulation and cell cycle control.

Keywords: acetyltransferase/*ESA1*/nucleosome/transcription

Introduction

Eukaryotic gene transcription can be regulated in several ways, many of which involve chromatin structure. Nucleosomes suppress basal transcription initiation, increasing the dependence on the function of sequence-specific activator proteins (reviewed in Owen-Hughes and Workman, 1994). Chromatin structures are remodeled prior to or concurrent with transcription activation, creating DNase I-hypersensitive sites at regulatory elements (reviewed in Steger and Workman, 1996). Genetic and biochemical studies have identified multiprotein complexes implicated in this remodeling. Complexes which use the energy of ATP hydrolysis to perturb chromatin structure include yeast and mammalian SWI/SNF complexes (Côté *et al.*, 1994, 1998; Kwon *et al.*, 1994; Owen-Hughes *et al.*, 1996; Wang *et al.*, 1996), *Drosophila* NURF, CHRAC and ACF complexes (Tsukiyama and Wu, 1995; Ito *et al.*, 1997; Varga-Weisz *et al.*, 1997) and the yeast RSC complex (Cairns *et al.*, 1996).

Another group of complexes modify histones in chromatin in order to regulate transcription. Histone acetyltransferases (HATs) and histone deacetylases (HDs) play a critical role for positive and negative regulation of transcription (reviewed in Grunstein, 1997; Struhl, 1998). A number of studies have correlated acetylation of specific lysine residues in the N-terminal tails of histones with the transcriptional activity of chromatin (reviewed in Turner and O'Neill, 1995). Hyperacetylated histones accumulate in actively transcribed chromatin (Hebbes *et al.*, 1994), while hypoacetylated histones are enriched in transcriptionally repressed domains (Braunstein *et al.*, 1993). Acetylation of histone N-terminal tails is thought to reduce their affinity for nucleosomal DNA (Hong *et al.*, 1993), and increase the binding of transcription factors to their binding sites within a nucleosome (Lee *et al.*, 1993; Vettese-Dadey *et al.*, 1996). Becker and colleagues showed that histone hyperacetylation increases DNase I sensitivity and conformational flexibility of chromatin, as well as facilitates transcription of the *Drosophila hsp26* gene in chromatin (Krajewski and Becker, 1998; Nightingale *et al.*, 1998).

Several transcriptional regulatory proteins have intrinsic HAT activity. These include coactivators like Gcn5 (Brownell *et al.*, 1996), p300/CBP (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996), p/CAF (Yang *et al.*, 1996), ACTR (Chen *et al.*, 1997), Src-1 (Spencer *et al.*, 1997) and $TAF_{II}250$ (Mizzen *et al.*, 1996). All these proteins are members of multisubunit complexes involved in transcriptional activation through interaction with sequence-specific activators. Similarly, HDs are present in corepressor complexes (see Struhl, 1998 and references therein). Other proteins having *in vitro* HAT activity include Tip60 (Yamamoto and Horikoshi, 1997) and Esa1 (Smith *et al.*, 1998; Clarke *et al.*, 1999), two members of the MYST family of putative acetyltransferases (Borrow *et al.*, 1996; Reifsnyder *et al.*, 1996; Hilfiker *et al.*, 1997), and Hat1, the cytosolic deposition-related HAT (Kleff *et al.*, 1995; Parthun *et al.*, 1996). The identification of those proteins as HATs also demonstrated their differing substrate specificities. Recombinant Gcn5, p/CAF, $TAF_{II}250$ and src-1 acetylate mainly histone H3, whereas Tip60, Esa1p and Hat1p prefer histone H4. CBP/p300 and

ACTR modify both histone H3 and H4 (see respective references above). Importantly, the ability to acetylate histone tails in the context of chromatin is thought to be a necessary characteristic of transcription-related HATs.

We have previously identified four native high molecular weight complexes from yeast extracts that contain HAT activities which function on polynucleosomal substrates (Grant *et al.*, 1997). Gcn5p is the catalytic subunit of two of these complexes, termed SAGA and Ada. Both complexes also contain ADA2 and ADA3 gene products, confirming their role as native transcription adaptor complexes, and modify primarily histone H3 in chromatin, and H2B to a lesser extent. The SAGA complex also contains Spt20/Ada5, Spt3 and Spt7, a group of transcriptional regulators thought to be involved in helping TBP function (Winston, 1992), and a subset of TBP-associated factors (TAF_{II}s) originally identified in the $TF_{II}D$ complex (Grant *et al.*, 1998a). The presence of ADA, SPT and TAF_{II} gene products in the SAGA complex indicates the importance of histone acetylation in transcription activation, a reaction mediated by interaction with transcription activators and general transcription factors. It is important to note that the presence of Gcn5p in native complexes stimulates its activity on chromatin substrates, suggesting the presence of subunit(s) involved in chromatin binding and/or stimulating Gcn5p activity. Finally, we have identified two other HAT activities, termed complexes 2 and 3 (Grant *et al.*, 1997), which acetylate nucleosomal histones H4 and H3, respectively. Since the subunit composition of these complexes was unknown, they were named NuA4 (Nucleosome acetyltransferase of histone H4) and NuA3 according to their substrate specificity.

In this report we show the purification and characterization of the NuA4 complex. Using genetic and biochemical approaches, we show that Esa1p is the catalytic subunit of this 1.3 MDa native HAT complex. We also demonstrate that stimulation of GAL4–VP16-driven transcription on chromatin templates by NuA4 requires a functional Esa1p subunit. Since the *ESA1* gene product is essential for cell cycle progression in yeast (Clarke *et al.*, 1999), this demonstrates the first purification of a native essential HAT complex. Furthermore, it is also the first native complex targeting mainly histone H4 in chromatin, which are the most thoroughly studied N-termini. Esa1p is a direct homolog of MOF, a putative acetyltransferase necessary for X chromosome hypertranscription and H4 acetylation in *Drosophila* males (dosage compensation) (Hilfiker *et al.*, 1997). We have also identified the ATM/PI-3 kinase-related protein Tra1p as a subunit of NuA4. Tra1p is essential for cell viability (Saleh *et al.*, 1998) and is the homolog of human protein TRRAP, a c-Myc- and E2F-interacting protein essential for their oncogenic activities (McMahon *et al.*, 1998). These facts and the finding that yeast NuA4 stimulates *in vitro* transcription from chromatin templates implicate NuA4 as a transcriptional adaptor/regulatory complex essential for cell cycle progression.

Results

Identification of a native yeast HAT complex that acetylates nucleosomal histone H4

In a previous report we have shown that nickel–agarose resin binds several native nucleosomal HAT activities from yeast extracts (Grant *et al.*, 1997). When the fraction was further separated over a monoQ column, four distinct native nucleosomal HATs were identified. An example of this fractionation is shown in Figure 1A. Two HATs, Ada and SAGA, preferentially acetylate N-termini of H3/H2B (Figure 1A, fractions 22–24 and 42) and were shown to contain Gcn5p as the catalytic subunit as well as other transcription regulatory proteins (Grant *et al.*, 1997). The two other nucleosomal HATs were not further characterized aside from their substrate specificities. NuA3, like Ada and SAGA, also preferentially acetylates N-termini of nucleosomal H3, but fails to modify H2B (Figure 1A, fractions 36–38). NuA4 is unique amongst these HATs as it targets mainly the N-termini of histone H4 in oligonucleosomes, and H2A to a lesser extent (Figure 1A, fractions 24–28). Further fractionation of NuA4 by gel filtration over Superose 6 indicated an apparent molecular weight of ~1.3 MDa (Figure 1B, fraction 21). Therefore, like the SAGA and Ada complexes, NuA4 is also a high molecular weight multisubunit complex.

NuA4 can produce fully tetra-acetylated histone H4 in oligonucleosomes

To further characterize the acetyltransferase specificity of NuA4, we compared its activity toward HeLa oligonucleosomes, HeLa free histones and yeast free histones. As shown above, NuA4 efficiently acetylates H4/H2A in oligonucleosomes, while the Ada complex targets H3/H2B (Figure 2A, lanes 2 and 3). When oligonucleosomes were replaced by free histones as substrate in the reaction, the specificity of NuA4 was still mainly histone H4, while Ada targeted mainly H3 (Figure 2A, lanes 5 and 6). However, it is important to note that amongst free histones H3 can be acetylated by NuA4, while it is not modified in oligonucleosomes (compare lanes 3 and 6, see also Figure 4D, lanes 1 and 5). NuA4 activity was also tested on yeast free histones and gave very similar results to the ones obtained with human free histones, as expected from the strongly conserved lysine residues in the N-terminal tails of histones (Figure 2A, compare lanes 6 and 9).

Histone H4 isoforms acetylated at specific lysines (K5, K8, K12 and K16) are selectively localized on *Drosophila* chromosomes (Turner *et al.*, 1992). For example, acetylated lysine 16 of H4 on the male X chromosome is associated with dosage compensation in *Drosophila* (Bone *et al*., 1994), while heterochromatin seems to be acetylated only on lysine 12 (Braunstein *et al.*, 1996). Nevertheless, potentially active euchromatin can be modified at all the H4 acetylatable lysines, whereas mostly silent heterochromatin is hypoacetylated (O'Neill and Turner, 1995). Unlike H4 in chromatin, newly synthesized H4 is diacetylated at K5/K12, which is thought to be involved in chromatin assembly (Sobel *et al.*, 1995). Thus, it was important to determine which specific lysine in the N-terminal tails of nucleosomal H4 can be acetylated by NuA4. Oligonucleosomes were incubated in the presence or the absence of NuA4 and radioactive acetyl-CoA. Histones were then separated on a Triton–acetic acid–urea (TAU) gel which can resolve the different levels of H4 acetylation (0–4 acetylated lysines). In the left panel of Figure 2B, in the absence of NuA4, H4 migrates mainly as a non-acetylated form but 10–15% of the population is monoacetylated. In the presence of NuA4, mono-, di-, tri- and tetra-acetylated

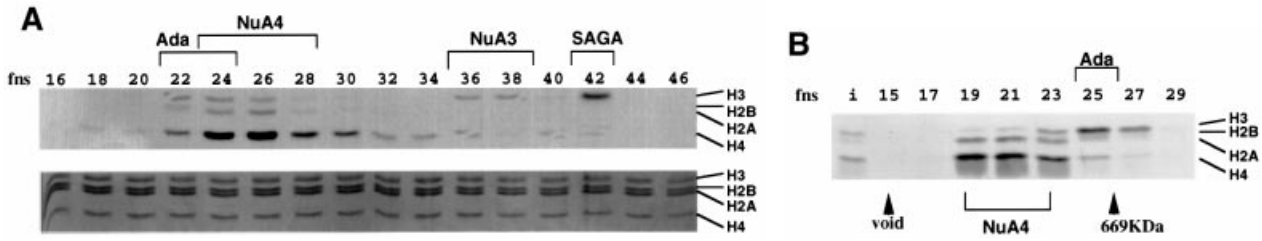

Fig. 1. Fractionation of four different native nucleosomal HATs from yeast. (**A**) Gel HAT assay on fractions from a monoQ column using oligonucleosomes as substrate. One microgram of oligonucleosomes is incubated with 1 µl of each fraction in the presence of radioactive acetyl-CoA for 30 min at 30°C; each reaction is then loaded on an 18% SDS–polyacrylamide gel, Coomassie Blue stained and treated for fluorography to detect histone acetylation (upper panel). The four different activities are identified showing their respective histone specificity (corresponding histone labeled on the right). The lower panel shows the equal amount of histones present in each reaction as revealed by Coomassie Blue staining of the gel. (**B**) Gel filtration over calibrated Superose 6 of the H4-specific monoQ pool. HAT activity of the fractions on oligonucleosomes is shown in the fluorogram. NuA4 is a high molecular weight complex of ~1.3 MDa. The H3-specific Ada complex is ~0.8 MDa. Two of the markers used for calibration are indicated.

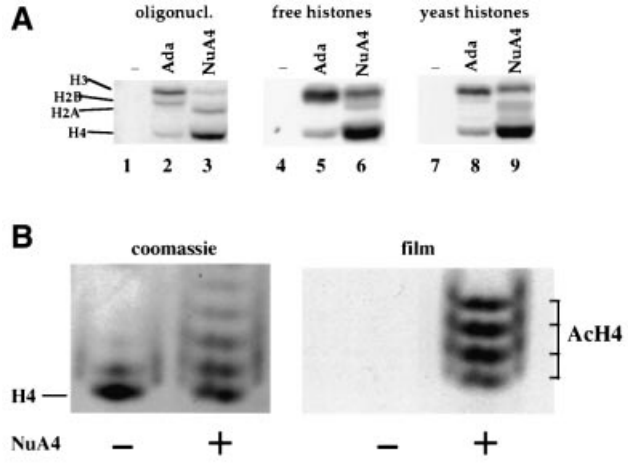

Fig. 2. Substrate specificity of the NuA4 HAT complex. (**A**) Comparison of NuA4 activity toward mammalian oligonucleosomes (lanes 1–3) and free histones (lanes 4–6), and yeast free histones (lanes 7–9). The Ada complex is also shown for comparison. One microgram of substrate and 0.5 µl of Superose 6 respective peak fractions were used in the HAT assay. NuA4 specificity is unchanged when yeast histones are used instead of human ones. Also H3-specific acetylation by NuA4 is not apparent when chromatin is used as substrate. (**B**) TAU gel HAT assay showing that NuA4 is able to create fully tetra-acetylated histone H4 in chromatin. Oligonucleosomes (8 µg) were incubated in the absence or presence of purified NuA4 (over five columns, see Figure 5A) and radioactive acetyl-CoA for 1 h at 30°C. Reactions were then loaded on a TAU gel to distinguish the N-terminal acetylation levels of histone H4. The left panel shows the Coomassie Blue-stained gel and the position of the non-acetylated H4. Note that oligonucleosomes already contain 10–15% of monoacetylated H4 and traces of the diacetylated form. The right panel shows the NuA4-dependent incorporation of radioactive acetate in H4, producing mono-, di-, tri- and tetra-acetylated forms, which were visualized by Coomassie Blue staining (left panel).

forms were produced. This is clearly shown in the autoradiography (right panel, Figure 2B), which demonstrates the NuA4-dependent incorporation of radioactive acetate in each of the four levels of H4 acetylation. These data reveal that NuA4 is able to create fully tetra-acetylated histone H4 in nucleosomes. Specific acetylation of the lysines at position 5, 8, 12 and 16 was also confirmed by microsequencing as reported recently (Ohba *et al.*, 1999). Since each of the four lysine residues in H4 tails can be acetylated by NuA4, we next wanted to determine if this was due to a cascade of lysine acetylation in a specific order on the same histone tail. This was partially answered by using synthetic peptides corresponding to the first 24 amino acids of H4. By using lysine or acetylated lysine during chemical synthesis, six different peptides were produced, each one having different lysines available for acetylation. Incubation with NuA4 and liquid assay quantification showed that each of the four conserved lysine residues on the H4 tail could be independently acetylated (data not shown).

Esa1p functions as the catalytic subunit of NuA4

Besides NuA4, only three proteins have been shown to acetylate primarily the H4 N-termini *in vitro*. These include yeast Hat1p, the major deposition-related cytoplasmic activity (Parthun *et al.*, 1996), human HIV Tat-interacting protein Tip60 (Yamamoto and Horikoshi, 1997) and the very recently identified yeast essential protein Esa1 (Smith *et al.*, 1998; Clarke *et al.*, 1999). A *hat1*-null mutant strain is known to have wild-type level of NuA4 activity (L.Duggan, P.Grant, J.Savard, J.Côté, J.Workman and S.Berger, unpublished data). While these proteins have weak activities on histones within nucleosomes, both recombinant Tip60 and Esa1p have very similar substrate specificity on free histones, i.e. H4>>H3>H2A, which is identical to NuA4 specificity on the same substrate (Figures 2A and 4D). We decided to investigate the possibility that Esa1p contributed to the catalytic activity of the NuA4 HAT complex. We produced an antiserum against the first 14 amino acids of Esa1p and analyzed our fractions by Western analysis. A monoQ fractionation of the nickel–agarose eluate was used to look for potential cofractionation (Figure 3A). As previously shown, the Ada and SAGA complexes were found to co-elute tightly with Ada2p while Swi2p, a subunit of the SWI/SNF complex, did not co-elute with any HAT activity. The antiserum directed against Esa1p detected strict cofractionation of a 55 kDa band with NuA4 activity. From the predicted molecular weight of Esa1p (52.5 kDa) and the fact that our antibody specifically recognized recombinant Esa1p (data not shown), we concluded that this 55 kDa signal was likely to represent Esa1 protein.

To confirm the NuA4–Esa1p relationship, we prepared resins containing cross-linked pre-immune or immune serum. When a NuA4 fraction from a Superose 6 column was incubated with the pre-immune resin, no depletion of HAT activity from the supernatant was detected (Figure 3B). By contrast, when the anti-Esa1p immune

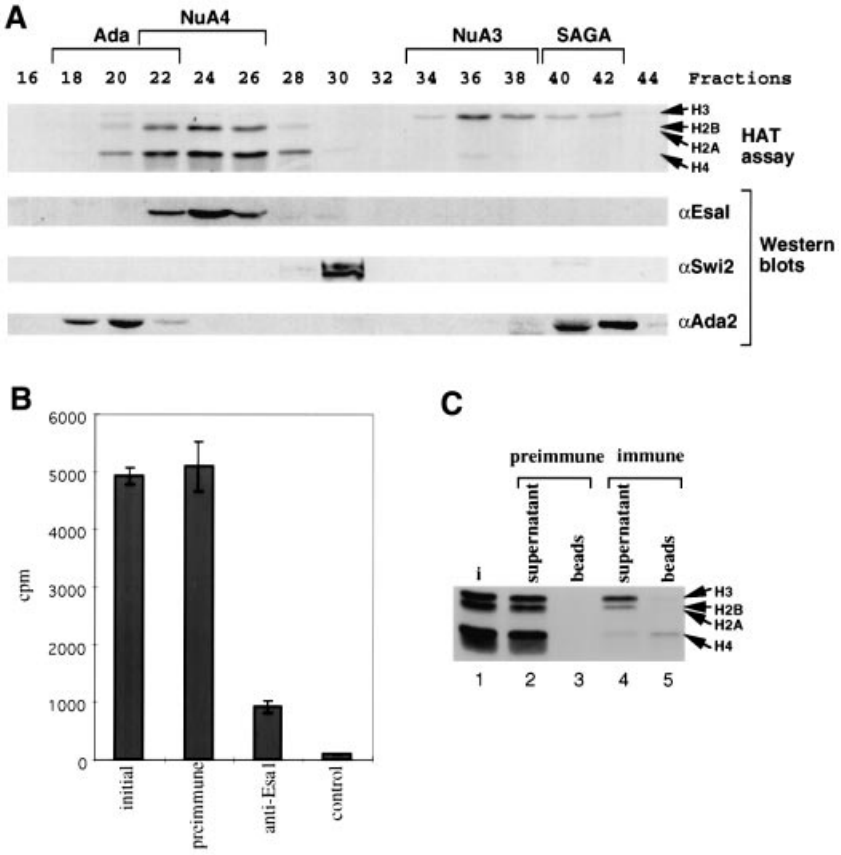

Fig. 3. Esa1p co-elutes with the NuA4 complex and anti-Esa1p serum immunoprecipitates NuA4 activity. (**A**) Fluorogram of a HAT assay on monoQ fractions similar to Figure 1 (upper panel) and immunoscreening for Esa1p, Ada2p and Swi2p in corresponding fractions as indicated (lower panels). One microliter of fractions was used in the HAT assay and 5 µl in Western blot analysis. As shown previously the Ada and SAGA complexes co-elute with Ada2p, which is in direct interaction with Gcn5p in these complexes (Grant *et al.*, 1997). Interestingly, Esa1p signal by Western blotting co-elutes perfectly with NuA4 activity. (**B**) Liquid HAT assay showing that the purified NuA4 activity is depleted by anti-Esa1p serum but not the pre-immune serum. NuA4-containing fraction 21 from a Superose 6 column as in Figure 1B was incubated with pre-immune serum cross-linked to protein A beads. After incubation at 4°C the supernatant was then incubated with anti-Esa1p beads in similar conditions. Equivalent amounts of initial, pre-immune and immune supernatants were then assayed for HAT activity on oligonucleosomes by liquid scintillation counting. The results are presented in a histogram with the standard deviation of three independent experiments. (**C**) Gel HAT assay showing the depletion of NuA4 activity and partial recovery on the anti-Esa1p beads. Experiment similar to (B) except that the sample used was a side fraction containing both NuA4 and Ada activities. Also the beads themselves were tested by HAT assay and the results were visualized by fluorography. As expected the pre-immune beads did not deplete any HAT activity from the initial sample (lane 2 versus 1). In contrast, H4-specific acetylation (NuA4) was clearly depleted by the anti-Esa1p beads (lane 4 versus 1), while H3-specific acetylation (Ada) was unaffected. Some NuA4 activity could be detected when the immune beads were tested for acetylation (lower activity than the amount depleted presumably because of less efficient assay and/or steric hindrance when using beads, lane 5).

resin was used, a very strong depletion of NuA4 activity was observed. We confirmed the specificity of this depletion by using a cruder fraction containing both NuA4 and Ada HATs and looked further for activities on the antibody beads after the binding reaction (Figure 3C). Again, NuA4 activity on histones H4/H2A was not affected by the incubation with the pre-immune beads while incubation with anti-Esa1p beads resulted in depletion (Figure 3C, lanes 1, 2 and 4). Importantly, H3/H2B HAT activity of Ada was unaffected by the incubation with either resins. Moreover, we could partially detect the specific recovery of NuA4 activity on the anti-Esa1p beads (Figure 3C, lane 5, the weaker activity is likely due to the less efficient HAT assay with beads in suspension, steric hindrance due to IgG binding to Esa1). These data strongly suggest that Esa1p is part of the NuA4 complex.

The next step was to determine the role of Esa1p in the nucleosomal HAT activity of NuA4. The most direct way would be to purify the HAT activity from a yeast bearing a disrupted copy of the candidate gene (Grant *et al.*, 1997). However, since Esa1p is essential for growth in yeast (Smith *et al.*, 1998; Clarke *et al.*, 1999), we used a strain in which the *ESA1* ORF was deleted, and carried either a wild-type or a temperature-sensitive allele of *ESA1* on a plasmid. The mutation is a frameshift at amino acid 414 of Esa1p (Clarke *et al.*, 1999). Mutant and wild-type strains were grown at permissive temperature up to early log phase. At that time half of each culture was shifted to the non-permissive temperature and incubations were continued for 4 h. As expected, the temperature-sensitive culture placed at 37°C rapidly stopped growing while the wild-type culture was unaffected (data not shown; Clarke *et al.*, 1999). Whole cell extracts were prepared and fractionated over nickel–agarose resin. Equivalent amounts of proteins from each growth condition were then fractionated over the monoQ column and assayed for HAT activity (Figure 4A). When the wild-type strain was analyzed, the typical successive elution of

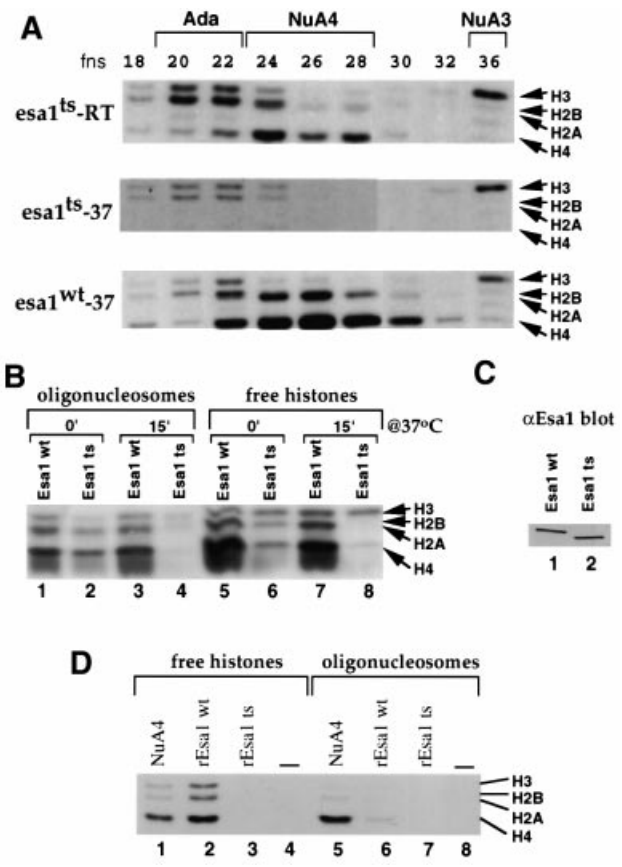

Fig. 4. Temperature-sensitive mutation of Esa1p destroys NuA4 HAT activity at the non-permissive temperature. (**A**) *In vivo* temperature sensitivity of NuA4 in an *Esa1-414* ts strain. Gel HAT assays of monoQ fractions prepared from yeast strains producing wild-type or ts-Esa1p. Equivalent amounts of proteins for corresponding fractions in all strains/growth conditions were assayed; incubation was at room temperature (RT). Cultures were grown at RT to exponential growth and were then put at 37°C or RT for 4 h as indicated. Ada and NuA3 activity are given as internal controls. Note that NuA4 in the ts strain grown at RT (upper panel) is already 10 times less active than NuA4 from the wt strain (lower panel). Shifting the ts strain to 37°C provokes the loss of NuA4 activity while the other two complexes are unaffected (middle panel). (**B**) *In vitro* temperature sensitivity of NuA4 in fraction purified from an *esa1*-ts strain. MonoQ peak fractions from *ESA1* wt and ts mutant strains grown at RT were incubated at the non-permissive temperature for the time indicated before performing the HAT assay at RT on both oligonucleosomes (lanes 1–4) and free histones (lanes 5–8) substrates. Three times more ts fraction was used than wt to achieve closer levels of NuA4 activity. Note that, while NuA4 activity is specifically lost in the ts-Esa1 mutant fraction by treatment at 37°C, weaker H3-specific HAT activity coming from contaminating Ada complex remains unaffected (lane 8 versus 6). (**C**) Anti-Esa1p Western blotting shows that the amounts of wt and ts Esa1p are comparable between monoQ fractions; thus the NuA4 complex is not physically destabilized by the mutation. The same total amounts of proteins were loaded from monoQ fraction 26 of *ESA1* wt and ts mutant strains grown at RT. Note that ts-Esa1p (Esa1-414p) migrates more quickly than the wt because of the frameshift mutation making it 22 amino acids shorter. (**D**) Comparison of NuA4 activity of bacterially produced Esa1p and Esa1-414p. Clarified bacterial extracts containing approximately the same amount of recombinant Esa1 proteins were incubated with 1 μg of HeLa free histones or oligonucleosomes in HAT assay conditions. Unlike purified NuA4 (after five columns, see Figure 5A), recombinant Esa1p is not able to acetylate H4 in chromatin. Also the ts-mutant is completely inactive in bacteria even on free histones, suggesting that another subunit(s) of NuA4 gives Esa1p chromatin capabilities.

Ada, NuA4 and NuA3 HAT complexes was seen (Figure 4A, lower panel). The ts-*esa1* mutant strain grown only at room temperature gave similar results, except for the fact that NuA4 HAT activity was weaker while Ada and NuA3 were equivalent to the activity detected in the wild-type strain (Figure 4A, upper panel). When the extract was prepared with the mutant strain that had been shifted to 37°C, Ada and NuA3 activities were again unchanged compared with the permissive growth condition or the wild-type strain (middle panel). However, no NuA4 activity was detected. This demonstrates clearly the essential role of Esa1p for the nucleosomal HAT activity of NuA4. The variation in NuA4 activities between the wild-type and mutant strains was not due to different levels of episomal Esa1 protein expression, since Western analysis with anti-Esa1p serum showed equivalent amounts of protein between samples (Figure 4C). Also the equivalent mutation in a bacterially expressed recombinant Esa1p was shown to destroy HAT activity on free histones (Figure 4D, lanes 2 and 3; Clarke *et al.*, 1999). In fact, we could not further purify the NuA4 complex from the ts-*esa1* mutant strain grown at room temperature since the weak remaining HAT activity was highly unstable and lost after only a couple of freeze–thaw treatments (data not shown).

The *in vivo* inactivation of Esa1p indicates that it is directly or indirectly necessary for NuA4 activity *in vivo*. In fact, no Esa1p could be detected by Western blotting in the fractions prepared for the mutant strain grown at non-permissive temperature (data not shown). To distinguish between these possibilities we tested whether the temperature sensitivity of the mutant Esa1p could be reproduced on the native protein *in vitro*, using partially purified fractions. We used NuA4 monoQ fractions from Esa1 wild-type or mutant strains grown at room temperature and incubated them at 37°C for 15 min before performing the HAT assay (Figure 4B). Using the wild-type fraction, no obvious decrease of NuA4 HAT activity was detected after treatment at 37°C (Figure 4B, compare lanes 1 and 3). In contrast, the ts-Esa1 fraction completely lost its NuA4 HAT activity after the same treatment at 37°C (Figure 4B, compare lanes 2 and 4). Since the mutant NuA4 complex could be falling apart during the heat treatment as suggested by the *in vivo* data, we performed the same assay using free histones to determine if other HAT proteins were present in the complex (Figure 4B, lanes 5–8). This is based on previously published data showing that monomeric HAT proteins can acetylate free histones easily (e.g. Gcn5p, Hat1p, Esa1p, p300/CBP, p/CAF, TAFII250, SRC-1) while they may need to be part of a native complex to work efficiently on chromatin (e.g. Esa1p in NuA4, see Figure 4D; and Gcn5p in SAGA/Ada, see Grant *et al.*, 1997). NuA4 activity on free histones in the mutant fraction was also lost upon heat treatment while the wild-type complex was unaffected (compare lanes 6 with 8 and 5 with 7). As a convenient internal control, the weaker H3-specific HAT activity present in the same fraction (from contaminating Ada complex) remained unaffected by the heat treatment. Taken together, the data obtained with the anti-Esa1p antibodies along with the effect of Esa1 ts-mutant on NuA4 activity, demonstrate the function of Esa1p as a true nucleosomal HAT within the NuA4 complex. More-

over, the essential requirement of functional Esa1p for both nucleosomal and free HAT activity from the native NuA4 complex strongly argues that it is the primary catalytic subunit of NuA4.

Since NuA4 binds to the nickel column even without the presence of a His-tagged protein in the complex, it was possible that a portion of cellular Esa1p was not associated with NuA4 and would not bind to the column (we cannot detect Esa1p by Western blotting on whole cell extract, data not shown). We answered that question by using a strain expressing a His-tagged version of Esa1p (i.e. all Esa1p molecules would bind to the nickel column). Since we did not see any new Esa1p Western blot signal or stronger NuA4 activity appearing in our subsequent fractions, we concluded that all cellular Esa1p is associated with the NuA4 complex (data not shown). As Esa1p is required for cell cycle progression, this makes NuA4 the first essential nucleosomal HAT identified in yeast.

Unlike NuA4, recombinant Esa1p is unable to acetylate H4 in nucleosomes under these conditions (Figure 4D, compare lanes 1 and 5 with 2 and 6) (Smith *et al.*, 1998; Clarke *et al.*, 1999). This suggests that other proteins in the NuA4 complex give nucleosomal capabilities to Esa1p HAT activity by presenting the H4/H2A N-termini from within nucleosomes. Obvious candidates for this kind of histone binding activity were Hat2 and Cac3/Msi1 proteins, respectively subunits of the cytoplasmic HAT complex and the chromatin assembly factor-1 complex, that were proposed to be involved in histone binding (Parthun *et al.*, 1996; Kaufman *et al.*, 1997). We purified NuA4 from yeast strains bearing wild-type and disrupted *HAT2* or *MSI1* genes, but failed to detect any effect on NuA4 nucleosomal HAT activity (data not shown). In agreement with this, it was recently suggested that Hat2 and Cac3/Msi1 are involved in binding core histones in their soluble form, whereas nucleosomal DNA inhibited this binding (Verreault *et al.*, 1998).

To demonstrate further the association of Esa1p with NuA4 and to visualize its subunit complexity, we purified the complex over seven columns (Figure 5A). We found continued co-fractionation of Esa1p with the HAT activity of NuA4 over all these columns. Figure 5B shows the protein profile of the NuA4-containing fractions from the final column, heparin–Sepharose. No further purification was obtained by binding NuA4 to glutathione *S*-transferase (GST)–H4 tail or GST–VP16 resins (data not shown). As expected from the large size of the complex, there were numerous polypeptides in the most purified fractions. Importantly, many of the NuA4 co-eluting bands appear to be present in similar amounts, including a band of the predicted size of Esa1p (55 kDa) which corresponds to the Western blot signal (arrowhead in Figure 5B) and follows the H4-specific HAT activity.

Essential ATM-related cofactor Tra1p is a component of NuA4

Interestingly, a very high molecular weight protein was detected in the most purified fraction (asterisk in Figure 5B). Only a few yeast ORF could produce that size of a protein (~400 kDa). The SAGA complex was very recently shown to contain Tra1p, an essential ATM-related 433 kDa protein homologous to human TRRAP (transformation/transcription domain-associated protein; 27.3% identity/58.9% similarity), a necessary cofactor for c-Myc or E2F-mediated cell transformation (Grant *et al.*, 1998b; McMahon *et al.*, 1998; Saleh *et al.*, 1998). Using a Tra1p antiserum, we determined whether the 400 kDa band in the heparin fraction was in fact Tra1p. As shown in the lower panel of Figure 5B, Tra1p is indeed the high molecular weight band and is perfectly co-eluting with NuA4 activity and Esa1p. Since the serum was raised against the N-terminal peptide sequence of Tra1p, potential cross-reactivity with other ATM-related proteins is avoided (Grant *et al.*, 1998b). The region of homology of Tra1p and its human homolog to other ATM/PI-3 kinase-related proteins is limited to ~300 amino acids at the C-terminus (McMahon *et al.*, 1998). Interestingly, the amino acids required for the PI-3 related kinase activity within this region are not present in Tra1p or TRRAP (McMahon *et al.*, 1998).

Strict co-elution of Tra1p with Esa1p and NuA4 activity was confirmed also on earlier columns including DNA–cellulose, histone–agarose and Superose 6 (Figure 5C). Physical interaction of Tra1p with NuA4 was confirmed by immunoprecipitation experiments (Figure 6). Affinity-purified Esa1p IgG was produced and cross-linked to protein A–Sepharose. A partially purified NuA4 fraction (Ni^{2+}–agarose/monoQ/Superose 6 fraction 20) was pre-cleared and then incubated with the αEsa1p resin. After several washes, equivalent amounts of initial sample, bead supernatant (FT) and beads were tested for the presence of Esa1p and Tra1p by Western blotting (Figure 6A). As expected from the 80–90% depletion of NuA4 activity (data not shown), the Esa1p signal seems almost completely recovered in the bound fraction (Figure 6A, compare lane 3 versus lane 1, lower panel). Strikingly, Tra1p signal follows exactly the same pattern, where all the initial signal is found in the Esa1p-bound fraction. This shows that all the Tra1p present in this fraction has a direct or indirect interaction with Esa1p. To confirm further this interaction, we performed the reciprocal co-immunoprecipitation experiments. We prepared Superose 6 NuA4 fractions (as in Figure 6A) from yeast strains expressing natural Tra1p or an N-terminal Myc-tagged version, which is capable of sustaining growth (Saleh *et al.*, 1998). When the fractions were incubated with anti-myc beads and then assayed for NuA4 activity, very different results were obtained (Figure 6B). With the untagged fraction (Figure 6B, compare lanes 1–3) all NuA4 activity stayed in the supernatant fraction as expected. On the other hand, using the myc-tagged fraction, the majority of NuA4 activity disappeared from the supernatant and was recovered on the anti-myc beads (Figure 6B, compare lanes 4–6; note that a HAT activity physically linked to beads can be underestimated compared with an equivalent fully soluble one). The remaining NuA4 activity present in the supernatant is most likely explained by the presence of endogenous untagged Tra1p expressed by the cell (myc–Tra1p is expressed by a centromeric plasmid in a wild-type background) (Saleh *et al.*, 1998). These immunoprecipitation experiments confirm the stable association of Tra1p within the NuA4 complex. The relative amount of Tra1p in the NuA4 complex versus the SAGA complex could not be addressed. This was due to the fact that Tra1p seems to be part of at least two other complexes

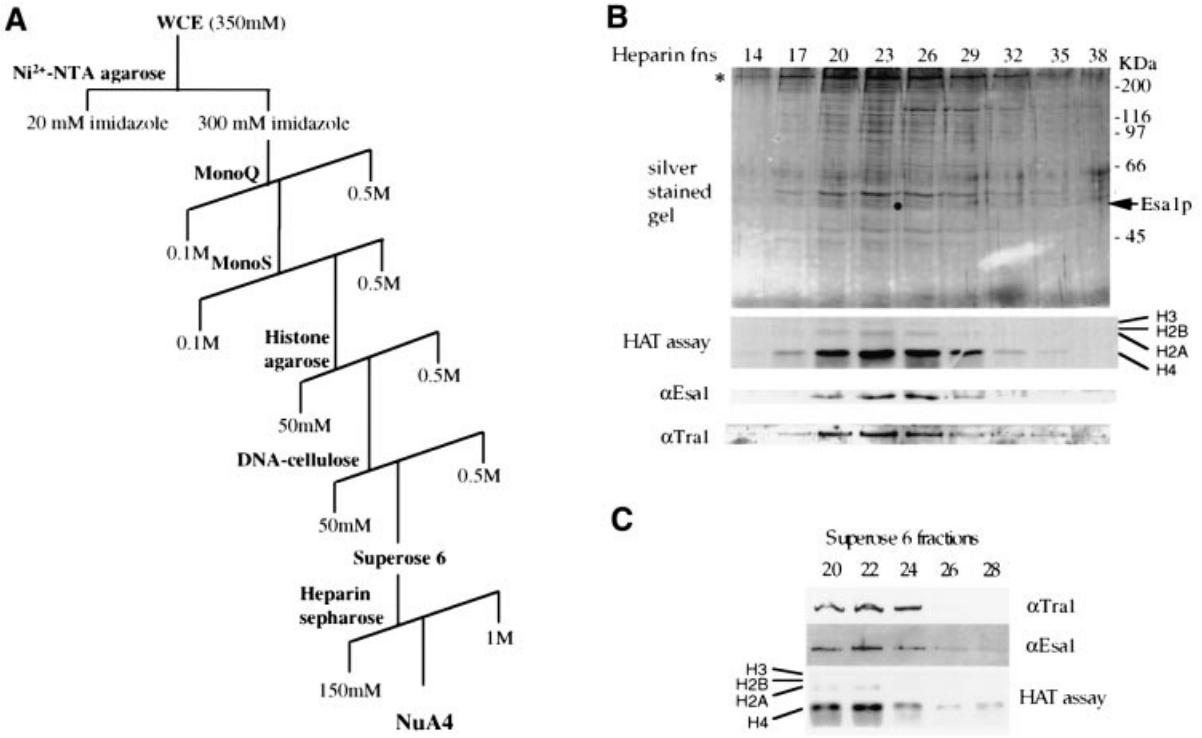

Fig. 5. Purification of the NuA4 complex. (**A**) The NuA4 complex purification scheme with seven chromatographic steps. (**B**) Silver stained SDS–PAGE, fluorogram of the HAT assays on oligonucleosomes and anti-Esa1p and anti-Tra1p Western blot analysis of the fractions from the last column, heparin–Sepharose. Twenty-four microliters of the fractions were used for gel staining while 15 and 2 μl were used for Western blot and HAT assay, respectively. Based on the Western signal, the position of the 55 kDa Esa1p band is shown by a closed circle and an arrow. The asterisk shows the co-eluting 400 kDa band corresponding to the Tra1p Western signal. Positions of the molecular weight markers are indicated. (**C**) Gel HAT assay and Western blots on the sixth column (Superose 6) to show that the size of the NuA4 complex is not changed during the purification and that Esa1p and Tra1p are again closely co-eluting.

also binding to the nickel and monoQ columns, and partially co-eluting with NuA4 (data not shown).

To further characterize the subunit composition of NuA4, the immunoprecipitations were scaled up to visualize true Esa1p-associated polypeptides. NuA4 fraction from Superose 6 as in Figure 6A was incubated with affinity-purified Esa1p antibodies cross-linked to protein A beads. After binding, the beads were washed extensively in 350 mM NaCl. The bound complex was eluted with 100 mM glycine–HCl pH 2.5 and loaded on a SDS–polyacrylamide gel (Figure 6C). This allowed us to visualize a complete set of Esa1p-interacting polypeptides, subunits of the NuA4 complex. Importantly, all protein bands appear in equivalent amounts, including Esa1p and Tra1p as indicated. This experiment was repeated several times using NuA4 fractions at different stages of conventional purification and the same protein bands in equivalent stoichiometry were repeatedly obtained. Migration over a gradient gel showed that p59 seems to contain in fact two different proteins, which explains the apparently stronger signal in Figure 6C (data not shown). A shorter migration showed at least two more subunits of smaller molecular weights (p32 and p36, data not shown). These 11 bands add up to >1 MDa in good agreement with NuA4 size determined by gel filtration. Finally, the presence of both Tra1p and Esa1p in affinity-purified NuA4 complex (as in Figure 6C) was further confirmed by a first attempt at mass spectrometry analysis. MS/MS peptide sequences were obtained from trypsin digestion and included five peptides derived from Tra1p (amino acids 150–164, 603–617, 798–813, 3217–3236 and 3529–3545) and three peptides derived from Esa1p (amino acids 98–118, 334–350 and 351–366).

A fully functional Esa1p is required for NuA4-mediated stimulation of transcription

Recently, we presented biochemical evidence that interactions between two native HAT complexes, SAGA and NuA4, and the VP16 activation domain target GAL4–VP16-bound nucleosomes for acetylation (Utley *et al.*, 1998). Moreover, GAL4–VP16-driven transcription from chromatin templates was stimulated by both HAT complexes in an acetyl-CoA-dependent manner. These data showed transcription activation domain targeting of NuA4 and its participation as nucleosomal acetyltransferase in transcription activation. Importantly, stimulation of transcription by the NuA4 complex was only apparent on nucleosomal templates (Utley *et al.*, 1998) and can be targeted by the VP16 activation domain (Ikeda *et al.*, 1999). We further analyzed the role of Esa1p in the transcriptional stimulatory activity of the NuA4 complex. Figure 7A shows an *in vitro* transcription assay by primer extension using a naked DNA HIV promoter as internal control and a chromatin reconstituted E4 promoter containing five GAL4 binding sites. Since the E4 promoter containing five GAL4 sites is within a spaced array of 5S rDNA nucleosome positioning sequences, the reconstituted template has two nucleosomes formed over the promoter region, in phase with the repeated 5S nucleosomes (Utley *et al.*, 1998). GAL4–VP16 was present in all the lanes and,

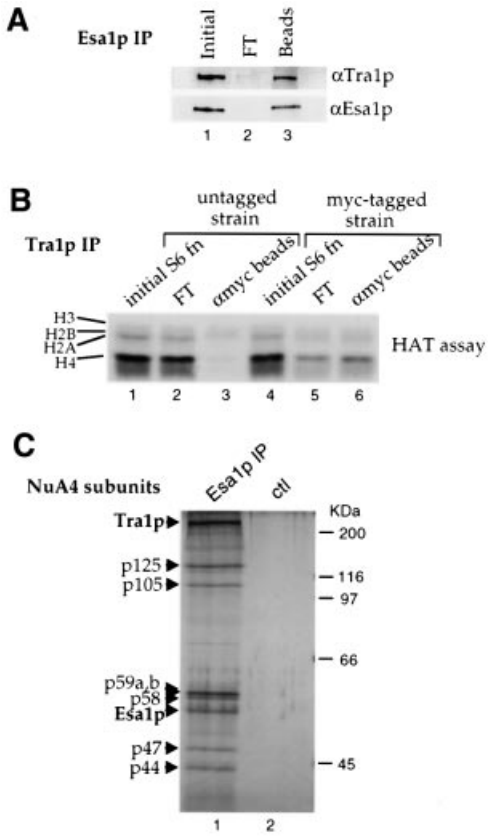

Fig. 6. Tra1p is a component of the purified NuA4 complex. (**A**) Anti-Esa1p antibodies co-immunoprecipitate Tra1p. Affinity purified anti-Esa1p antibodies were used to prepare an immunoaffinity resin. Pre-cleared partially purified NuA4 complex (Ni^{2+}–agarose/monoQ/Superose 6 fraction 20) was incubated with the resin and beads were then washed twice with 500 mM NaCl and twice with 150 mM NaCl. Equivalent amounts of initial, bound (beads) and unbound (FT) fractions were analyzed by Western blotting using Esa1p and Tra1p antiserum. (**B**) Antibodies against Tra1p specifically immunoprecipitate NuA4 activity. Superose 6 NuA4 fractions as in (A) were prepared from yeast strains FY23 and KY320 expressing an episomal myc-tagged version of Tra1p. Pre-cleared Superose 6 NuA4 fractions were incubated with anti-myc–protein G–Sepharose for 2.5 h at 4°C, washed several times and equivalent amounts of initial, unbound (FT) and bound (αmyc beads) fractions were tested for nucleosomal HAT activity. NuA4 activity was recovered on the beads from the myc-Tra1p-expressing strain (lane 6), while all NuA4 activity remained in the supernatant when using the wild-type Tra1p-expressing strain (compare lanes 1 and 2). (**C**) Pre-cleared Superose 6 NuA4 fraction as in (A) was incubated with affinity-purified anti-Esa1p–protein A–Sepharose protein A. Beads were then washed twice with 300 mM and twice with 150 mM NaCl. The bound NuA4 complex was eluted with 100 mM glycine–HCl pH 2.5, neutralized and precipitated with TCA. The sample was then loaded on a 10% SDS–polyacrylamide gel followed by silver staining. Positions of Esa1p, Tra1p and seven other protein bands that were reproducibly obtained from several independent experiments are indicated by arrowheads. Positions of molecular weight marker bands are also indicated (kDa).

as expected, was able on its own to produce a low level of transcription from chromatin template (Figure 7A, lanes 3 and 4). However, GAL4–VP16-driven transcription was greatly stimulated by the NuA4 complex (Figure 7A, lane 2). Importantly, this NuA4-dependent stimulation required the presence of acetyl-CoA, indicating the importance of the acetyltransferase activity (Figure 7A, compare lanes 1 and 2). On the other hand, recombinant Esa1p is unable to stimulate transcription in this assay, again

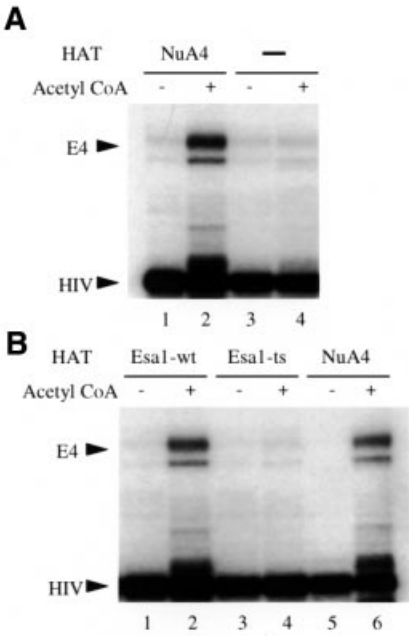

Fig. 7. Chromatin-specific and acetyl-CoA-dependent stimulation of transcription by NuA4 requires a fully functional Esa1p subunit. (**A**) *In vitro* transcription assay of a chromatin-reconstituted E4 promoter bearing five GAL4 sites. Neighboring 5S rDNA nucleosome-positioning sequences force the formation of in-phase positioned nucleosomes on both GAL4 sites and E4 promoter and downstream sequence (Utley *et al.*, 1998). Unreconstituted HIV LTR transcription is shown as control. Five nanomolar GAL4–VP16 is present in all lanes. Acetyl-CoA is present in lanes 2 and 4 while 1 μl of purified NuA4 (fifth column) is present in lanes 1 and 2. Note the acetyl-CoA dependence of NuA4 stimulation of the E4 transcript. (**B**) Comparison of the NuA4 monoQ fractions obtained from the *ESA1* wt and ts strains. *In vitro* transcription was performed as in (A). The NuA4 monoQ peak fraction from the ESA1 wild-type strain (1 μl, lanes 1 and 2) is able to stimulate transcription on the chromatin template as efficiently as the purified NuA4 complex (lanes 5 and 6). In contrast, even three times more proteins from the NuA4 monoQ peak fraction from the *esa1* temperature-sensitive mutant strain is not able to stimulate transcription (4 μl, lanes 3 and 4).

supporting the need for other subunits in NuA4 for efficient targeting and action on chromatin (data not shown; Ohba *et al.*, 1999). We then used the NuA4 monoQ fraction prepared from the yeast strain with episomal expression of wild-type *ESA1* gene. The level of acetyl-CoA-dependent stimulation of transcription by this NuA4 fraction was very similar to the one obtained with purified natural NuA4 (Figure 7B, compare lanes 1and 2 with 5 and 6). In contrast, when a NuA4 fraction obtained from the temperature-sensitive *esa1* mutant strain was used, no obvious stimulation of transcription was detected (Figure 7B, lanes 3 and 4). Since the amounts of Esa1 protein present in the different NuA4 fractions used in these studies were very similar (see Figure 4C), we can conclude that a fully functional Esa1 acetyltransferase subunit was required for NuA4 to stimulate transcription in this assay.

Discussion

NuA4 acetylates the H4 N-termini of nucleosome cores through its catalytic subunit Esa1p

There is a striking correlation between the presence of acetylated lysines in core histone N-termini and transcrip-

tional activity of chromatin (Turner and O'Neill, 1995). The discovery of multiple yeast HATs having different core histone substrate specificities may contribute to the distinct functions of different histone tails in transcription activation and repression *in vivo* (Fisher-Adams and Grunstein, 1995). Acetylation of histone tails is thought to reduce interactions of transcriptional repressors with nucleosomes (Hecht *et al.*, 1995; Edmonson *et al.*, 1996), and to enhance binding of transcriptional activators or basal transcription factors (Imbalzano *et al.*, 1994; Vettese-Dadey *et al.*, 1996). The fact that H4 N-terminal acetylation is the histone modification most frequently implicated in those proposed mechanisms suggested the presence of a very important nucleosomal H4-specific HAT *in vivo*. *In vitro* binding experiments have even demonstrated that the transcription activator USF binds preferentially to acetylated H4-containing nucleosomes compared with those containing acetylated H3 (Vettese-Dadey *et al.*, 1996). In our initial fractionation of native yeast nucleosomal HATs we found three different activities targeting mainly H3 tails but only one preferentially acetylating H4 in chromatin, NuA4 (Figure 1A) (Grant *et al.*, 1997).

The discovery that the Esa1 protein functions as a HAT preferentially modifying H4 tails in free histones (Smith *et al.*, 1998; Clarke *et al.*, 1999) suggested that it might be involved in NuA4 activity. Esa1p is the first essential HAT and clearly functions in the cell cycle (Clarke *et al.*, 1999). It is a member of the MYST family of proteins, which includes putative acetyltransferases Sas2p and Sas3p, which affect gene silencing (Reifsnyder *et al.*, 1996), and MOZ, which is involved in a chromosomal translocation resulting in a form of acute myeloid leukemia (Borrow *et al.*, 1996). Two other members of this family have even stronger homology with Esa1 by sharing a chromo-like domain, identified in several proposed chromatin-binding transcription regulators (Koonin *et al.*, 1995). Tip60 was cloned by two-hybrid screening with HIV Tat transactivator and was recently shown to acetylate free histones but not chromatin *in vitro*, with a specificity very similar to Esa1p (Yamamoto and Horikoshi, 1997). MOF was discovered in a genetic screen for genes necessary for dosage compensation in *Drosophila* (Hilfiker *et al.*, 1997). It is thought to be part of the dosage compensation complex and is required for male X chromosome hypertranscription and its associated H4 acetylation at Lys16. Significantly, the *mof* mutant obtained in the screen contained a single point mutation in the putative HAT domain of the protein (Hilfiker *et al.*, 1997).

NuA4 is an essential transcription coactivator complex

Although recombinant Esa1p is a potent acetyltransferase of free histones, it has little ability to access and/or modify core histones associated with DNA in oligonucleosomes, the expected relevant substrate for nuclear HATs (Figure 4D) (Smith *et al.*, 1998). In this report we show that Esa1p functions as a nucleosomal HAT in the context of a native multisubunit high molecular weight complex, i.e. NuA4. Biochemical data have illustrated targeting of NuA4 activity by transcription activators (Gcn4p, VP16) and that it stimulated transcription on chromatin templates (Steger *et al.*, 1998; Utley *et al.*, 1998; Ikeda *et al.*, 1999). These data, along with the homology of Esa1p with transcription regulators and the colocalization of acetylated H4 with transcriptionally competent chromatin, strongly support a primary role for NuA4 in transcription mechanisms *in vivo*. The importance of NuA4 is further emphasized by the fact that the NuA4 catalytic subunit, Esa1p, is essential for cell growth (Smith *et al.*, 1998; Clarke *et al.*, 1999). Such an important role in transcription implies that NuA4 might be generally involved in the activation of a great number of yeast genes, or in a subset of genes whose expression is essential for cell cycle progression. A recent study also suggests that the acetyl-CoA binding domain of Esa1p is necessary for Esa1p function *in vivo* (Clarke *et al.*, 1999), as it is in the case of Gcn5p (Kuo *et al.*, 1998; Wang *et al.*, 1998). Temperature-sensitive *esa1* mutant strains replicate their DNA but fail to proceed normally through mitosis in a manner dependent on the *RAD9* checkpoint gene, a phenotype similarly obtained by mutations of H4 N-terminal lysines (Megee *et al.*, 1995; Clarke *et al.*, 1999).

The specificity of NuA4 activity is also shared by a very recently identified nucleosomal HAT in *Tetrahymena*, which seems to work as a single 80 kDa polypeptide (Ohba *et al.*, 1999). The ability of NuA4 to fully modify the H4 and H2A N-termini in chromatin could have several structural effects on chromatin. NuA4 increases the cutting efficiencies of restriction endonucleases on HIV-1 chromatin *in vitro*, independently of transcription (Steger *et al.*, 1998). The Tup1 repressor protein was shown to bind preferentially to underacetylated forms of H4 (Edmonson *et al.*, 1996) and acetylation of H4 Lys16 is thought to destabilize interaction with Sir3, another repressor protein (Hecht *et al.*, 1995). USF was shown to preferentially bind nucleosomes with H4 tails bearing acetylated Lys5 (Vettese-Dadey *et al.*, 1996). Indeed, specific acetylation of this lysine seems to be decreased in the temperature-sensitive *esa1* mutant strains at non-permissive temperature (Clarke *et al.*, 1999). The X-ray crystal structure of the nucleosome core particle illustrates that residues 16–25 of H4 tails interact with a negatively charged pocket of the H2A-H2B dimer in the adjacent nucleosome (Luger *et al.*, 1997). This raises the possibility that H4 acetylation at Lys16 (and probably Lys5, 8, 12) could have a negative effect on internucleosomal interactions. The acetylation of the histone H2A N-terminal tail has been relatively poorly studied. Nevertheless, this domain of H2A seems to bind DNA at two defined locations within nucleosome cores, centered around a position 40 bp from the nucleosomal dyad (Lee and Hayes, 1997). The defined structure adopted by this tail and its interaction with DNA would certainly be greatly affected by acetylation of its lysine residues.

Potential recruitment of NuA4 through its essential Tra1p subunit

We identified Tra1p as a stably associated subunit of the NuA4 complex. Like Esa1p, Tra1p is essential for cell growth (Saleh *et al.*, 1998). It is an ATM/PI-3 kinase-related protein homologous along its entire length to the human protein TRRAP, a cofactor interacting with c-Myc and E2F transactivation domains and essential for their oncogenic activities (McMahon *et al.*, 1998). Unlike the other members of the ATM superfamily, Tra1p does not seem to have the specific motifs conserved in the catalytic

site of PI-3 kinases, but this does not rule out such activity. Tra1p is also a component of the SAGA and SAGA-like complexes (Grant *et al.*, 1998b; Saleh *et al.*, 1998) and TRRAP is part of the homologous human P/CAF complex (Vassilev *et al.*, 1998). Tra1p could have a structural role in multisubunit complexes as suggested by the fact that it interacts separately and independently with Spt7p and Ada2p in the SAGA complex (Saleh *et al.*, 1998). More importantly, the transcription activator-binding abilities of its mammalian homolog suggest that it might play a similar role in yeast multisubunit complexes. This could explain the fact that both SAGA and NuA4 complexes interact with VP16 and GCN4 transcription activation domains *in vitro* while Ada and NuA3 complexes do not (Utley *et al.*, 1998). Interactions with transcription activators promoting cell cycle progression (as c-Myc and E2F) are definitely plausible in the light of the cell cycle phenotype of temperature-sensitive *esa1* strains (Clarke *et al.*, 1999). The need for Tra1p for cell growth might reflect primarily its presence in NuA4 since the HAT subunit of SAGA, Gcn5p, is non-essential, like several other subunits (Ada, Spt). This will be best understood by analyzing temperature-sensitive mutants of the *TRA1* gene.

In conclusion, we have purified and characterized a native transcription-related nucleosomal H4 HAT complex, NuA4. Identification of its primary catalytic subunit as essential Esa1 protein supports a necessary link between histone H4 acetylation in chromatin, transcriptional activation and cell cycle control. Furthermore, the presence of Tra1p in NuA4 suggests a role for this cofactor in transcriptional regulation through the recruitment of NuA4 HAT activity to an activator-bound promoter important for cell cycle progression. Tra1p being a member of the ATM superfamily with similarity to PI-3 kinases suggests a possible function in transduction of stimulatory and/or inhibitory signals. Identification of other subunits of this 1.3 MDa complex will provide further important insights into its regulation and role in gene expression linked to cell cycle control.

Materials and methods

Yeast strains and reagents

The yeast strain CY396 has been described (Peterson *et al.*, 1994). The yeast strains LPY3317 and LPY3123 with episomal wild-type *ESA1* or temperature-sensitive mutant *esa1-414* in an *esa1* null mutant genomic background are described elsewhere (Clarke *et al.*, 1999). Recombinant wild-type Esa1p and mutant Esa1-414p were produced in *Escherichia coli* as described (Clarke *et al.*, 1999). The yeast KY320 strain expressing an N-terminal myc-tagged version of the *TRA1* gene on a centromeric plasmid has been described (Saleh *et al.*, 1998).

HeLa core histones and H1-depleted oligonucleosomes were isolated as described (Côté *et al.*, 1995). Yeast core histones were generously provided by Dr Sharon Roth, University of Texas MD Anderson Cancer Center and prepared as described (Edmonson *et al.*, 1996). Sera against a peptide corresponding to the N-terminal first 14 amino acids of the yeast Esa1 protein were produced in rabbits. Partial purification of the IgG fraction from the sera was performed by ammonium sulfate precipitation and chromatography over CM-Affigel-blue resin (Bio-Rad). For immunopurification of the NuA4 complex, anti-Esa1p antibodies from partially purified serum were obtained by affinity-purification over a resin (Sulfolink, Pierce) harboring the immunizing peptide following standard protocols (Harlow and Lane, 1988). Anti-HA antibody for Swi2p detection was purchased from Boehringer Mannheim. Anti-Ada2p serum was kindly provided by Dr Shelley Berger, The Wistar Institute. Anti-Tra1p serum produced against the N-terminal first 20 amino acids of the protein has been described (Grant *et al.*, 1998b). Anti-myc monoclonal antibodies were obtained from Babco.

HAT assays

Liquid HAT assays were performed essentially as described (Grant *et al.*, 1997) using 0.5 μg of free core histones or oligonucleosomes, protein fractions and 0.125 μCi of [^{3}H]acetyl-CoA (4.7 Ci/mmol) in HAT buffer [50 mM Tris–HCl pH 8.0, 50 mM KCl, 0.1 mM EDTA, 5% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium butyrate] for 30 min at 30°C. Quantification was performed by spotting the reactions on Whatman P81 filters and liquid scintillation counting as described (Brownell and Allis, 1995). Visualization of histone specificity was obtained by loading the reactions on an 18% SDS–polyacrylamide gel, followed by Coomassie Blue staining to ensure equivalent loading of histones in each lane, destaining and fluorography with Enhance (DuPont NEN). Assays with H4 N-terminal peptides were performed with 70 ng and quantified by scintillation. The wild-type peptide sequence is SGRG**K**GG**K**GLG**K**GCA**K**RHRRVLRDC. Lysine residues in bold were replaced by acetylated lysines in the mutant peptides to allow only one remaining free lysine in each peptide.

For the TAU gel in Figure 2B, HAT assays were performed using 8 μg of oligonucleosomes with 0.075 μCi of [^{14}C]acetyl-CoA (51 mCi/mmol) at 30°C for 1 h. Electrophoresis was performed as described (Braunstein *et al.*, 1993), the gel was Coomassie Blue stained, dried and exposed.

Purification of the NuA4 HAT complex

Preparation of whole-cell extract from 20 l of yeast strain CY396 and fractionation over Ni^{2+}–NTA agarose (Qiagen) and a monoQ HR 5/5 column (Pharmacia) were described (Côté *et al.*, 1994; Grant *et al.*, 1997). Typically 1 μl of fraction was tested for HAT activity on 0.5 μg of oligonucleosomes for 30 min at 30°C. Peak nucleosomal H4/H2A acetyltransferase activity from the salt gradient elution from the monoQ was pooled (fractions 24–28), diluted to 100 mM NaCl with buffer A (20 mM HEPES 7.5, 0.5 mM DTT, 0.1% Tween-20, 10% glycerol and protease inhibitors) and loaded directly on a monoS HR 5/5 column (Pharmacia). Bound proteins were eluted with a 20 ml linear gradient of 100–500 mM NaCl. Peak fractions of NuA4 activity were pooled, diluted to 50 mM NaCl and loaded on a 1 ml histone–agarose column (Sigma). Bound proteins were eluted with a 10 ml linear gradient of 50–500 mM NaCl. Peak fractions of NuA4 activity were pooled, diluted to 50 mM and loaded directly on a 1 ml DNA–cellulose column (Sigma). NuA4 peak fractions from a 10 ml 50–500 mM NaCl gradient were pooled and concentrated down to 0.6 ml with a Centriprep-30 concentrator (Amicon). The sample was then split and run successively on a calibrated Superose 6 HR 10/30 column (Pharmacia) in buffer A plus 350 mM NaCl and 20% glycerol. Pooled NuA4 fractions (19–23) were then diluted to 150 mM NaCl and loaded directly on a 1 ml heparin–Sepharose column (Pharmacia). Purified NuA4 was then eluted with a 7.5 ml linear gradient from 0.15 to 1 M NaCl. Fractions (0.25 ml) were supplemented with 50 μg/ml of insulin, frozen in liquid nitrogen and stored at –80°C.

Whole-cell extracts were prepared from 2–4 l of other yeast strains grown as described in the text, bound to Ni^{2+}–NTA agarose, eluted with 300 mM imidazole and fractionated on monoQ and Superose 6 columns as described for the larger cultures (Grant *et al.*, 1997).

Western blotting and immunoprecipitations

Aliquots of 10–15 μl of fractions were typically used for Western blotting after electrophoresis on a 10% SDS–polyacrylamide gel. Immunoblotting with anti-Esa1p partially purified serum was carried out as a 1:3000 dilution in 1% non-fat dry milk in PBS–0.1% Tween-20 for 4 h at room temperature. For immunoprecipitation studies, partially purified antisera were cross-linked to protein A–Sepharose resin (Pharmacia) with dimethylpimelimidate (Pierce) following standard protocols (Harlow and Lane, 1988). Samples were diluted in buffer B (40 mM HEPES pH 7.5, 10% glycerol) plus 150 mM NaCl, 1% Triton buffer and precleared with protein A–Sepharose. Precleared supernatant was incubated with preimmune or immune cross-linked beads for at least 2 h at 4°C as a 30% slurry. After incubation, beads were washed twice with each of the following buffers: buffer B containing 150 mM NaCl–1% Triton then buffer B containing 350 mM NaCl and finally buffer B with 150 mM NaCl. HAT assays were performed directly on equivalent amounts of supernatant or resuspended beads. For the immunoprecipitations using anti-myc monoclonal antibodies (Figure 6B), protein G–Sepharose was used instead of protein A–Sepharose. For immunopurification of the

NuA4 complex shown in Figure 6A and C, affinity-purified anti-Esa1p antibodies were used (0.5–1 mg Ab/ml of resin) and the complex was eluted by resuspending the beads in 100 mM glycine–HCl pH 2.5, neutralized, followed by TCA precipitation and SDS–PAGE. Sequence analysis of the obtained peptides was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (uLC/MS/MS) on a Finnigan LCQ quadrupole ion trap mass spectrometer.

In vitro transcription assay

Protocol and reagents for *in vitro* transcription assays on chromatin templates are described thoroughly elsewhere (Steger *et al.*, 1998; Utley *et al.*, 1998). Reactions containing 15–21 ng of nucleosome reconstituted template DNA were incubated with 5 nM of GAL4–VP16 in the absence or presence of NuA4 complex in final conditions (60 mM NaCl, 10 mM HEPES pH 7.8, 5 mM DTT, 0.5 mM PMSF, 5% glycerol, 0.25 mg/ml bovine serum albumin, 1.25 μM acetyl CoA, 10 mM sodium butyrate) at 30°C for 20 min. HeLa nuclear extracts and nucleoside triphosphates were added and transcription/primer extension assays were carried out according to Steger *et al.* (1998).

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