Human Histone Acetyltransferase GCN5 Exists in a Stable Macromolecular Complex Lacking the Adapter ADA2†

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ABSTRACT: Acetylation of core histones is an important regulatory step in transcriptional activation from chromatin templates. The yeast transcriptional coactivator protein GCN5 was recently shown to be a nuclear histone acetyltransferase (HAT). Genetic and biochemical studies in yeast suggest that GCN5 functions with the adapter proteins ADA1, ADA2, ADA3, and ADA5 in a heteromeric complex. We have established conditions for chromatographic fractionation of HATs and ADA2 from human K562 erythroleukemia cells. Gel-filtration chromatography revealed two populations of GCN5 with Stokes' radii of 67 and 33 Å, consistent with a large macromolecular complex and a monomer, respectively. The GCN5-related HAT, PCAF, was resolved as a stable complex with a Stokes' radius of 74 Å. The HAT complexes were resistant to 0.3 M NaCl and DNase I. ADA2 was characterized by a Stokes' radius of 35 Å, consistent with a monomer. Thus, in contrast to the stable GCN5−adapter complex in yeast, human GCN5 and ADA2 are not *stably* associated with each other. The implications of this result are discussed *vis-a-vis* the mechanism of recruitment of GCN5 to regulatory regions of genes.

The organization of DNA into chromatin plays an important role in transcriptional regulation. The wrapping of the DNA duplex around an octamer of histones to form the nucleosome can selectively occlude the binding of certain transcription factors to cognate DNA recognition sites (*1*). In contrast to the repressive effects of chromatin, nucleosomes can also facilitate transcription by bringing together distant DNA sequences, allowing for protein−protein interactions between transcription factors (*2*, *3*). On the basis of the impact of chromatin structure on protein−DNA interactions, it is not surprising that chromatin-modifying enzymes play a critical role in gene regulation.

The binding of transcription factors to sites occluded by nucleosomes can be facilitated by the multiprotein complex SWI/SNF (*4*). The SWI/SNF complex consists of approximately 12 proteins and appears to have a molecular mass of at least 2 megadaltons (*5*−*7*). Although the exact mechanism of SWI/SNF-induced chromatin remodeling is unknown, it has been proposed that SWI/SNF may enhance histone octamer mobility, thus exposing factor binding sites (*8*).

In addition to SWI/SNF, nuclear type-A histone acetyltransferases regulate chromatin structure and transcription. Acetylation of conserved lysine residues on the amino-terminal tails of core histones has long been correlated with gene activation (*9*). Genetic studies have provided strong evidence that the lysine acetylation sites are important in transcriptional activation (*10*). By neutralizing the positive charge of the lysine residue, acetylation may disrupt electrostatic interactions between the histone tail and the phosphodiester backbone of DNA. Thus, a functional consequence of acetylation can be increased access of transcription factors to nucleosomal recognition sites (*11*, *12*).

Four type-A HATs[1] have recently been identified. Brownell et al. (*13*) discovered a *Tetrahymena* HAT with strong sequence homology to the yeast transcriptional coactivator GCN5. Yeast GCN5 physically interacts with the DNA binding protein GCN4 and is necessary for transcriptional enhancement by GCN4 (*14*). The mammalian coactivator, CBP, which is necessary for transcriptional activation by the cyclic AMP response element binding protein (CREB) (*15*), nuclear receptors (*16*), and certain other transactivators (*17*), was recently shown to be a HAT (*18*). CBP physically associates with a GCN5-related human protein, PCAF, which is also a HAT (*19*). Moreover, a component of the basal transcription machinery, $TAF_{II}250$, has intrinsic HAT activity (*20*). These studies have led to a model in which recruitment of HATs to genes is accomplished through protein−protein interactions with DNA-bound activator proteins (*13*, *18*, *21*). This model assumes that the HATs are recruited as part of large multimeric protein complexes.

Genetic and biochemical studies have provided evidence that yeast GCN5 functions with the "adapter" proteins ADA1, ADA2, ADA3, and ADA5 (*22*−*27*). GCN5 physi-

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[1] Abbreviations: BSA, bovine serum albumin; CBP, CREB binding protein; CREB, cyclic AMP response element binding protein; DTT, dithiothreitol; f-GCN5, Flag epitope-tagged GCN5; f-PCAF, Flag epitope-tagged PCAF; FPLC, fast pressure liquid chromatography; HAT, histone acetyltransferase; LCR, locus control region; PCAF, p300/CBP-associated factor; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; R_s, Stokes' radius.

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cally interacts with ADA2 and ADA3, as shown by binding studies with recombinant GST fusion proteins, the yeast two-hybrid assay, and coimmunoprecipitation. The multimeric GCN5−adapter complex has been postulated to constitute a coactivator that mediates communication between upstream activator proteins and the basal transcription machinery (*28*). An obvious question is whether a major role for HATs is to facilitate loading of the preinitiation complex by disrupting chromatin structure only in the vicinity of a promoter. This seems inconsistent with the finding that heavily acetylated histones are concentrated throughout the entire chicken β-globin locus (*29*).

We previously hypothesized that chromatin-modifying enzymes, such as SWI/SNF and HATs, may be crucial for long-range gene activation by LCRs (*30*, *31*). LCRs are complex genetic elements that are often positioned several thousand bases away from an associated gene. A defining feature of LCRs is their ability to confer position-independent and copy number-dependent gene expression when linked to a gene integrated into chromosomal DNA (*32*). LCRs appear to be necessary for generating transcriptionally active chromosomal domains (*32*, *33*), which requires unfolding of higher-order chromatin structure. Our model is analogous to the GCN4−GCN5 paradigm, in which LCR-bound activators recruit regulatory enzymes necessary for chromatin decondensation. The β-globin LCR contains an abundance of recognition sites for ubiquitous and erythroid-specific factors (*34*−*37*) that could mediate such interactions. Consistent with this model, it was recently reported that the important LCR-binding protein NF-E2 (*38*) physically interacts with recombinant CBP (*39*).

To begin testing the recruitment hypothesis, conditions were established for biochemical fractionation of type-A HATs from human K562 erythroleukemia cell nuclear extracts. These cells are routinely used to study transcriptional activation by the β-globin LCR (*30*, *40*−*43*). Surprisingly, most of the ADA2 was resolved as a monomer by gel-filtration chromatography, while PCAF and GCN5 were present in large macromolecular complexes. Thus, in contrast to the stable GCN5−ADA2 complex in yeast, human GCN5 and ADA2 are not *stably* associated with each other.

EXPERIMENTAL PROCEDURES

Cell Culture. The human erythroleukemia cell line K562 was propagated in Iscove's modified Eagle's medium (Biofluids) containing 10% fetal calf serum (Life Technologies, Inc.), 2 mM glutamine, and gentamycin (25 μg/mL). HeLa cells derived from a human ovarian carcinoma were propagated in Dulbecco's modified Eagle's medium containing 5% fetal calf serum, 4 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cell lines were grown in a humidified incubator at 37 °C, in the presence of 5% carbon dioxide. K562 cells were treated with 20 μM hemin for 72 h before isolation of nuclear extract.

Nuclear Isolation and Preparation of Nuclear Extracts. Nuclear extracts were prepared from cell lines as described previously (*37*, *44*). Briefly, cells were harvested by centrifugation for 15 min at 150*g*. Cells were washed once in 50 volumes of ice-cold phosphate-buffered saline and resuspended in 2 volumes of 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.2% Nonidet P-40, and 5 mM DTT. Cells were lysed with 8 gentle strokes of a type B Dounce homogenizer. Nuclei were collected by centrifugation for 6 min at 600*g*. Nuclei were washed by gently resuspending in 2 volumes of 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, and 5 mM DTT and then collected by centrifugation for 4 min at 600*g*. Nuclei were immediately resuspended in an equal volume of 20 mM HEPES (pH 7.5.), 25% glycerol, 20 mM KCl, 0.2 mM EDTA, and 10 mM DTT, and 1.33 volumes of the same buffer containing 1.2 M KCl was added dropwise. Nuclei were extracted for 30 min at 4 °C with constant rotation. The suspension was then centrifuged for 20 min at 150000*g*. The supernatant was dialyzed against 500 mL of 20 mM HEPES (pH 7.8), 25% glycerol, 100 mM KCl, 0.2 mM EDTA, and 5 mM DTT for 1 h at 4 °C. Aliquots were frozen on dry ice and stored at −70 °C. The protein concentration as measured by the Bradford assay, with γ-globulin as a standard, ranged from 7.2 to 12.1 mg/mL. Phenylmethanesulfonyl fluoride (PMSF, 0.5 mM) was included in all buffers.

Analytical Gel-Filtration Chromatography. K562 nuclear extract (0.2 mL) was chromatographed on a Superdex 200 HR 10/30 column (Pharmacia Biotech Inc.) with a Pharmacia FPLC system. The column was equilibrated in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% glycerol, 0.2 mM EDTA, and 5 mM DTT. Before being loaded on the column, the extract was subjected to centrifugation for 1 min at 18700*g*. Proteins were eluted with a flow rate of 0.5 mL/min, and 0.5 mL fractions were collected. Aliquots of alternate fractions were assayed for GCN5, PCAF, and ADA2 by immunoblotting. In certain experiments, adjacent fractions were pooled, and the pooled fractions were analyzed by immunoblotting.

The column was calibrated by applying protein standards (5 μL each of a 10 mg/mL solution, diluted to 0.2 mL with equilibration buffer) and eluting with equilibration buffer. Proteins were detected by measuring the absorbance at 280 nm with an on-line detector. The void volume (V_0) was determined by measuring the eluted volume (V_e) of blue dextran. The V_e for protein standards and the V_0 were used to calculate K_{av} using the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$ as described previously (*37*, *45*). K_{av} values were plotted against the appropriate Stokes' radius (R_s) to obtain a linear calibration plot used to determine the R_s for GCN5, PCAF, and ADA2. As identical R_s values were measured in multiple independent chromatographic separations, the values are indicated with no deviation.

For the experiment of Figure 4, K562 nuclear extract (220 μL) was mixed with 20 μg of Bluescript SK+ plasmid DNA and incubated with 1000 units/mL of DNase I (Promega, RQ1 RNase-free), in the presence of 1 mM $MgCl_2$ and 1 mM $CaCl_2$, for 30 min at 30 °C. An aliquot of the extract (20 μL) was removed, and plasmid DNA was purified by phenol/chloroform extractions. DNA was analyzed by agarose gel electrophoresis to ensure complete digestion by DNase I. The remaining portion of the extract was applied to the Superdex 200 column and analyzed as described above.

SDS−PAGE and Western Blot Analysis. Aliquots of unfractionated or fractionated nuclear extract were boiled for 10 min in SDS−PAGE sample buffer and resolved on SDS−9% polyacrylamide gels as described previously (*46*). Gels were equilibrated in transfer buffer (25 mM Tris base, 192 mM glycine, and 20% methanol) for 20 min, and then proteins were transferred to Immobilon P membranes at 106 V for 1 h. Membranes were blocked by incubation in TBS

buffer (25 mM Tris base, 13.7 mM NaCl, and 2.7 mM KCl, pH 7.4) containing 0.1% Tween-20 and 10% nonfat dry milk for 1 h at 23 °C. Blocked membranes were incubated with primary antibody in TBS/0.1% Tween-20/1% BSA (1/1000 dilution of antibody) for at least 12 h at 23 °C. After three washes of 15 min each with TBS/0.1% Tween-20, [^{125}I]-protein A (0.25 μCi) (New England Nuclear) was diluted in TBS/0.1% Tween-20 and incubated with the membrane for 2 h at 23 °C. Membranes were washed three times for 15 min each with TBS/0.1% Tween-20 to remove nonspecifically associated protein-A. Immunoreactive bands were visualized by analysis of the membrane with a Phosphorimager.

Histone Acetyltransferase Assay. Aliquots (10 μL) of combined fractions from the Superdex 200 H/R column were assayed for HAT activity in a final volume of 18 μL. HAT assays contained 50 mM Tris (pH 8.0), 0.1 mM EDTA, 5% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, 5.9 μM [^{3}H]acetyl-CoA (0.5 μCi) (Amersham, 4.7 Ci/mmol), and 8 μg of purified calf thymus core histones (Sigma, H9250). Reactions were terminated by the addition of 10 μL of SDS-sample buffer and were then incubated at 100 °C for 10 min. Histones were resolved by SDS−PAGE (18% polyacrylamide) and visualized by Coomassie blue staining. After treatment of the gel with Enhance (New England Nuclear), acetylated histones were detected by autoradiography. Acetylation assays were also performed in reactions containing only purified H3/H4 or H2A/H2B histones (provided by Dr. Jeffrey Hayes, University of Rochester), which yielded identical results *vis-a-vis* the core histone specificity of acetylation.

RESULTS AND DISCUSSION

GCN5 Exists in a Large Macromolecular Complex in K562 Nuclear Extract. As a first step toward determining whether the β-globin LCR recruits chromatin-modifying enzymes that mediate long-range chromatin unfolding, we have characterized endogenous HATs in K562 cells. The human genes encoding the HATs GCN5 (*19*, *47*) and PCAF (*19*) and the adapter protein ADA2 (*47*; Yang and Nakatani, unpublished data) were recently cloned. Two distinct clones of human GCN5 have been isolated, encoding for proteins with 476 (*19*) and 427 (*47*) amino acids and predicted M_r of 54 031 and 48 889 Da. The long and short forms of GCN5 arise from the utilization of two distinct translation initiation codons. PCAF consists of 832 amino acids (*19*) and has a predicted molecular weight of 92 867 Da. Polyclonal antibodies specific for PCAF, GCN5, and ADA2 have been generated from the purified recombinant proteins (Yang and Nakatani, unpublished data). However, a biochemical characterization of the endogenous antigens in human cells has not been reported. The GCN5 antibody was produced against the long form of GCN5 that shows strong sequence homology with the carboxy-terminal domain of PCAF (*19*) and therefore cross-reacts with PCAF (Yang and Nakatani, unpublished data).

Conditions were established for detecting endogenous PCAF, GCN5, and ADA2 in unfractionated and fractionated K562 nuclear extracts using a sensitive Western blot assay. Gel-filtration chromatography was performed to determine whether these components form high molecular weight complexes, as has been shown in yeast (*24*). K562 nuclear

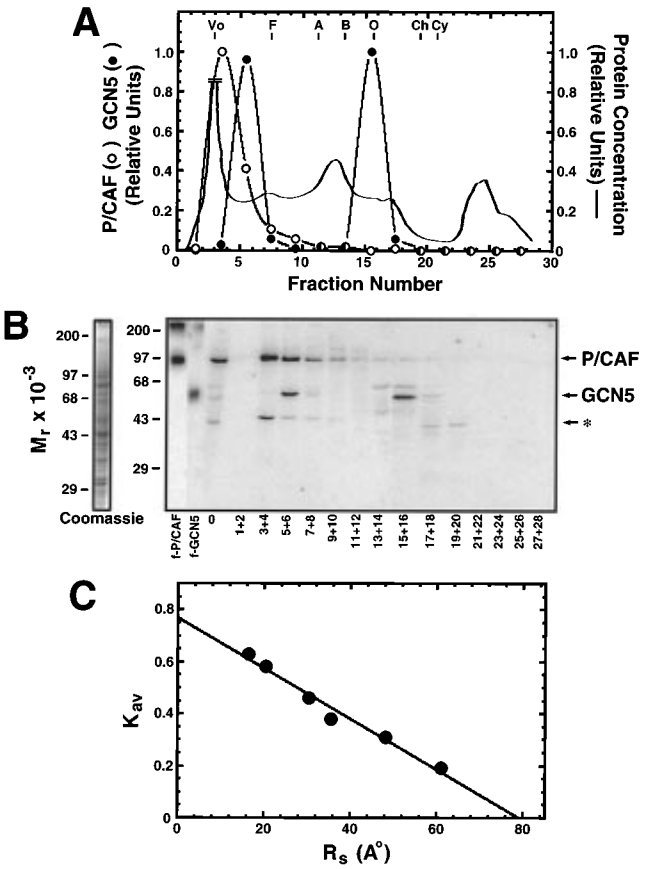

FIGURE 1: Evidence that GCN5 and PCAF exist in large macromolecular complexes. K562 nuclear extract (0.2 mL) was fractionated on a Superdex 200 HR 10/30 column equilibrated in buffer containing 0.15 M NaCl. Aliquots of combined fractions (0.12 mL) were concentrated by lyophilization. The resuspended proteins were resolved by SDS−PAGE and analyzed by immunoblotting with an anti-human GCN5 antibody. (A) The relative amounts of PCAF (○) and GCN5 (●) were determined by analysis of the blot with a Phosphorimager and plotted against the fraction number. The solid line shows the relative concentration of total protein eluting from the column. The elution positions of standard proteins of known Stokes' radius (R_s), used to calibrate the column, are indicated at the top. F, ferritin; A, aldolase; B, bovine serum albumin; O, ovalbumin; Ch, chymotrypsinogen; Cy, cytochrome *c*. (B) A Coomassie stain of the nuclear extract is shown on the left. The positions of PCAF and GCN5 are indicated on the immunoblot. The asterisk denotes an immunoreactive band that may represent a short version of GCN5, resulting from usage of an alternative translation initiation site. The positions of molecular weight markers are indicated on the left. Fraction 0 represents the crude nuclear extract applied to the column. f-PCAF and f-GCN5 represent purified flag epitope-tagged recombinant proteins. (C) K_{av} values for protein standards shown in panel A were plotted against their respective R_s values to yield a linear plot.

extract was resolved on a Superdex 200 HR 10/30 gel-filtration column equilibrated in buffer containing 0.15 M NaCl (Figure 1). Aliquots of fractions were concentrated by lyophilization and resolved by SDS−PAGE. Lanes were stained with Coomassie blue or analyzed by immunoblotting with the anti-human GCN5 antibody (Figure 1B). As expected, many proteins were detected in the nuclear extract by Coomassie blue staining. Three specific proteins (M_r of 46 300, 63 900, and 95 100) were detected in the unfractionated nuclear extract by the GCN5 antibody. These proteins are consistent with the known molecular masses of PCAF (92.9 kDa) and the two forms of GCN5 (54.0 and 48.9 kDa). No proteins were detected if the immunoblot

was incubated with either [^{125}I]-protein A alone or preimmune antibody, followed by [^{125}I]-protein A (data not shown).

PCAF was resolved on the gel-filtration column with a Stokes' radius of 74 Å ($n = 4$). Endogenous PCAF comigrated with recombinant human PCAF containing a Flag-epitope tag (f-PCAF). An additional antibody directed against amino acids 125−397 of PCAF (*19*) reacted with endogenous PCAF, but not GCN5 (data not shown). The 63.9-kDa band, which comigrated with Flag-epitope-tagged GCN5 (f-GCN5), is consistent with the long form of GCN5 (referred to as GCN5) (*19*). The 46.3-kDa band was also reproducibly observed and may represent the short form of GCN5 (*47*). Other weak bands were occasionally seen and may represent weakly cross-reactive proteins and/or proteolytic products.

GCN5 was detected in fractions 5.5 and 15.5, corresponding to Stokes' radii of 67 and 33 Å, respectively ($n = 4$). The relative amount of GCN5 with a Stokes' radius of 67 and 33 Å was 49.5 ± 5.5 and 50.5 ± 5.5 ($n = 4$, mean ± standard error), respectively. The immunoreactive band eluting at 33 Å exhibited a reproducible slight increase in mobility on the gel compared to the band eluting at 67 Å. Peak levels of the 46.3 kDa protein were detected in fractions 3 and 4, corresponding to a Stokes' radius of 74 Å ($n = 4$).

The molecular masses estimated from Stokes' radii for PCAF, GCN5 eluting at 67 Å, GCN5 eluting at 33 Å, and putative short GCN5 are 1.38 MDa, 0.80 MDa, 53.0 kDa, and 1.38 MDa, respectively. These estimates would only be valid for macromolecules with a prototypical globular conformation. If the overall shapes deviate from a sphere and are rodlike, the molecular masses would likely be overestimates. Nevertheless, on the basis of the known molecular masses of PCAF and GCN5, the gel-filtration results provide evidence that both HATs exist in large macromolecular complexes. As peak levels of GCN5 and PCAF occur in different fractions, it is likely that multiple HAT complexes exist in the K562 extract. The presence of PCAF and absence of GCN5 in fractions 3 and 4 suggest that one PCAF-containing complex lacks GCN5. Quantitative analysis reveals that virtually all of the immunodetectible PCAF exists as a high molecular weight species in the nuclear extract. As indicated above, GCN5 resolves into two populations, consistent with approximately equal amounts of monomer and a high molecular weight complex.

The gel-filtration chromatography was also performed in the presence of 0.3 M NaCl to assess the salt stability of the PCAF- and GCN5-containing complexes (data not shown). The elution profiles of PCAF and GCN5 were not significantly affected by 0.3 M NaCl, suggesting that the macromolecular interactions mediating complex formation may have a significant hydrophobic component. PCAF eluted as a single peak (74 Å, $n = 2$) and GCN5 eluted as two peaks (67 and 33 Å, 43.6% and 56.4%, respectively, $n = 2$), identical to that measured in low salt. In contrast to GCN5, high salt resulted in the elution of the putative short GCN5 as a smaller molecular species. The Stokes' radii of this species in low and high salt were 74 Å ($n = 3$) and 44 Å ($n = 2$), respectively, suggesting that the salt dissociated the antigen from associated components.

In Contrast to GCN5, the Majority of ADA2 Is Not Stably Associated with a Large Macromolecular Complex. The fractions from the Superdex 200 column equilibrated in 0.15 M NaCl were also analyzed for the presence of ADA2. On the basis of genetic and biochemical studies with yeast ADA2, it was expected that ADA2 would also be present in a large complex. Aliquots of fractionated K562 nuclear extract were resolved by SDS−PAGE and analyzed by Western blotting with anti-ADA2 antibodies (Figure 2). The anti-human ADA2 antibody reacted specifically with three proteins (M_r of 51 000, 85 500, and 100 600). Human ADA2 consists of 443 amino acids (*47*) and has a predicted M_r of 51 462. The 51-kDa band is in excellent agreement with the expected M_r of ADA2. The cross-reacting bands may represent proteins homologous to ADA2, although their identity is unknown.

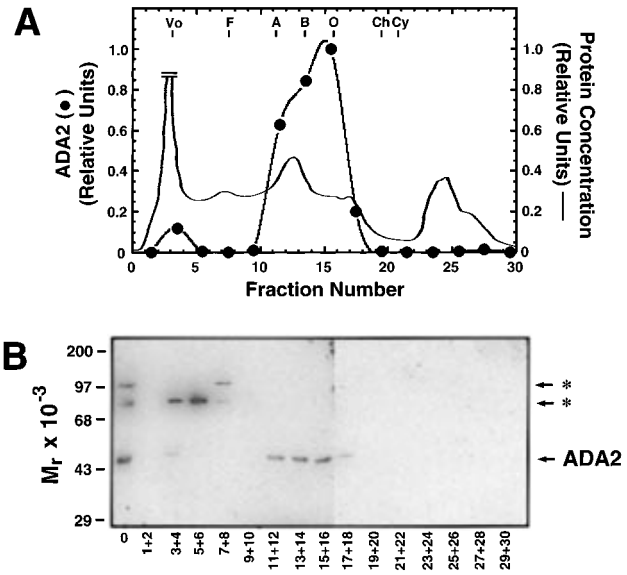

FIGURE 2: In contrast to GCN5, the majority of ADA2 exists as a monomer in K562 nuclear extract. K562 nuclear extract (0.2 mL) was fractionated on a Superdex 200 HR 10/30 column equilibrated in buffer containing 0.15 M NaCl. Aliquots of combined fractions (20 μL) were resolved by SDS−PAGE and analyzed by immunoblotting with anti-ADA2 antibodies. (A) The relative amount of ADA2 was determined by analysis of the blot with a Phosphorimager and plotted against the fraction number (●). The solid line shows the relative concentration of total protein eluting from the column. (B) Immunodetection of ADA2. The positions of ADA2 and two cross-reacting bands (asterisks) are indicated on the right. Fraction 0 represents the crude nuclear extract applied to the column. The positions of molecular weight markers are indicated on the left.

Peak levels of ADA2 were detected in fraction 15, corresponding to a Stokes' radius of 35 Å ($n = 4$). The Stokes' radius of 35 Å for ADA2 would be equivalent to an M_r of 64 900, assuming a globular conformation. If ADA2 has a rodlike conformation, the M_r would be even less. Regardless of the conformation, the results suggest that ADA2 exists as a monomer. As ADA2 associates with multiple adapter proteins and GCN5 in yeast, it is intriguing that approximately half of the GCN5 was recovered in a complex lacking ADA2 and that ADA2 was predominantly monomeric. On the basis of the strong sequence homology of PCAF and GCN5, it was expected that PCAF would also interact with ADA2. The gel-filtration results are inconsistent with a stable association of PCAF and ADA2.

To determine whether the monomeric state of ADA2 is unique to the K562 system, the hydrodynamic properties of ADA2 were also analyzed using nuclear extracts from human HeLa cells. HeLa nuclear extract was fractionated on the Superdex 200 column equilibrated in buffer containing 0.15

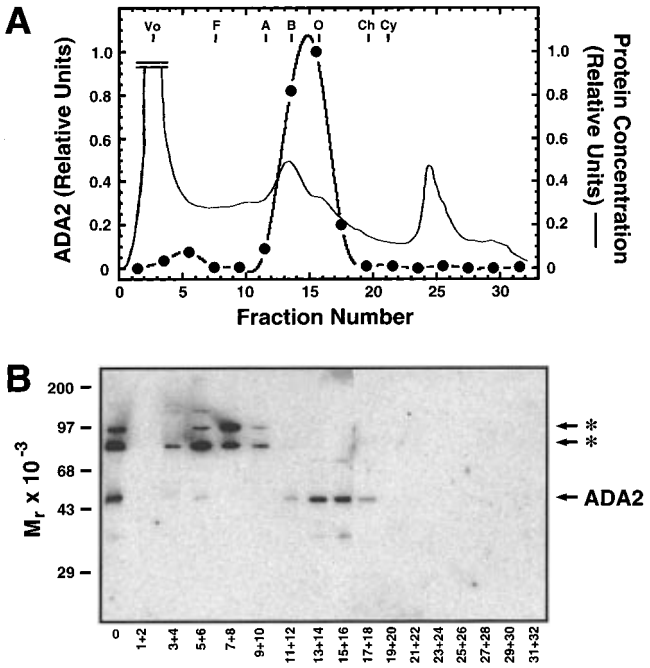

FIGURE 3: The majority of ADA2 exists as a monomer in HeLa nuclear extract. HeLa nuclear extract (0.2 mL) was fractionated on a Superdex 200 HR 10/30 column equilibrated in buffer containing 0.15 M NaCl. Aliquots of alternate fractions (20 μL) were combined, resolved by SDS−PAGE, and analyzed by immunoblotting with anti-ADA2 antibodies. (A) The relative amount of ADA2 was determined by analysis with a Phosphorimager and plotted against the fraction number (●). The solid line shows the relative concentration of total protein eluting from the column. (B) Immunodetection of ADA2. The positions of ADA2 and two cross-reacting bands (asterisks) are indicated on the right. Fraction 0 represents the crude nuclear extract applied to the column. The positions of molecular weight markers are indicated on the left.

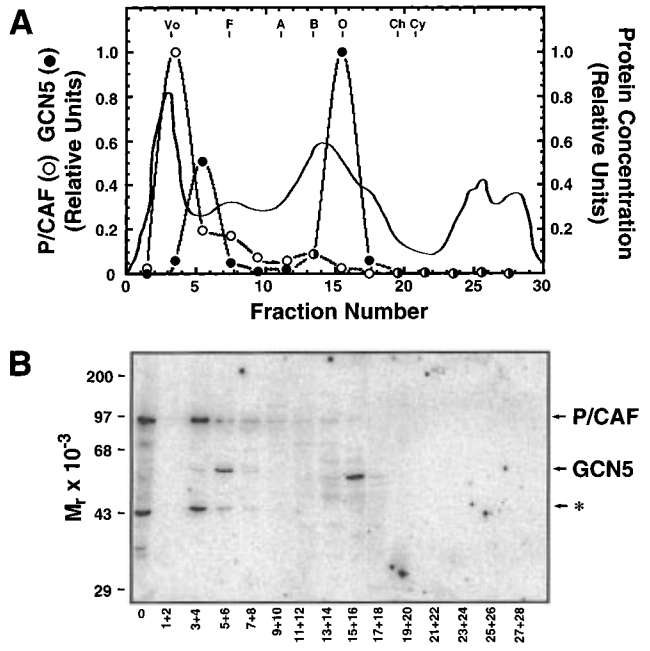

FIGURE 4: A high concentration of DNase I does not affect the elution properties of HAT complexes. K562 nuclear extract was incubated with 1000 units/mL of DNase I in the presence of 1 mM $MgCl_2$ and 1 mM $CaCl_2$ for 30 min at 30 °C. The extract (0.2 mL) was fractionated on a Superdex 200 HR 10/30 column equilibrated in buffer containing 0.15 M NaCl. Aliquots of alternate fractions (0.12 mL) were concentrated by lyophilization, resolved by SDS−PAGE, and analyzed by immunoblotting with an anti-human GCN5 antibody. (A) The relative amounts of PCAF (○) and GCN5 (●) were determined by analysis of the blot with a Phosphorimager and plotted against the fraction number. The solid line shows the relative concentration of total protein eluting from the column. The elution positions of standard proteins of known Stokes' radius (R_s), used to calibrate the column, are indicated at the top. (B) The positions of PCAF and GCN5 are indicated on the immunoblot. The asterisk denotes an immunoreactive band that may represent a short version of GCN5, resulting from usage of an alternative translation initiation site. The positions of molecular weight markers are indicated on the left. Fraction 0 represents the crude nuclear extract applied to the column.

M NaCl. Aliquots of fractions were resolved by SDS−PAGE and analyzed by Western blotting with the anti-ADA2 antibody. As shown in Figure 3, the anti-ADA2 antibody recognized three proteins in HeLa nuclear extract that had similar mobilities as the immunoreactive proteins in K562 extract. Furthermore, the elution profile of HeLa ADA2 was identical to the elution profile of K562 ADA2 (Figure 2). Thus, the majority of K562 and HeLa ADA2 elute from the Superdex column as a homogeneous molecular species, consistent with a monomer. HeLa nuclear extract resolved on the Superdex column was also analyzed by immunoblotting with the GCN5 antibody. The results were indistinguishable from the experiment of Figure 1B with K562 nuclear extract (data not shown).

It is intriguing that the 85.5-kDa protein detected with ADA2 antibodies in K562 and HeLa extracts peaks in fractions 5 and 6, which contain maximal levels of GCN5. The cross-reactivity may suggest that the 85.5-kDa protein is an ADA2 homologue. Further purification of the GCN5 complex will be necessary to determine whether the 85.5-kDa protein is a component of the GCN5 complex.

The presence of GCN5 in a large macromolecular complex lacking ADA2 was surprising, on the basis of the evidence that ADA2 is an integral component of the yeast GCN5−adapter complex. A simple explanation for our results is that the complex containing GCN5 and ADA2 is unstable after cell lysis. However, a considerable amount of GCN5 is stably associated with the large complex even under dilute conditions in the presence of high salt. To begin to investigate the composition of the GCN5-containing complex, we asked whether the complex contained DNA.

If the PCAF and GCN5 complexes contain DNA, extensive digestion with DNase I should alter the elution properties of the complexes on the gel-filtration column. K562 nuclear extract was treated with a high concentration of DNase I (1000 units/mL) for 30 min at 30 °C. Bluescript plasmid DNA (20 μg) was added to the extract to assess the DNase I activity. The plasmid was quantitatively degraded by the DNase I treatment (data not shown). The extract was immediately applied to the Superdex 200 column after the incubation with DNase I. Aliquots of the fractions were resolved by SDS−PAGE and analyzed by Western blotting with the anti-GCN5 antibody. As shown in Figure 4, DNase I did not significantly affect the elution profiles of PCAF, long-GCN5, or putative short-GCN5 (compare with Figure 1). Thus, it is unlikely that the large molecular sizes of the HAT complexes can be explained by an association with DNA. Of course, we cannot rule out the possibility that DNase I-resistant nucleic acid is present in the complex.

We have unsuccessfully attempted to measure HAT activity of the fractionated HATs using an in-gel acetylation assay (*48*). The assay appeared to be insufficiently sensitive to detect very low amounts of endogenous HATs. However,

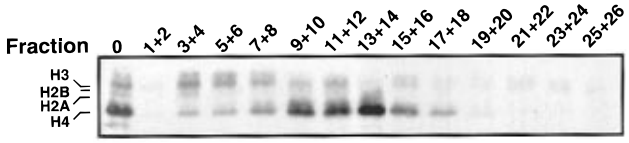

FIGURE 5: HAT activity of fractionated K562 nuclear extract. K562 nuclear extract (0.2 mL) was fractionated on a Superdex 200 HR 10/30 column equilibrated in buffer containing 0.15 M NaCl. Aliquots of combined fractions (10 μL) were assayed for HAT activity using [^{3}H]acetyl-CoA and purified core histones as described under Experimental Procedures. Histones were resolved by SDS−PAGE and visualized by Coomassie blue staining. Acetylated histones were detected by autoradiography. The positions of core histones identified by Coomassie staining are indicated.

HAT activity of the endogenous proteins was measured with a liquid-phase assay and purified core histone substrate. As shown in Figure 5, histone H3 and H4 acetyltransferase activity was detected in fractions 3−6, which contain PCAF and GCN5. A strong H4 acetyltransferase activity peaked in fraction 13.5, which did not correlate with the elution positions of GCN5 and PCAF. Equivalent amounts of all four core histones were detected in each lane by Coomassie staining (data not shown). Further purification of PCAF and GCN5 will be required to prove whether these antigens are responsible for the HAT activity observed in fractions 3−6. Additional purification and a detailed analysis of the substrate specificity of the endogenous proteins will be presented elsewhere.

Recruitment of GCN5 to Regulatory Regions of Genes by DNA-Bound Activator Proteins in Yeast and Humans. The studies of Brownell et al. (*13*, *49*) and Yang et al. (*19*) have led to a simple model for recruitment of HATs to regulatory regions of genes. The model assumes that DNA-bound activator proteins interact with HATs through specific protein−protein interactions. The association of adapter proteins with GCN5 and their ability to interact with basal transcription factors suggest that the adapter proteins may be important for the recruitment step. An alternative activity of adapter proteins may be to modulate the catalytic activity of HATs.

While purified yeast GCN5 is competent to acetylate free histone H3 and H4, it cannot acetylate histones in a nucleosomal substrate. In contrast, purified yeast GCN5 complex can acetylate nucleosomal histones (*24*), suggesting that components of the complex modulate the substrate specificity of GCN5. Obvious candidates for such factors are the adapter proteins such as ADA2. However, our results show that human GCN5 exists in a stable complex lacking ADA2. The anchoring of GCN5 to the complex, therefore, does not require an association of GCN5 with ADA2. This raises the possibility that another factor, which is tightly associated with GCN5, modulates the catalytic activity of GCN5.

Based on studies in yeast described earlier and the sequence conservation of yeast and human GCN5 and ADA2 (*47*), one would have predicted that human GCN5 and ADA2 would physically interact. Human GCN5 and ADA2 do interact in the yeast two-hybrid assay, but the interaction has not been shown by other methods (*47*). Taken together with our work, these results can be explained by a model in which the human GCN5−ADA2 interaction is of low affinity. A sustained GCN5−ADA2 association would not be required in a hit-and-run mechanism, in which GCN5

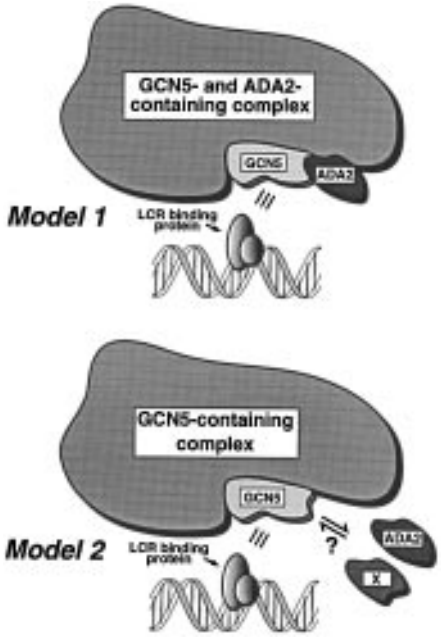

FIGURE 6: Models of the GCN5-containing HAT complex. Model 1 assumes that GCN5 and ADA2 physically interact in a stable complex. ADA2 would be tethered to the complex through high-affinity protein−protein interactions with GCN5 and potentially other components. Model 2 assumes that GCN5 is stably associated with a complex lacking ADA2. A regulated assembly process would determine whether ADA2 or additional factors would associate with the complex. This may serve to generate complexes of diverse functionality. Both models 1 and 2 assume that GCN5 engages in protein−protein interactions with an LCR binding protein, resulting in recruitment of the HAT complex to DNA.

transiently interacts with ADA2. As described above, the interaction could modulate the catalytic activity of GCN5 or allow the recruitment of the GCN5 complex to a DNA regulatory region. An alternative explanation of why GCN5 and ADA2 are not stably associated in human cell extracts is that factors present in the human, but not the yeast, system may modulate the stability of the GCN5−ADA2 complex.

Teleologically, one might ask why ADA2 is stably associated with the GCN5 complex in yeast but not humans. A relevant observation is that human GCN5 and ADA2 are incapable of complementing null mutations of the respective yeast genes (*23*, *47*). It seems reasonable to propose that in the human system greater functionality would be attained if multiple proteins recognized and functioned through the ADA2 docking site on the GCN5 complex (Figure 6, model 2). This could be accomplished by incorporating a regulated recruitment step to determine which component would associate with the complex. In yeast, ADA2 may be the sole component that interacts with the GCN5 complex (Figure 6, model 1). Thus, in yeast, it may be beneficial to tether ADA2 to GCN5 via a high-affinity protein−protein interaction. This simple model could explain the lack of genetic complementation, as the GCN5 interaction domain of human ADA2 might have diverged in such a way that it only weakly associates with yeast GCN5. The necessary regulatory components to generate a functional yeast GCN5−human ADA2 complex would be lacking in the yeast system.

In summary, we have performed the first biochemical analysis of the endogenous HAT and adapter proteins in human cells. In contrast to the stable yeast GCN5−adapter complex, in which ADA2 is an integral component, ADA2 does not exist as a large complex in human cell extracts. As

GCN5 is present as a higher-order molecular species, ADA2 does not mediate the association of GCN5 with the complex. Identification of the protein components of the HAT complexes and determining which components engage in protein−protein interactions with the HATs should further our understanding of how HATs are regulated and recruited to specific genes.

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