Eukaryotic genes are under the control of regulatory complexes acting through chromatin structure to control gene expression. Here we report the identification of a family of multiprotein corepressor complexes that function through modifying chromatin structure to keep genes silent. The polypeptide composition of these complexes has in common a core of two subunits, HDAC1,2 and BHC110, an FAD-binding protein. A candidate X-linked mental retardation gene and the transcription initiation factor II-I (TFII-I) are components of a novel member of this family of complexes. Other subunits of these complexes include polypeptides associated with cancer causing chromosomal translocations. These findings not only delineate a novel class of multiprotein complexes involved in transcriptional repression but also reveal an unanticipated role for TFII-I in transcriptional repression.

The genome of eukaryotes is packaged into chromatin, the fundamental unit of which is the nucleosome. The higher order chromatin structure is formed by arrangement of nucleosomes into an array. Such a higher order chromatin structure presents a barrier to cellular processes such as transcription, DNA replication, and DNA repair. Therefore, controlling accessibility to the nucleosomal DNA provides an important regulatory point in these processes (1). One way to modulate nucleosomal structure is through enzymatic modification of histones by acetylation, phosphorylation, or methylation.

A number of transcriptional regulatory complexes have been identified that contain histone acetylation or deacetylation activity. It was previously shown that the hyperacetylated chromatin correlates with active genes whereas the repressed genes exhibit a pattern of hypoacetylation (2, 3). This contention was strengthened by the discovery of the association of a number of transcriptional corepressors with histone deacetylation activity, exemplified by the silencer protein, REST (also called NRSF) (14, 16–19). In addition, a number of groups reported the isolation and characterization of a complex termed NuRD (also NURD and NRD) that not only contains histone deacetylases 1 and 2 but also a DNA-dependent ATPase subunit (20–22).

Here, we report the isolation of a new family of HDAC1,2 complexes that also contain the FAD-binding protein BHC110 (23). Unique to this family of corepressor complexes is the presence of a distinct structural DNA-binding subunit defining different HDAC1/2-containing complexes.

**MATERIALS AND METHODS**

**Immunoprecipitation of the BHC110/HDAC2-containing Complex**—HeLa nuclear extract was fractionated according to the protocol described above using P11. Anti-BHC110 and anti-HDAC2 antibodies (500 μg each) were cross-linked to Protein A-Sepharose (1 ml, Repligen) using standard techniques for affinity purification. The P11 0.3 M KCl fraction was incubated with 1 ml of antibody-Protein A beads for 4–5 h at 4 °C. The beads were washed first with 1 M KCl in buffer A (20 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol, 0.2 mM PMSF) followed by a wash with 0.5 M KCl in buffer A with 0.5% Tween 20. The beads were then washed with 100 mM KCl in buffer A, and the proteins were eluted with 0.1 M glycine, pH 2.5, and neutralized with 0.1 volume of 1 M Tris-HCl, pH 8.0.

**Affinity Purification of FLAG-XFIM—FLAG-XFIM and a selectable marker for puromycin resistance were co-transfected into 293 human embryonic kidney cells by calcium phosphate co-precipitation. Transfected cells were grown in the presence of 10 μg/ml puromycin, and individual colonies were isolated and analyzed for FLAG-XFIM expression. A cell line expressing FLAG-tagged XFIM, F-XFIM, was used for the affinity purification of the XFIM-containing complex as previously described for the FLAG-BRAF35 cell line (23). Chromatographic Purification of TFII-I Complex from HeLa Nuclear Extract—HeLa nuclear extract (3 g) was loaded on a 500-ml column of phosphocellulose (P11, Whatman) and fractionated stepwise by the indicated KCl concentration in buffer A. The P11 0.3 M KCl fraction (700 mg) was loaded on a 80-ml DEAE-Sepharose column (Amersham Bio-
FIG. 1. HDAC2 and BHC110 are components of multiple complexes. The purification scheme is shown. HeLa nuclear extract was fractionated by chromatography as described under “Materials and Methods.” The 0.3 M KCl elution of P11 was fractionated using antibody columns as indicated. The bound proteins were washed with buffer containing 1 M KCl and eluted using 0.2 M glycine, pH 2.5. The affinity-purified α-HDAC2 (lane 1), α-BHC110 (lane 2), and α-IgG (lane 3) were separated on an SDS-polyacrylamide gel (4–12%), and proteins were visualized by colloidal blue staining. Molecular masses of marker proteins (kDa) are indicated on the left, and the proteins analyzed by ion trap mass spectrometry for each complex are indicated. NE, nuclear extract; FT, flow-through.

FIG. 2. Diagrammatic depiction of the novel HDAC1,2/BHC110-associated proteins and description of their reported domain structure and association with human disease. aa, amino acids. PHD, plant homology domain; SANT, SWI3, ADA2, N-COR, and TFIIIB B' domain.
The column was resolved using a linear 10-column volume gradient of 700 to 0 mM KCl in buffer A and loaded on a 70-ml Bio-Gel HT column (hydroxyapatite, Bio-Rad). The column was resolved by using a linear 10-column volume gradient of 700 to 0 mM NH₄SO₄ in Buffer HB. TFII-I-containing fractions 12–30 were dialyzed to 0.35M KCl in Buffer A. The 0.35M KCl elution (500 mg) was dialyzed to 100 mM Hepes, pH 7.6, 1 mM dithiothreitol, 0.5 mM PMSF, 10% glycerol, 0.5 mM EDTA, 10% glycerol, 0.5 mM PMSF) and loaded on a butyl-Sepharose column (Amersham Biosciences). The column was resolved using a linear 10-column volume gradient of 700 to 0 mM NH₄SO₄ in Buffer HB. TFII-I-containing fractions 12–18 were dialyzed to 100 mM KCl in Buffer A and loaded on Heparin-5PW (TosoHaas). The column was resolved using a linear 20-column volume gradient of 100–500 mM KCl in Buffer A. TFII-I-containing fractions 12–16 was fractionated on a Superose 6 HR 10/30 column (Amersham Biosciences) and eluted with 0.5M KCl in buffer A. TFII-I containing fractions 12–30 were dialyzed to 700 mM NH₄SO₄ in Buffer HB. TFII-I containing fractions 12–30 were dialyzed to 0.35M KCl in Buffer A. TFII-I containing fractions 12–30 were dialyzed to 100 mM Hepes, pH 7.6, 4 mM dithiothreitol, 0.5 mM EDTA, 10% glycerol, 0.5 mM PMSF) and loaded on a butyl-Sepharose column (Amersham Biosciences). The column was resolved using a linear 10-column volume gradient of 700 to 0 mM NH₄SO₄ in Buffer HB. TFII-I-containing fractions 12–18 were dialyzed to 100 mM KCl in Buffer A and loaded on Heparin-5PW (TosoHaas). The column was resolved using a linear 20-column volume gradient of 100–500 mM KCl in Buffer A. TFII-I-containing fractions 12–16 was fractionated on a Superose 6 HR 10/30 column (Amersham Biosciences) equilibrated in 0.5 mM KCl in buffer A. Superose 6 was calibrated using molecular weight standards from Amersham Biosciences. The void was determined according to the manufacturer’s guidelines (one-third of column volume or 7 ml). Fractions 16–20 and 24–28 were used for immunoaffinity purification of the TFII-I-containing complexes.

Mass Spectrometric Peptide Sequencing—Excised bands were subjected to in-gel reduction, carboxymidemethylation, and tryptic digestion (Promega). Multiple peptide sequences were determined in a single run by microcapillary reverse-phase chromatography (a custom New Objective 50-μm column terminating in a nanospray 15-μm tip), directly coupled to a Finnigan LCQ Deca quadrupole ion trap mass spectrometer. The ion trap was programmed to acquire successive sets of three scan modes consisting of: full scan MS over alternating ranges of 395–800 m/z or 800–1300 m/z, followed by two data-dependent scans on the most abundant ion in those full scans. These dependent scans allowed the automatic acquisition of a high resolution (zoom) scan to determine charge state and exact mass and MS/MS spectra for peptide sequence information. MS/MS spectra were acquired with a relative collision energy of 30%, an isolation width of 2.5 daltons, and dynamic exclusion of ions from repeat analysis. Interpretation of the resulting MS/MS spectra of the peptides was facilitated by programs developed in the Harvard Microchemistry Facility (24) and by data base correlation with the algorithm SEQUEST (25).

Immunoblot Analysis—Anti-BHC110, anti-BHC80, and anti-BRAF35 antibodies were developed according to the manufacturer’s instructions (Promega). Anti-TFII-I and XFIM antibodies were developed to a peptide corresponding to the amino acids IKETDGSQKQEFDPTW and DPLTLEKPLAGDLHPF for TFII-I and XFIM, respectively. Immunoblotting was performed with alkaline phosphatase.

RESULTS

BHC110 Defines a New Family of HDAC-containing Complexes—We recently reported the isolation from HeLa nuclear extract of a BRAF35-HDAC complex (BHC) containing flavin adenine dinucleotide (FAD)-binding subunit, BHC110 (23). This complex contains subunits similar to the CoREST complex described previously (26, 27). To determine whether there are other BHC110-containing complexes in HeLa cells, we developed anti-BHC110 antibodies and affinity-purified the BHC110-
containing complexes following the scheme in Fig. 1. The anti-BHC110 affinity eluate was subjected to ion trap mass spectrometric sequencing. In addition to other components of the BHC complex (23), this analysis revealed the stable association of BHC110 with \( \text{ZNF261} / \text{XFIM} \), a candidate gene for X-linked mental retardation in Xq13.1 (28, 29), \( \text{ZNF198} / \text{FIM} \), a gene related to XFIM that is associated with myeloproliferative disorder that involves myeloid hyperplasia and eosinophilia (29, 30), \( \text{KIAA0182} \), a proline-rich protein of unknown function, and TFII-I, the initiator binding protein and a transcriptional coactivator (31, 32) (Fig. 1, lane 2, and Fig. 2). The association of these polypeptides and BHC110 is specific as the affinity eluate from a control antibody column was devoid of their presence (Fig. 1, lane 3).

To determine whether these polypeptides are also stable components of an HDAC-containing complex, we affinity-purified HeLa HDAC2-containing complexes from the 0.3M KCl eluate of phosphocellulose chromatography (P11) (Fig. 1). Ion trap mass spectrometric sequencing of the anti-HDAC2 affinity eluate revealed that, in addition to components of the previously described complexes of Mi2 (20–22), Sin3 (13–15), and BHC (23, 26, 27), the anti-HDAC2 eluate contained \( \text{ZNF261/XFIM} \), \( \text{ZNF198/FIM} \), \( \text{KIAA0182} \), and TFII-I (Fig. 1, lane 1, and Fig. 2). These results indicate that these novel subunits are associated with both HDAC2 and BHC110, although these polypeptides most likely represent multiple distinct HDAC2/BHC110-containing complexes. To test this hypothesis we embarked on isolating other BHC110-containing complexes that are distinct from the BHC complex.

**TFII-I Is a Component of an XFIM Complex**—To isolate other BHC110-containing complexes, we developed a 293-derived cell line stably expressing a FLAG-tagged XFIM (F-XFIM). FLAG-XFIM was affinity-purified from F-XFIM nuclear extract using anti-FLAG antibodies followed by elution of bound material with FLAG peptide (Fig. 3a). A combination of ion trap mass spectrometry and Western blot analysis demonstrated the presence of TFII-I, BHC110, and HDAC1/2 polypeptides (Fig. 3a). These polypeptides were absent in affinity-purified eluate of the parent 293 cell line (Fig. 3a). Further analysis of the XFIM complex by Superose 6 gel filtration confirmed the association of XFIM, TFII-I, BHC110, and HDAC2 as a component of a single complex of about 1 MDa, although a small percentage of HDAC2 and TFII-I eluted at a smaller molecular mass (Fig. 3c). This complex was termed the XFIM complex (Fig. 3a).

Moreover the XFIM complex displayed HDAC activity toward core histones (Fig. 3c). It is noteworthy that analysis of the XFIM protein following gel filtration by colloidal staining revealed a higher stoichiometry for XFIM (4–11 XFIMs per complex) to other subunits of the complex. Taken together, these results establish BHC110 and HDAC2 as common subunits of at least two distinct (BHC and XFIM) histone deacetylase complexes (23).

To determine the fraction of TFII-I that associates with BHC110 we purified TFII-I by conventional column chromatography (Fig. 4a). Analysis of TFII-I on the gel filtration, the last step of purification, revealed a higher stoichiometry for XFIM (~4 XFIMs per complex) to other subunits of the complex. Taken together, these results establish BHC110 and HDAC2 as common subunits of at least two distinct (BHC and XFIM) histone deacetylase complexes (23).
HDAC2 only with the TFII-I derived from the larger molecular mass fraction (Fig. 4a, see fractions 18–20). Furthermore, immunoprecipitation using anti-HDAC2, anti-BHC110, and anti-TFII-I antibodies demonstrated the specific association of TFII-I with HDAC2 and BHC110 (Fig. 4a, b and c). These results indicate that although TFII-I is predominantly monomeric, a fraction of TFII-I is in a stable complex with BHC110 and HDAC2.

**XFIM Complex Is Recruited to the c-fos Promoter**—Because TFII-I was reported as a transcriptional coactivator for serum response factor (SRF) at the c-fos promoter (33), we analyzed the c-fos promoter as a target of the XFIM complex. We first confirmed the responsiveness of the c-fos promoter to inhibitors of histone deacetylation. Consistent with previous reports c-fos displayed an increased transcription level following either sodium butyrate or trichostatin A treatment (34) (Fig. 5a). Moreover, although the histone H3 acetylation state is not affected by EGF stimulation, there is an increase in acetylated histone H4 coincident with the absence of HDAC1,2 complex 30 min following EGF stimulation (Fig. 5d). Moreover, although the histone H3 acetylation state is not affected by EGF stimulation, there is an increase in acetylated histone H4 coincident with the absence of HDAC1,2 complex 30 min following EGF stimulation (Fig. 5d). However, although the SRF levels at the promoter were enhanced 30 min following the stimulation of c-fos transcription, HDCA2 and BHC110 were no longer detectable (Fig. 5d). BHC110 and HDAC2 were returned to the promoter as the repressed state was reestablished (Fig. 5d, 60 and 90 min). Interestingly, TFII-I occupancy of the promoter was unchanged following EGF stimulation (Fig. 5d). Moreover, although the histone H3 acetylation state is not affected by EGF stimulation, there is an increase in acetylated histone H4 coincident with the absence of HDAC1,2 complex 30 min following EGF stimulation. These results suggest a role for the XFIM complex as a corepressor at the c-fos promoter and are consistent with the contention that TFII-I may play a dual role in that it participates as a component of a corepression complex in the basal repressed state of the promoter, but once the gene is activated it remains bound to the promoter to form a stable complex with the activator as previously described (33, 34).
Recruitment of TFII-I to the Promoter Results in Transcriptional Repression.—To directly assess the role of TFII-I in transcription, we tethered TFII-I to the GAL4 DNA-binding domain and tested its activity using a promoter containing five GAL4-binding sites (Fig. 6a). Interestingly, although GAL4-VP16 resulted in a potent activation of transcription from this promoter, GAL4-TFII-I caused a moderate (∼50%) repression of transcription (Fig. 6b). However, the transcriptional repression by GAL4-TFII-I was smaller than that obtained with either GAL4-SAP30 or GAL4-HP1α, two previously characterized transcriptional corepressors (35, 36). Taken together, our results point to a role for TFII-I in transcriptional repression.

To further assess the role of TFII-I in transcription of an endogenous c-fos promoter, we utilized small interfering RNA-mediated depletion (RNAi) specific for TFII-I to inhibit its synthesis. Two rounds of RNAi treatment were necessary to see a substantial (larger than 80%) decrease in TFII-I mRNA levels (Fig. 6b). Analysis of c-fos transcription following TFII-I RNAi indicated a pronounced and specific de-repression of basal transcription in the absence of TFII-I (Fig. 6b). Moreover, the EGF-mediated activation of c-fos promoter still persisted. It is difficult to assess the change in the -fold stimulation following TFII-I RNAi because there is no basal activity in the absence of the RNAi treatment. These results point to a role for TFII-I in the maintenance of the basal repressed state of the c-fos promoter.

DISCUSSION

We identify a new family of HDAC1,2-associated complexes containing BHC110. Moreover, we define the polypeptide composition of a novel member of this family containing the candidate gene for X-linked mental retardation XFIM, ZNF261/XFIM, ZNF198/FIM, and BRAF35 have close homologs in Drosophila melanogaster, indicating that similar corepressor complexes may also be involved in gene-specific repression in D. melanogaster. Finally, the close association of a number of HDAC-associated subunits with specific disease states (Fig. 2) attests to the importance of this family of corepressor complexes in human health.

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