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Isolation and Characterization of a Novel H1.2 Complex That Acts as a Repressor of p53-mediated Transcription*

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Linker histone H1 has been generally viewed as a global repressor of transcription by preventing the access of transcription factors to sites in chromatin. However, recent studies suggest that H1 can interact with other regulatory factors for its action as a negative modulator of specific genes. To investigate these aspects, we established a human cell line expressing H1.2, one of the H1 subtypes, for the purification of H1-interacting proteins. Our results showed that H1.2 can stably associate with sets of cofactors and ribosomal proteins that can significantly repress p53-dependent, p300-mediated chromatin transcription. This repressive action of H1.2 complex involves direct interaction of H1.2 with p53, which in turn blocks p300-mediated acetylation of chromatin. YB1 and PURα, two factors present in the H1.2 complex, together with H1.2 can closely recapitulate the repressive action of the entire H1.2 complex in transcription. Chromatin immunoprecipitation and RNA interference analyses further confirmed that the recruitment of YB1, PURα, and H1.2 to the p53 target gene *Bax* is required for repression of p53-induced transcription. Therefore, these results reveal a previously unrecognized function of H1 as a transcriptional repressor as well as the underlying mechanism involving specific sets of factors in this repression process.

Histones are the major protein components to compact genomic DNA into the limited volume of the nucleus as a highly organized chromatin structure. The basic element of chromatin is the nucleosome, which consists of 146 base pairs of DNA wrapped around an octameric core of histones containing two molecules each of H2A, H2B, H3, and H4 (1–4). This repeating unit of chromatin is associated with another type of histone called linker histone H1 to achieve an additional level of compaction, making genes inaccessible to transcription factors and preventing their expression (5–9). Mammalian cells have at least eight histone H1 subtypes including H1.1 through H1.5 and somatic cell-specific H1o as well as germ cell-specific H1t and H1oo, all consisting of a highly conserved globular domain and less conserved N- and C-terminal domains (6, 8, 10). The existence of multiple H1 subtypes and the diversity of their amino acid sequences raise the possibility that individual subtypes have nonredundant functions in various cellular processes. In addition, the expression of each H1 subtype depends on the tissue, phase of the cell cycle, and developmental stage, further suggesting the specific contribution of linker histone subtypes for regulation of various cellular processes (6, 8, 11).

Although most studies have focused on the contribution of H1 as a structural component of the nucleosome, it is becoming apparent that H1 also acts as a repressor for specific gene transcription (12–15). This repressive capacity of H1 on transcription appears to be accomplished by its localization at particular chromosomal domains with specific transcription regulators. Msx1 recruits a linker histone H1 to the *MyoD* gene, and this selective localization correlates with a repressive chromatin state and gene repression (16). Simultaneous inactivation of three H1 subtype genes (H1.2, H1.3, and H1.4) in mouse embryonic cells significantly affects the expression of a subset of genes, supporting a rather specific action of H1 in gene regulation (17). A recent study demonstrating that specific sets of ribosomal proteins interact with H1 to suppress transcription also provides support for a rather complex mechanism for the effect of H1 in gene regulation (18). It thus appears that the linker histone H1 requires other regulatory factors to retain its optimal capacity and specificity for epigenetic gene regulation.

To understand the molecular mechanisms by which transcription is down-regulated by H1, we purified factors stably associated with H1.2, one of the human H1 subtypes, by using an epitope tagging and stable cell line approach. Our functional analysis demonstrated that the purified H1.2 complex represses p53-dependent, p300-mediated chromatin transcription by blocking chromatin acetylation. The result that H1.2 alone is defective in repression underscores the significance of factors associated with H1.2 for repressive action of H1.2 in transcription. Furthermore we found that the physical interaction of the H1.2 complex with p53, most likely through H1.2 present in the complex, provides a novel mechanism for the transcriptional repression by the H1.2 complex. Therefore, apart from a role of H1 in maintaining higher order chromatin structure, our results provide new insights into the molecular mechanism of action of linker histone H1 in specific transcription events.

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EXPERIMENTAL PROCEDURES

Generation of H1.2 Cell Line—To construct pIRES-FHH1.2 vector used for mammalian expression of H1.2, human H1.2 gene was amplified by PCR and inserted into NotI and EcoRI sites of pIRESneo containing FLAG and HA[2] tags. HeLa-S cells (1×10^6) were transfected with 3 μg of pIRES-FHH1.2 using Lipofectamine (Invitrogen), and positive clones were selected with G418 (500 μg/ml, Invitrogen) for 3 weeks. H1.2 expression within the isolated clones was confirmed by 4–20% gradient SDS-PAGE and Western blotting with anti-FLAG (Sigma) and anti-HA antibodies (Santa Cruz Biotechnology, Inc.). The selected H1.2 cell line was grown in an 8-liter spinner culture in Dulbecco's modified Eagle's-PO_4 medium (Irvine Scientific), and nuclear extract (0.5 g) was prepared as described recently (19).

Purification and Identification of H1.2 Complex—To purify the H1.2 complex, the nuclear extract prepared from the H1.2 cell line was first loaded onto a phosphocellulose P11 column (Whatman) equilibrated with BC150 buffer (20 mM HEPES-KOH (pH 7.9), 0.5 mM EDTA, 0.05% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, protease inhibitors, 150 mM KCl). The bound proteins were step-eluted with BC300, BC500, BC850, BC1200, and BC2000 buffers. The BC1200 fraction containing the H1.2 complex was dialyzed in BC300 buffer and further purified by using M2 agarose affinity chromatography (Sigma) (19). The preparation was confirmed by SDS-PAGE with a 4–20% linear gradient and immunoblotting using either anti-FLAG or anti-HA antibody. A portion of the H1.2 complex isolated after M2 agarose affinity chromatography (0.2 ml) was applied to a 5-ml 15–40% glycerol gradient in BC250 buffer containing 0.1% Nonidet P-40. After centrifugation at 150,000 $\times$ *g* for 20 h in an SW 55Ti rotor at 4 °C, fractions (150 μl) were collected from the top of the tube. The distribution of the H1.2 complex was determined by silver staining and Western blotting of 4–20% SDS-PAGE gels. For mass spectrometry analysis, purified factors were resolved by 4–20% gradient SDS-PAGE, and proteins were visualized by Coomassie Blue staining. Bands were excised from the gel and submitted to the protein sequencing facility at the University of Southern California core mass spectrometry facility for in-gel trypsin digestion followed by peptide sequencing according to facility protocols. The presence of the identified proteins within the purified H1.2 complex was further confirmed by Western blot analysis. Antibodies used for Western blot analysis were as follows: anti-nucleolin and anti-lamin A/C from Santa Cruz Biotechnology, Inc., anti-CAPERα from Bethyl Laboratories, anti-WDR5 from Abcam, anti-tubulin from Calbiochem, anti-H2B from Upstate Biotechnology, anti-DNA-dependent protein kinase from Dr. Lieber (20), anti-PARP1 from Dr. Comai (21), anti-FIR from Dr. Levens (22), anti-YB1 from Dr. Kohno (23), and anti-PURα from Dr. Johnson (24). Anti-ASXL1 was from Dr. Brock.

Construction and Expression of Recombinant H1.2—Wild type and C terminus-deleted H1.2 constructs were generated by subcloning H1.2 gene fragments encoding amino acids 1–213 and amino acids 1–109. *Escherichia coli* Rosetta 2 (DE3) pLysS cells (Novagen) were transformed with the resulting pET-H1.2 construct, grown in 1 liter of Luria-Bertani broth at 37 °C. Harvested bacteria were lysed in 25 ml of lysis buffer (20 mM Tris (pH 8.0), 10% glycerol, 3 mM dithiothreitol, 0.5 M KCl) by sonication, and the cleared lysate (125 mg) was bound to Ni-NTA affinity resin (Novagen) in batch by rocking at 4 °C for 1 h. After binding, the resin was washed five times with lysis buffer and eluted with BC300 buffer containing 0.25 M imidazole. The eluted protein was loaded onto a 1-ml CM-Sephadex C-25 column (Amersham Biosciences) pre-equilibrated with BC300 buffer. After extensive washing with BC300 buffer, the bound proteins were step-eluted with BC300, BC500, BC800, and BC1200 buffers. The combined BC800 fractions containing H1.2 were dialyzed against BC300 buffer and applied to an SP-HP column (Amersham Biosciences) pre-equilibrated with BC300 buffer. Elution was again carried out by using a four-phase salt gradient (0.3, 0.5, 0.8, and 1.2 M) in BC buffer. H1.2-containing 0.8 M fractions were collected and dialyzed against BC100 buffer. Protein concentrations were determined by BCA protein assay (Pierce) using BSA as a protein standard.

Glutathione S-Transferase (GST) Pulldown Assays—To generate H1.2 fused to GST, H1.2 DNA sequence was subcloned into the EcoRI and BamHI sites of pGEX-2T (Amersham Biosciences). Recombinant GST and GST-H1.2 proteins were expressed in *E. coli* Rosetta 2 (DE3) pLysS and purified by affinity chromatography on glutathione-Sepharose 4B beads (GE Healthcare) according to the manufacturer's protocol. Vectors encoding ASXL1, β-catenin, TGase7, CAPERα, YB1, and WDR5 were prepared by subcloning their cDNAs into pcDNA3.1/His vector (Invitrogen). Vectors encoding GST-PURα and HA-FIR were as described recently (22, 25). ASXL1, β-catenin, TGase7, CAPERα, YB1, and WDR5 were synthesized by *in vitro* translation using the TnT coupled transcription-translation system with conditions as described by the manufacturer (Promega). FLAG-tagged PARP1 was expressed in insect Sf9 cells and purified on M2 agarose according to standard protocol. HA-tagged FIR and GST-fused PURα were purified as described recently (22, 25). The GST tag was removed from GST-PURα by thrombin cleavage kit (Novagen). *In vitro* binding experiments were carried out using purified recombinant GST or GST-H1.2 (2 μg) proteins bound to glutathione-Sepharose 4B beads and one of the prepared components of the H1.2 complex in 0.5 ml of binding buffer (25 mM HEPES (pH 7.8), 0.2 mM EDTA, 20% glycerol, 150 mM KCl, 0.1% Nonidet P-40). Glutathione beads were washed four times in binding buffer and boiled in SDS sample buffer to elute bound proteins, which were then analyzed by SDS-PAGE and Western blot analysis with antibodies as described in Fig. 2*B*. For identification of the p53 interaction domain of H1.2, expression vectors for H1.2 deletion mutants, *i.e.* H1.2 N-terminal domain (H1.2 NT), H1.2 globular domain (H1.2 GD), and H1.2 C-ter-

[2] The abbreviations used are: HA, hemagglutinin; Ni-NTA, nickel-nitrilotriacetic acid; BSA, bovine serum albumin; GST, glutathione *S*-transferase; RT, reverse transcription; ChIP, chromatin immunoprecipitation; shRNA, short hairpin RNA; ASXL1, additional sex combs-like protein 1; PARP1, poly(ADP-ribose) polymerase-1; TGase7, tissue transglutaminase 7; CAPERα, coactivator of activating protein-1 and estrogen receptors α; FIR, far upstream element-binding protein 1-interacting repressor; YB1, Y box-binding protein 1; WDR5, WD repeat domain 5; PURα, purine-rich element-binding protein A; NAP1, nucleosome assembly protein 1; CT, C-terminal domain; NT, N-terminal domain.

minal domain (H1.2 CT) were also generated by inserting PCR-amplified cDNA fragments encoding amino acids 1–34 (for NT), 35–109 (for globular domain, GD), and 110–213 (for CT) of H1.2, respectively, into pGEX4T-1 (Amersham Biosciences). The GST-p53 fusion proteins comprising full-length p53, its first 83 residues (for NT), residues 120–290 (for DNA binding domain), and its final 104 residues (for CT) were also prepared as described previously (26).

Immunoprecipitation Assays—The genes encoding H1.2 and PURα were prepared by PCR amplification and subcloned into pcDNA3.1/His. Construction of pCMV-Taq2 PARP1 vector was described previously (27). All other expression vectors were identical to those used in the TnT coupled transcription-translation system. 293T cells (3×10^6) were transiently transfected with 3 μg of an expression vector for FLAG-PARP1, Xpress-β-catenin, Xpress-TGase7, HA-FIR, Xpress-CAPERα, Xpress-YB1, Xpress-PURα, or Xpress-WDR5 along with 3 μg of an expression vector for FLAG- or Xpress-H1.2. Total amounts of the expression vectors were kept constant by adding empty vectors. Two days after transfection, cells were harvested, and total cell extracts were clarified by centrifugation. Immunoprecipitation was performed with anti-FLAG, anti-HA, and anti-Xpress antibodies (Invitrogen) as described previously (28). Co-precipitated proteins were detected by Western blot analysis with anti-FLAG, anti-HA, and anti-Xpress antibodies. To generate H1.2 deletion mutants, human H1.2 gene fragments encoding amino acids 35–213 (ΔNT), amino acids 1–109 (ΔCT), and amino acids 110–213 (CT) were subcloned into pcDNA3.1/His. A construct expressing amino acids 290–393 of p53 was generated by inserting a corresponding gene fragment into pIRESneo with the FLAG and HA tags. Other p53-expressing constructs have been described recently (28). Transfection and immunoprecipitation assays with mutant p53 and H1.2 were performed as described above. For co-immunoprecipitation of endogenous proteins, 293T cell lysates were immunoprecipitated with anti-p53 monoclonal antibody (DO-1, Santa Cruz Biotechnology, Inc.) followed by immunoblotting with antibodies against H1.2 (Abcam) and p53.

Immunofluorescence Microscopy Analysis—The vector pEGFP-H1.2 was generated by subcloning H1.2 gene into the EcoRI and BamHI sites of pEGFP-C1, and other expression plasmids were prepared as described under "Immunoprecipitation Assays." HeLa cells were grown on 18-mm glass coverslip to 40% confluency with Dulbecco's modified Eagle's-PO_4 medium supplemented with 10% fetal bovine serum and transfected with 0.3 μg of mammalian expression vectors for EGFP-H1.2 along with 0.3 μg of an expression vector for Xpress-ASXL1, FLAG-PARP1, HA-FIR, Xpress-CAPERα, Xpress-YB1, Xpress-PURα, and Xpress-WDR5 as indicated in Fig. 2*D*. Two days after transfection, cells were briefly washed with phosphate-buffered saline, fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 min, and permeabilized with 0.3% Triton X-100 in phosphate-buffered saline for 15 min at room temperature. For localization of other proteins, fixed cells were blocked in 3% BSA and incubated with the anti-FLAG, anti-Xpress, or anti-HA antibody, diluted in phosphate-buffered saline containing 3% bovine serum albumin, and subsequently with the Cy3-conjugated secondary antibodies (The Jackson Laboratory). All incubations were at room temperature for 2 h. Confocal laser microscopy was performed with a Zeiss LSM 510 dual photon confocal microscope at 63× magnification, and digital images were analyzed with Adobe Photoshop software.

Transcription and Histone Acetyltransferase Assays—The assembly of chromatin templates with recombinant ATP-utilizing chromatin assembly and remodeling factor, recombinant NAP1, recombinant core histones, and p53RE/G-less plasmid DNA was performed as described recently (19). FLAG-tagged human p300 and p53 proteins were expressed and purified on M2 agarose (Sigma) according to standard procedures and as described previously (29). For construction of the YB1 expression vector, coding sequence of YB1 was PCR-amplified and inserted into the NdeI and BamHI sites of pET-15b. The recombinant YB1 protein was expressed in *E. coli* Rosetta 2 (DE3) pLysS cells and purified with Ni-NTA affinity resin (Novagen). The recombinant PURα was prepared as described above. Transcription assays were performed with 40 ng of chromatin or free DNA as described recently (19) except that H1.2 or H1.2 complex was added together with p300 and acetyl-CoA. When H1.2 (40 and 80 ng) was used in transcription, BSA (160 and 320 ng) was also included to make the final concentration similar to that of H1.2 complex (200 and 400 ng). For histone acetyltransferase assays, chromatin template (100 ng) was incubated with H1.2 (80 ng with 320 ng of BSA) or H1.2 complex (400 ng) in the presence of p53 (30 ng), p300 (40 ng), and 2.5 μM [^{3}H]acetyl-CoA. Transcription assays with wild type/C terminus-deleted H1.2, YB1, and PURα were as described above except that H1.2 (50 ng), YB1 (100 ng), and PURα (100 ng) were added together with p300. For reporter gene assays with wild type/C terminus-deleted H1.2, YB1, and PURα, H1299 cells were grown to 50% confluency (1×10^5) on 12-well plates in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Transfection assays were performed, as indicated, with reporter plasmid (200 ng) bearing p53 response element, p53 expression vector (100 ng), and expression vector (200 ng) of H1.2, YB1, or PURα. The total amount of plasmid DNA was adjusted to 1 μg by adding empty vector. Cells were harvested at 48 h and analyzed for luciferase activity as described previously (28).

RT-PCR and Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed essentially as described previously (28, 30) by using H1299 cells after transfection either with the plasmids expressing p53, Xpress-PURα, FLAG-YB1, and GAL4-H1.2 or with empty control vector. The following primers were used for PCR amplification: *Bax*, 5′-TATCTCTTGGGCTCACAAG-3′ and 5′-ACTGTCCAATGAGCATCTCC-3′; and glyceraldehyde-3-phosphate dehydrogenase, 5′-CAGCACAGCCCACAGGTTTCC-3′ and 5′-CCTGGCTCCTGGCATCTCTGG-3′. Anti-p53 (FL393, Santa Cruz Biotechnology, Inc.), anti-GAL4 DNA binding domain (Santa Cruz Biotechnology, Inc.), anti-FLAG, and anti-Xpress antibodies were used to immunoprecipitate DNA. Total RNA was also isolated with the RNeasy minikit (Qiagen) and subjected to RT-PCR as described previously (28). The following primers were used for RT-PCR: *Bax*, 5′-CGTCCACCAAGAAGCTGAGCG-3′ and 5′-AGCACTCCCGCCACAA AGATG-3′; and

actin, 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′. The PCR products were resolved on a 1.5% agarose gel containing ethidium bromide.

Construction of H1.2, YB1, and PURα shRNA Plasmids and Stable Transfection—The design and construction of the shRNA clones against H1.2, YB1, and PURα were performed according to the manufacturer's protocol (Ambion). shH1.2 (gatccAGAGCGTAGCGGAGTTTCTttcaagagaAGAAACTCCGCTACGCTCTTTttttggaaa and agcttttccaaaaAAAGAGCGTAGCGGAGTTTCTctcttgaaAGAAACTCCGCTACGCTCTg), shYB1 (gatccGAAGGTCATCGCAACGAAGttcaagagaCTTCGTTGCGATGACCTTCTTttttggaaa and agcttttccaaaaAAGAAGGTCATCGCAACGAAGtctcttgaaCTTCGTTGCGATGACCTTCg), and shPURα (gatccgCCGCAAGTACTACATGGATttcaagagaATCCATGTAGTACTTGCGGTTttttggaaa and agcttttccaaaaAACCGCAAGTACTACATGGATtctcttgaaATCCATGTAGTACTTGTGGcg) (target sequences are capitalized) were subcloned into pSilencer 2.1-U6 neo plasmid (Ambion) and used for generation of stable cell lines. RNA was extracted from U2OS cells stably transfected with shRNA of H1.2, YB1, or PURα with or without adriamycin (0.5 mg/ml for 8 h, Fluka) treatment by using the RNeasy minikit. The cDNA was synthesized from purified RNA with the SuperScript III First-Strand kit (Invitrogen), and relative changes in expression of *Bax* gene were assessed by real time PCR.

RESULTS

Linker Histone H1.2 Stably Associates with Multiple Regulatory Factors in Living Cells—As a first step in exploring the repressive roles of H1 in transcription, we generated a HeLa-derived cell line that constitutively expresses FLAG- and HA-tagged H1.2 for the purification of the H1.2 complex (see "Experimental Procedures" for details). Our Western blot analysis with FLAG antibody confirmed that the major fraction of expressed H1.2 was present in the nucleus (Fig. 1*B*). Similar Western analysis of the cell line nuclear extracts with H1.2 antibody also confirmed comparable levels of ectopic H1.2 *versus* endogenous H1.2 (Fig. 1*C*), thus minimizing the possibility that ectopic H1.2 nonspecifically interacts with other factors due to its non-physiological concentration. To enhance the purity of the H1.2 complex in our purification, the nuclear extract prepared from the cultured H1.2 cell line was initially fractionated on a P11 phosphocellulose column with increasing salt concentrations (Fig. 1*A*). The 1.2 M fraction containing ectopic H1.2 was further purified by immunoaffinity chromatography using anti-FLAG antibody. An SDS-PAGE analysis of the purified H1.2 complex consistently identified 16 bands that were not observed with the control purification similarly conducted with HeLa nuclear extracts (Fig. 1*E*). In an effort to confirm the stability of the H1.2 complex, we further purified it by ultracentrifugation in a 15–40% glycerol gradient under stringent conditions (250 mM KCl, 0.1% Nonidet P-40). As shown in Fig. 1*G*, the complex sedimented as a single discrete peak in the glycerol gradient (fractions 21–25), suggesting that H1.2 forms a single complex. To define the functional role of H1 as a single protein, His-tagged H1.2 was also expressed in bacteria and purified by three consecutive chromatographies using Ni-NTA, CM-Sephadex C-25, and SP-HP as described under "Experimental Procedures" (Fig. 1*D*). The overall procedures for purification of the H1.2 complex and the recombinant H1.2 are summarized in Fig. 1, *A* and *D*.

To identify the factors present in the H1.2 complex, the major protein bands were excised from the gel and subjected to mass spectrometry analysis. The most prominent proteins identified in our analysis were four endogenous ribosomal proteins (L13a, L7a, L22, and S3) among the cluster of abundant low molecular weight proteins. These results are consistent with recent results from *Drosophila* indicating that H1 interacts with multiple nuclear ribosomal proteins for more efficient repression of transcription (18). In further support of a repressive role for H1 in transcription, four of the proteins (YB1, FIR, PARP1, and PURα) present in the purified complex also belong to the corepressor family of proteins (22, 31–33). Somewhat surprisingly, however, we also found among the pulldown factors ASXL1, nucleolin, β-catenin, and CAPERα, which were originally identified as coactivators in gene activation (34–37). In addition to transcription-related factors, mass spectrometry analysis also identified proteins that have a role in other cellular processes such as cellular protein shuttling (Importin7/90), chromatin signaling (WDR5), protein metabolism (TGase7), apoptosis (heterogeneous nuclear ribonucleoprotein K), and nucleosome formation (H2A/H2B) (38–42). Another interesting finding in the H1.2 complex is the presence of DNA-dependent protein kinase (DNA-PK) and protein phosphatase 1 (PP1), which are known to phosphorylate and dephosphorylate H1, respectively (20, 43). Thus our observations bear an important implication on a possible competitive action of these two activities to regulate H1.2-dependent processes. Our mass spectrometry results were further validated by immunoblot analysis using available antibodies (Fig. 1*F*).

H1.2 Forms a Stable Complex with Its Associated Factors via Direct or Indirect Interactions—To obtain a detailed interaction map of H1.2 with its associated factors, we next analyzed the ability of H1.2 to interact *in vitro* with individual factors. GST-H1.2 fusion proteins were prebound to glutathione-Sepharose beads and incubated with an equimolar amount of each of nine selected factors that were prepared as recombinant (PARP1, FIR, and PURα) or *in vitro* translated (ASXL1, TGase7, CAPERα, β-catenin, YB1, and WDR5) proteins containing FLAG, Xpress, or HA epitope tags at their N termini (see "Experimental Procedures" for details). After extensive washing of the beads, bound proteins were analyzed by Western blot analysis with anti-FLAG, anti-Xpress, or anti-HA antibody. As shown in Fig. 2*B*, H1.2 was able to directly interact with ASXL1, PARP1, FIR, CAPERα, YB1, PURα, and WDR5, but similar experiments with TGase7 and β-catenin did not show any binding to H1.2. The lack of interactions of any of the factors with GST alone further confirmed the specificity of their interactions.

To determine whether H1.2 and its associated factors are also capable of similar interactions *in vivo*, immunoprecipitation was performed with 293T cells transiently expressing FLAG- or Xpress-H1.2 and one of the following: FLAG-PARP1, Xpress-β-catenin, Xpress-TGase7, HA-FIR, Xpress-CAPERα, Xpress-YB1, Xpress-PURα, and Xpress-WDR5 (Fig. 2*C*). The second day after transfection, cell lysates were prepared and

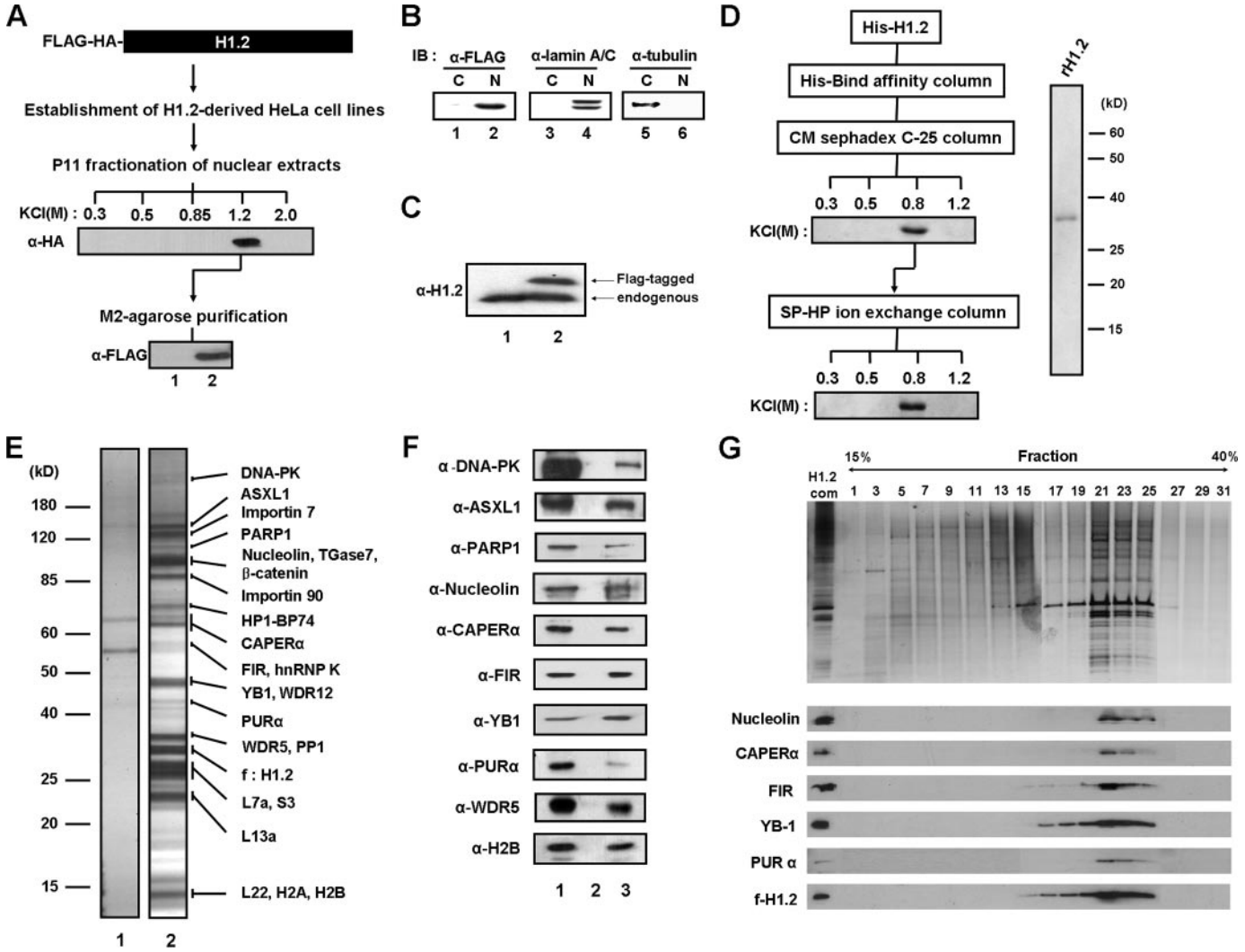
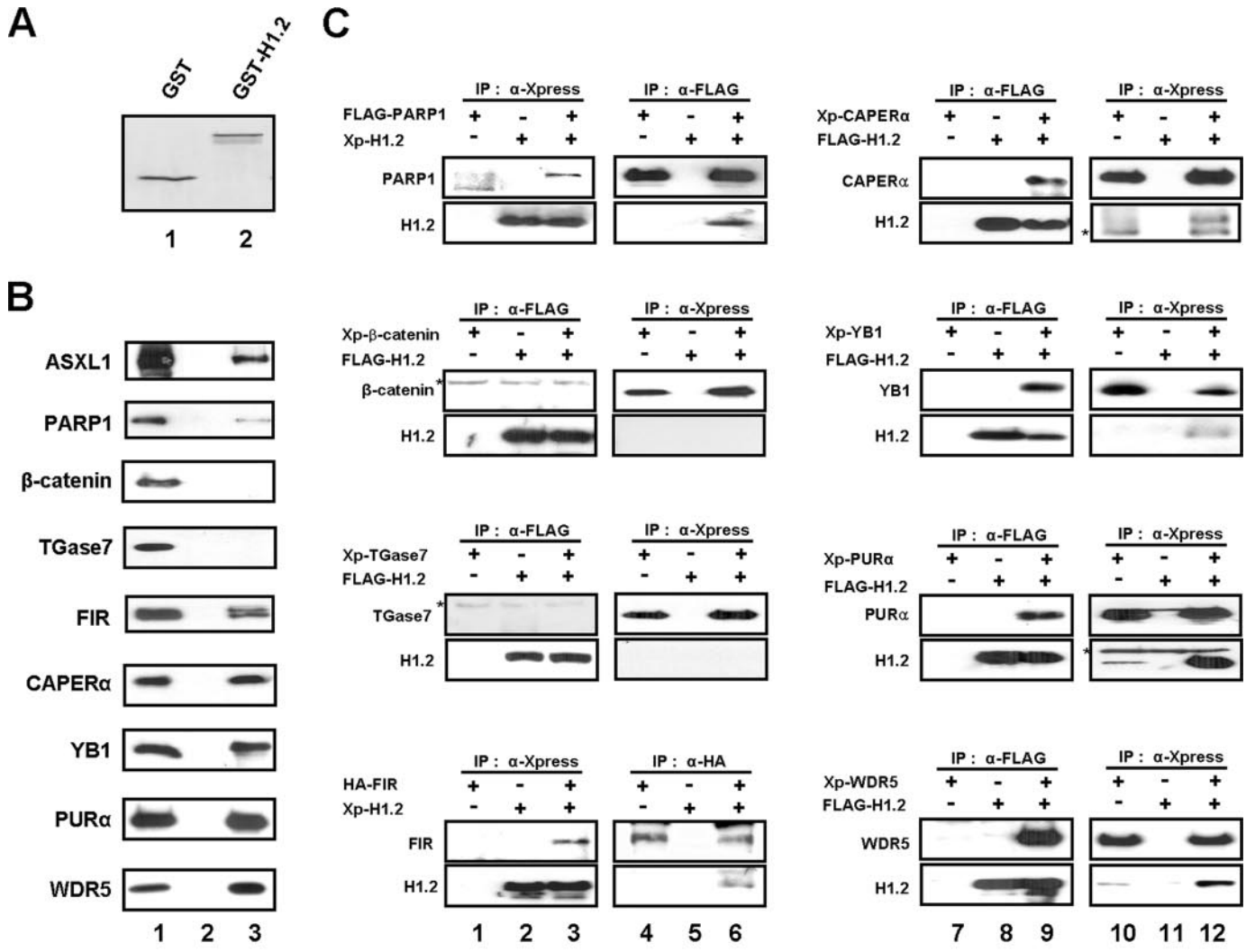


FIGURE 1. **Preparation of H1.2 complex and recombinant H1.2.** *A*, schematic diagram for the purification of H1.2 complex from the stable cell line. *Numbers* indicate the KCl concentration used to purify the individual fractions. Each elution was separated by 4–20% gradient SDS-PAGE and probed with HA and FLAG antibodies as indicated (*lane 2*). The control preparation with normal HeLa nuclear extract is also included (*lane 1*). *B*, subcellular localization of ectopic H1.2. Cytoplasmic and nuclear extracts were prepared as described recently (19) and analyzed by immunoblots with anti-FLAG, anti-lamin A/C, and anti-tubulin antibodies. *C*, relative levels of ectopic H1.2 *versus* endogenous H1.2. Nuclear extracts were prepared from control cells (*lane 1*) and H1.2-expressing cells (*lane 2*), and Western blot analysis was performed with anti-H1.2 antibody. *D*, purification of recombinant H1.2. Recombinant H1.2 (*rH1.2*) was purified as described under "Experimental Procedures." The purity of the purified H1.2 was confirmed by SDS-PAGE and Coomassie staining analysis. *E*, mass spectrometric identification of H1.2 complex. After H1.2 complex was fractionated by 4–20% gradient SDS-PAGE, bands were excised and subjected to mass spectrometry analysis as described under "Experimental Procedures." Identified components of H1.2 complex are indicated on the *right*. Molecular mass markers are indicated on the *left*. *Lane 1*, mock-purified control; *lane 2*, H1.2 complex. *F*, immunoblot confirmation of identified factors. Purified H1.2 complex was separated by 4–20% gradient SDS-PAGE, and the presence of selected factors was analyzed with the indicated antibodies. *Lane 1*, HeLa nuclear extract input; *lane 2*, mock-purified control; *lane 3*, H1.2 complex. *G*, glycerol gradient centrifugation of H1.2 complex. H1.2 complex, purified on phosphocellulose P11 and anti-FLAG antibody affinity columns, was separated by 15–40% glycerol gradient centrifugation as described under "Experimental Procedures." Fractions were loaded onto a 4–20% SDS-polyacrylamide gel, and proteins were detected by silver staining (*uppermost panel*) or Western blot (*six lower panels*). *IB*, immunoblot; *C*, cytoplasmic extract; *N*, nuclear extract; *DNA-PK*, DNA-dependent protein kinase; *com*, complex; *hnRNP K*, heterogeneous nuclear ribonucleoprotein K; *f*, FLAG.

subjected to immunoprecipitation of H1.2 with anti-FLAG or anti-Xpress antibody, and interactions of co-expressed factors were further analyzed by immunoblotting with antibodies specific to epitopes within the factors. As shown in Fig. 2*C*, immunoprecipitation of H1.2 resulted in the co-precipitation of PARP1, FIR, CAPERα, YB1, PURα, and WDR5 (*lanes 1–3* and *7–9*), thus confirming their physical interaction with H1.2 in cellular conditions. However, consistent with our *in vitro* results, Western blot analysis showed no detectable interaction of H1.2 with TGase7 and β-catenin. Reverse immunoprecipitation analysis using antibodies specific for associated factors (*lanes 4–6* and *10–12*) also showed the same interaction of H1.2 with the factors, further confirming the specificity of their interactions with H1.2.

To further support interaction between H1.2 and its associated factors, we next performed cellular co-localization analysis. Plasmids encoding EGFP-H1.2 and epitope-tagged factors (Xpress-ASXL1, FLAG-PARP1, HA-FIR, Xpress-CAPERα, Xpress-YB1, Xpress-PURα, and Xpress-WDR5) were constructed and co-transfected into HeLa cells, and their cellular localizations were analyzed by fluorescence confocal microscopy. Consistent with results from the H1.2 stable cell line (Fig.


A
GST
GST-H1.2
1
2
B
ASXL1
PARP1
β-catenin
TGase7
FIR
CAPERα
YB1
PURα
WDR5
1 2 3
C
IP : α-Xpress
IP : α-FLAG
FLAG-PARP1
Xp-H1.2
PARP1
H1.2
Xp-CAPERα
FLAG-H1.2
CAPERα
Xp-β-catenin
β-catenin
Xp-YB1
YB1
Xp-TGase7
TGase7
Xp-PURα
PURα
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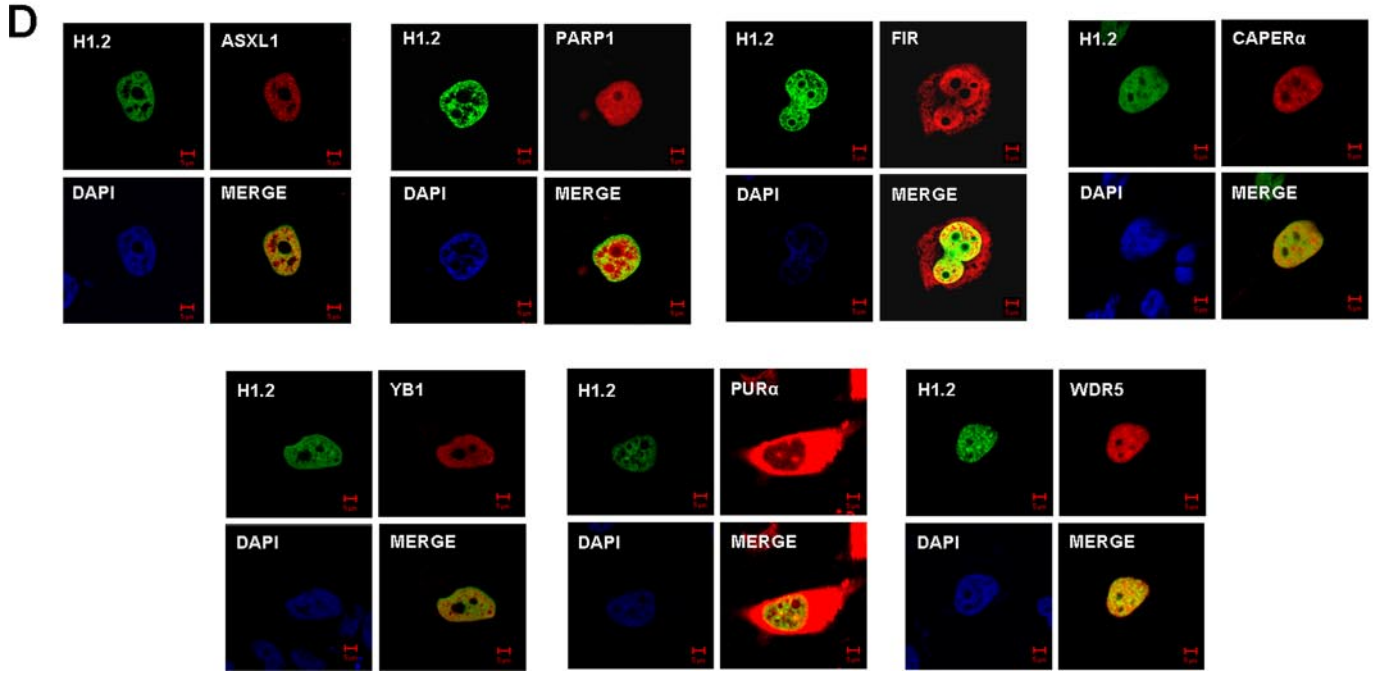
HA-FIR
FIR
Xp-WDR5
WDR5
1 2 3 4 5 6 7 8 9 10 11 12


D
H1.2
ASXL1
PARP1
FIR
CAPERα
YB1
PURα
WDR5
DAPI
MERGE

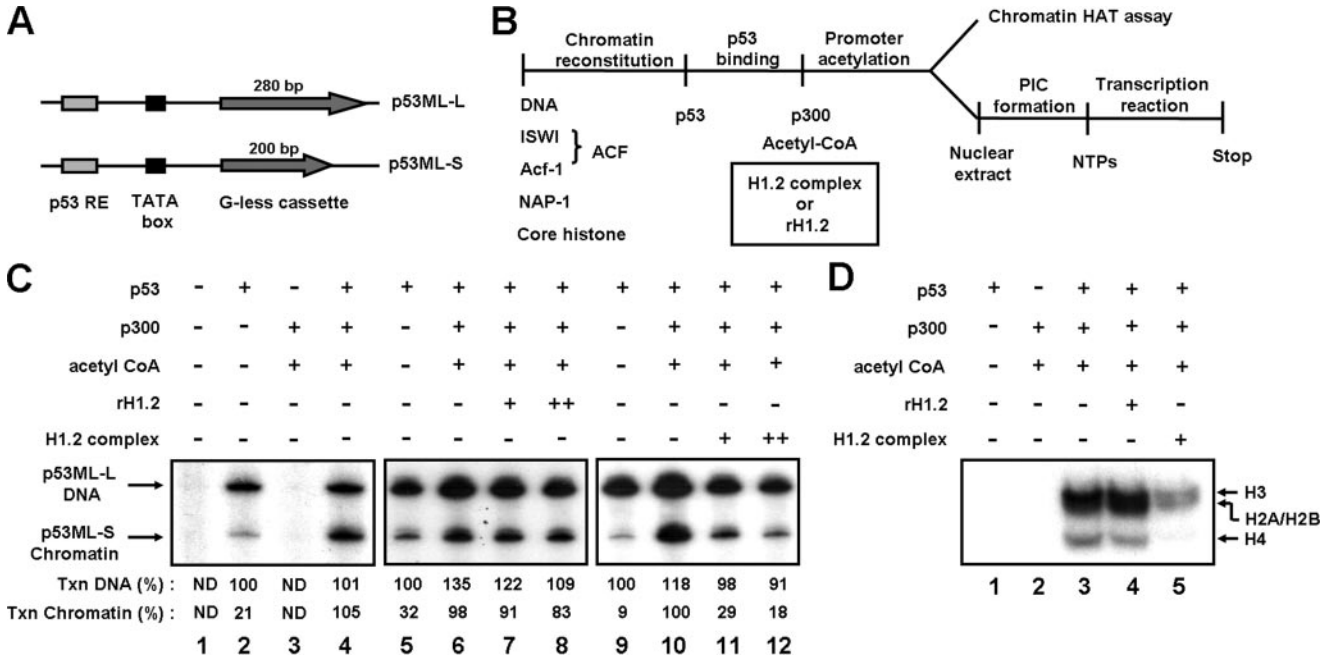


FIGURE 3. **Repressive effects of H1.2 complex in chromatin acetylation and transcription.** *A*, schematic representation of transcription templates. *Arrows* indicate the length of DNA to be transcribed. *p53 RE*, p53 response element. *B*, schematic summary of transcription and chromatin histone acetyltransferase (*HAT*) assays. *NTPs* and *PIC* indicate nucleotide triphosphates and preinitiation complex, respectively. *C*, transcription assays with recombinant H1.2 and H1.2 complex. p53ML-S chromatin template (40 ng) and p53ML-L DNA (40 ng) were transcribed with p53 (15 ng), p300 (20 ng), and/or acetyl-CoA (10 μM) as summarized in *B* and as described recently (29). H1.2 complex (200 and 400 ng) and recombinant H1.2 (40 and 80 ng of H1.2 mixed with 160 and 320 ng of BSA) were used in transcription. Note that results were obtained from three separate transcription experiments as indicated by *boxes*. Data were quantitated by PhosphorImager and normalized to reactions with p53ML-L DNA and p53 alone (100%). *D*, chromatin histone acetyltransferase assays with recombinant H1.2 and H1.2 complex. Chromatin template (100 ng) was incubated with recombinant H1.2 (80 ng of H1.2 mixed with 320 ng of BSA) or H1.2 complex (400 ng) in the presence of p53 (30 ng), p300 (40 ng), and 2.5 μM [^{3}H]acetyl-CoA. *Txn*, relative transcription levels; *ND*, nondetectable; *ACF*, ATP-utilizing chromatin assembly and remodeling factor.

1), our fluorescence microscopy specifically localized H1.2 to the cell nucleus as bright green spots in all cases. Indirect immunofluorescence studies also produced the positive red staining for ASXL1, PARP1, CAPER-α, YB1, and WDR5 mostly in the nucleus, whereas similar studies detected FIR in both the cytoplasm and nucleus. In contrast, minimal localization of PURα in nucleus was detected (Fig. 2*D*) possibly due to the lack of its phosphorylation, which is known to govern its nuclear localization (44). Next co-localization of H1.2 with expressed factors was examined by superimposing the green and red optical channels produced by H1.2 and its associated factors. H1.2 displayed localization patterns similar to those of ASXL1, PARP1, FIR, CAPERα, YB1, and WDR5 within the nucleus. Although PURα was visualized primarily in the cytoplasm, there was also considerable yellow staining in the nucleus, indicating that at least nuclear PURα can be co-localized with H1.2. These results suggest that H1.2 proteins can be associated with its associated proteins *in vivo*.

H1.2 Complex Represses p53-dependent Chromatin Transcription—The finding that specific regulatory factors are associated with H1.2 prompted us to determine whether these factors have any effect on transcription. To investigate the effect on DNA and chromatin transcription at the same time, two different transcription templates whose transcription is dependent on p53 were prepared: p53ML-S producing a 200-nucleotide long transcript and p53ML-L producing a 280-nucleotide long transcript (Fig. 3*A*). Chromatin was assembled only on p53ML-S DNA with recombinant core histones using recombinant ATP-utilizing chromatin assembly and remodeling factor (ACF) and NAP1. The transcription reaction contained equal amounts of p53ML-S chromatin (40 ng) and p53ML-L DNA (40 ng) templates to simultaneously monitor alteration of chromatin and

FIGURE 2. **Interaction of H1.2 with its associated factors.** *A*, purification of GST-H1.2 fusion protein. GST or GST-H1.2 was purified as described under "Experimental Procedures." The purity of the proteins was analyzed by 15% SDS-PAGE and Coomassie staining analysis. *Lane 1*, GST; *lane 2*, GST-H1.2. *B*, *in vitro* binding assay of H1.2 and its associated factors. *In vitro* translated proteins (Xpress-ASXL1, Xpress-β-catenin, Xpress-TGase7, Xpress-CAPERα, Xpress-YB1, and Xpress-WDR5) and recombinant proteins (FLAG-PARP1, HA-FIR, and PURα) were incubated with GST or GST-H1.2 as described under "Experimental Procedures." After extensive washing, bound proteins were analyzed by immunoblot with anti-Xpress (for ASXL1, β-catenin, TGase7, CAPERα, YB1, and WDR5), anti-HA (for FIR), anti-PARP1, and anti-PURα antibodies. *Lane 1*, 10% input; *lane 2*, GST alone; *lane 3*, GST-H1.2. *C*, *in vivo* binding assay of H1.2 and its associated factors. FLAG- or Xpress-tagged H1.2 was transiently transfected with its associated factors (FLAG-, HA-, or Xpress-tagged), and immunoprecipitation was performed as described under "Experimental Procedures." *Lanes 1*, *4*, *7*, and *10*, factor-only expression; *lanes 2*, *5*, *8*, and *11*, H1.2-only expression; *lanes 3*, *6*, *9*, and *12*, H1.2 and factor co-expression. *Asterisks* indicate nonspecific bands. *D*, cellular co-localization of H1.2 with its associated factors. HeLa cells were transfected with expression vectors encoding EGFP-H1.2 and Xpress-ASXL1, FLAG-PARP1, HA-FIR, Xpress-CAPERα, Xpress-YB1, Xpress-PURα, or Xpress-WDR5 as indicated. Cells were fixed with paraformaldehyde and immunostained with anti-FLAG, anti-Xpress, or anti-HA antibody followed by the Cy3-conjugated secondary antibodies. After mounting on glass slides with VECTASHIELD with 4′,6-diamidino-2-phenylindole (*DAPI*) (Vector Laboratories), confocal microscopy was performed as detailed under "EXPERIMENTAL Procedures." H1.2 is stained *green*, and its associated factor is stained *red*. Nucleus is stained *blue*, and co-localizations of H1.2 and its associated factors are shown in *MERGE*. The *scale bar* represents 5 μm. *IP*, immunoprecipitate; *Xp*, Xpress.

DNA transcription. Transcription assays were carried out with p53, p300, and acetyl-CoA as described recently (28) except that the H1.2 complex or H1.2 was added together with p300 and acetyl-CoA as summarized in Fig. 3*B*. Transcription from a chromatin template was completely dependent upon p53, p300, and acetyl-CoA, whereas transcription from a histone-free DNA template was activated only by p53, independent of p300 and acetyl-CoA (Fig. 3*C*, *lanes 1–4*). As shown in Fig. 3*C*, H1.2 alone showed only a slight inhibitory effect on transcription of both chromatin and DNA templates at the highest concentrations tested (*lanes 5–8*). In contrast, when we extended our assays to the H1.2 complex, a significant inhibitory effect on chromatin transcription was observed (*lanes 9–12*). Similar experiments with DNA templates failed to reveal any distinct effects of the H1.2 complex on DNA transcription (*lanes 9–12*). Addition of the H1.2 complex prior to p53 and/or p300 also reduced transcription to a level comparable to that observed following simultaneous addition of the H1.2 complex and p300 (data not shown).

Recent studies proved the contribution of histone acetylation *per se* in p300-mediated chromatin transcription (45). Thus, a possible interpretation of our transcription results is that the H1.2 complex represses p300-mediated acetylation at the promoter region after its recruitment by p53. This possibility was investigated by checking whether the H1.2 complex is an efficient repressor of p300-mediated acetylation of chromatin. Consistent with recent results, p300-mediated acetylation was completely dependent on p53, which is known to recruit p300 for promoter-targeted acetylation (Fig. 3*D*, *lane 3*) (45). In further analysis with the H1.2 complex, we observed a significant inhibition of p300-mediated acetylation of chromatin templates (*lane 5*). When the same concentration of recombinant H1.2 was examined, only a slight inhibitory effect was detected (*lane 4*). Collectively these results demonstrate that the H1.2 complex can repress p53-dependent transcription from chromatin by down-regulating p300-mediated acetylation of chromatin.

H1.2 Directly Interacts with p53 via Its C-terminal Domain—To investigate whether the H1.2 complex inhibits p53-mediated transcription by a possible interaction between H1.2 and p53, we also performed a series of *in vitro* protein-protein interaction assays. In initial experiments, purified FLAG-p53 protein was incubated with GST-H1.2 full length, GST-H1.2 N-terminal domain, GST-H1.2 globular domain, or GST-H1.2 C-terminal domain that was immobilized on glutathione-Sepharose beads. After rigorous washing, p53 binding was analyzed by Western blot analysis using anti-FLAG antibody. As shown in Fig. 4*A*, p53 can bind to the full-length and C-terminal domains (*lanes 10* and *13*), but not to the N-terminal and globular domains (*lanes 11* and *12*), of H1.2. In mapping the region of p53 required for H1.2 binding, we also found that H1.2 interacts with the p53 C-terminal domain (*lane 18*) but not with the p53 N-terminal and DNA binding domains (*lanes 16* and *17*). On the basis of ability of p53 to directly interact with H1.2, we also checked whether p53 can interact with the entire H1.2 complex via its recognition of H1.2 present in the complex. Thus immobilized GST-p53 was incubated with the purified H1.2 complex, and factors bound to p53 were identified by Western blot analysis. As expected, we found that the entire H1.2 complex is indeed able to bind to GST-p53 but not to GST alone (Fig. 4*B*).

To confirm these *in vitro* results *in vivo*, we transiently expressed Xpress-H1.2 and FLAG-p53 in 293T cells for immunoprecipitation. As shown in Fig. 4*C*, FLAG-p53 was co-immunoprecipitated from cells in an Xpress-H1.2-dependent manner (*lane 3*) but not from control cells that received the control empty plasmid (pcDNA3.1/His) (*lane 1*). These results were further confirmed by an inverse experiment in which the cell lysate was subjected to immunoprecipitation with an anti-FLAG antibody to precipitate p53 (*lane 6*). To define the region of H1.2 necessary for p53 binding, several H1.2 deletion mutants were also analyzed for their ability to interact with p53. Consistent with the *in vitro* binding data, H1.2 mutant in which the N-terminal region (amino acids 1–34) was deleted still retained the ability to bind to p53 (*lane 13*). However, when the C-terminal region (amino acids 110–213) of H1.2 was deleted (H1.2 ΔCT), no binding of H1.2 to p53 was observed (*lane 14*), indicating that the C-terminal domain (amino acids 110–213) of H1.2 is required for p53 association. Indeed an H1.2 mutant without both N-terminal and globular domains of H1.2 showed p53 binding comparable to that observed with full-length H1.2 (*lane 15*). To determine the H1.2-binding region of p53, we also analyzed one p53 mutant containing only C-terminal domain and two mutants lacking N- and C-terminal domains. Consistent with *in vitro* results (Fig. 4*A*), a p53 C-terminal deletion mutant (amino acids 1–300) showed no interaction with H1.2 (*lane 20*), whereas an N-terminal deletion mutant (amino acids 81–393) showed a wild type level of H1.2 binding (*lane 19*). p53 C-terminal domain also showed a strong binding to H1.2 similar to that of full-length p53 (*lane 21*), arguing that p53 C-terminal domain specifically interacts with H1.2 C-terminal domain. To further verify cellular interaction between H1.2 and p53 in physiological conditions, we immunoprecipitated 293T cell lysates with anti-p53 antibody and examined the co-immunoprecipitation of endogenous H1.2. In addition to p53, we also could confirm the presence of H1.2 in our immunoprecipitates (Fig. 4*D*).

We next checked whether p53 can interact with the entire H1.2 complex. FLAG-p53 was co-expressed with Xpress-H1.2 in 293 cells, and cell extracts were prepared and subjected to immunoprecipitation with FLAG antibody. We checked bound proteins by Western blot analysis. As shown in Fig. 4*E*, FLAG-p53 was co-immunoprecipitated with nucleolin, FIR, YB1, PURα, and WDR5 in Xpress-H1.2-expressing cells (*lane 3*), but none of these proteins could be found in the control precipitation with mouse IgG (*lane 2*) confirming specificity of the precipitation. Together these experiments demonstrate the interaction of the H1.2 complex with p53 *in vivo* and *in vitro* that seems to be mediated through a direct interaction between p53 C-terminal domain and H1.2 C-terminal domain.

Repression of p53-dependent Transcription by H1.2 Requires YB1 and PURα—Although the H1.2 complex could repress p53-dependent, p300-mediated chromatin transcription, it is unclear which factors are mainly involved in this repression. Recent studies indicated that YB1 can down-regulate p53-induced transactivation of genes involved in the apoptotic proc-

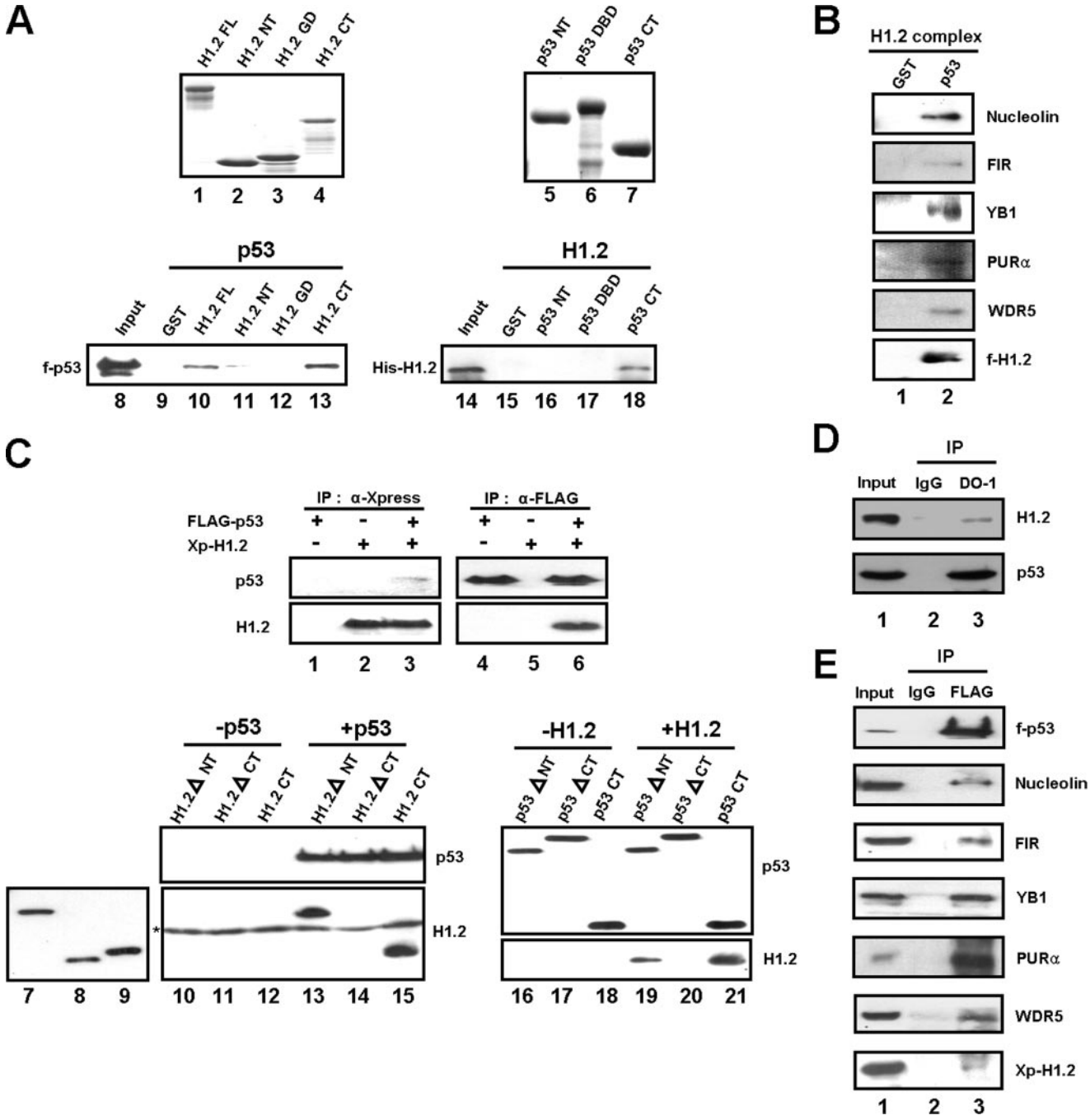
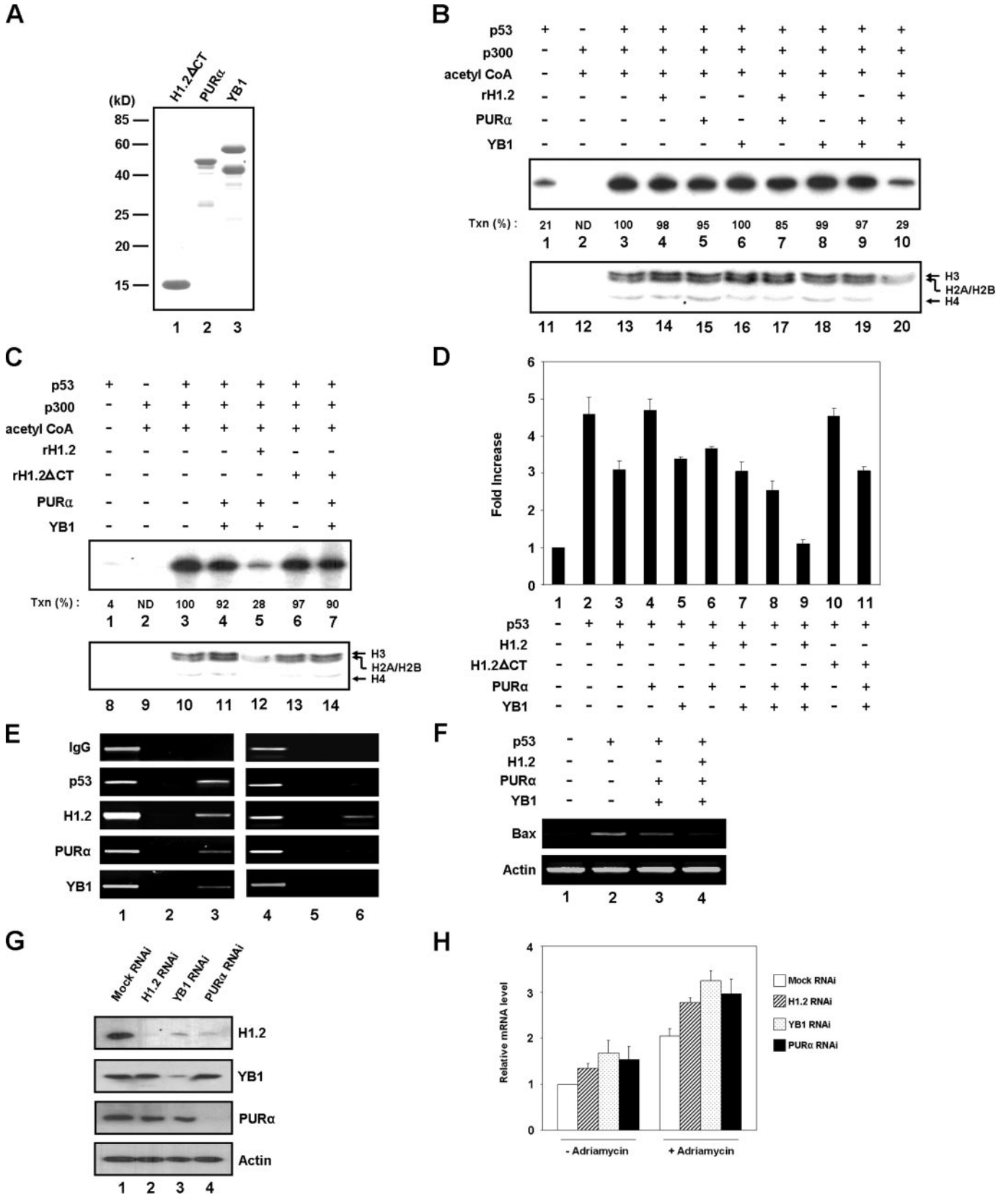


FIGURE 4. **Direct interaction of H1.2 with p53.** *A*, p53 interaction with H1.2 *in vitro*. GST-H1.2 mutants (*lanes 1–4*) or GST-p53 mutants (*lanes 5–7*) were analyzed by SDS-PAGE and Coomassie staining analysis. For interaction studies, GST-H1.2 mutants and GST-p53 mutants were incubated with FLAG-tagged p53 and His-tagged H1.2, respectively. After washing, binding of p53 and H1.2 was analyzed by Western blot analysis with anti-FLAG or anti-His antibody. *Lane 1*, GST-H1.2 full length (*FL*; amino acids 1–213); *lane 2*, GST-H1.2 NT (amino acids 1–34); *lane 3*, GST-H1.2 globular domain (*GD*; amino acids 35–109); *lane 4*, GST-H1.2 CT (amino acids 110–213); *lane 5*, GST-p53 NT (amino acids 1–83); *lane 6*, GST-p53 DNA binding domain (*DBD*; amino acids 120–290); *lane 7*, GST-p53 CT (amino acids 290–393); *lanes 8* and *14*, 10% input of FLAG-p53 and His-H1.2; *lanes 9* and *15*, GST control; *lanes 10–13*, p53 bound to GST-H1.2 mutants; *lanes 16–18*, H1.2 bound to GST-p53 mutants. *B*, p53 interaction with H1.2 complex *in vitro*. GST (*lane 1*) or GST-p53 full length (*lane 2*) was incubated with H1.2 complex, and pulldown fractions were analyzed by Western blot analysis using the indicated antibodies. *C*, p53 interaction with H1.2 *in vivo*. H1.2 and p53 were expressed in 293T cells and immunoprecipitated using anti-FLAG and anti-Xpress antibodies as indicated (*lanes 1–6*). Similar experiments were also performed after expression of p53 and H1.2 deletion mutants as described under "Experimental Procedures" (*lanes 10–21*). *Lanes 1* and *4*, p53-only expression; *lanes 2* and *5*, H1.2-only expression; *lanes 3* and *6*, p53 and H1.2 co-expression; *lanes 7–9*, expressed H1.2 mutants in whole cell lysates; *lanes 10–12*, H1.2-only controls; *lanes 16–18*, p53-only controls; *lanes 13–15* and *19–21*, H1.2 mutants and p53 mutants co-expressions. The *asterisk* indicates a nonspecific band containing IgG light chain. *D*, mutual interaction of endogenous p53 and H1.2. Whole cell extracts from 293T cells were immunoprecipitated with anti-p53 antibody (*DO-1*) and analyzed by Western blotting with anti-H1.2 and anti-p53 antibodies as indicated. *Lane 1*, whole cell lysate; *lane 2*, control IgG; *lane 3*, anti-p53 precipitates. *E*, p53 interaction with H1.2 complex *in vivo*. 293T cells were transfected with FLAG-tagged p53 and Xpress-tagged H1.2-expressing plasmids, and cell lysates were prepared 2 days after transfection. Lysates were immunoprecipitated with anti-FLAG and analyzed by Western blot analysis using the indicated antibodies. *Lane 1*, whole cell lysate; *lane 2*, control IgG; *lane 3*, anti-FLAG precipitates. *IP*, immunoprecipitate; *Xp*, Xpress; *f*, FLAG.

ess by its interaction with p53 (46). Results from co-immunoprecipitation analysis also suggested that YB1 can directly interact with PURα for their functional synergy (33, 47). Thus, having found that H1.2 can also interact with both YB1 and PURα in our studies (above), we asked whether YB1 and PURα together with H1.2 can repress p53-dependent, p300-mediated



chromatin acetylation and transcription as observed with the entire H1.2 complex. We first checked the effects of H1.2, YB1, and/or PURα on chromatin transcription by using recombinant H1.2, YB1, and PURα (Figs. 1*D* and 5*A*). As shown in Fig. 5*B*, the p300 stimulatory effect on p53-induced transcription was unaffected by YB1, PURα, or H1.2 (*lanes 4–6*). Similar experiments with pairwise combinations of H1.2, YB1, and PURα also showed no detectable change in transcription (*lanes 7–9*). However, simultaneous addition of H1.2, YB1, and PURα resulted in a significant repression of transcription, supporting functional cooperativity of H1.2, YB1, and PURα for transcription repression (*lane 10*). To test the possibility that transcription repression by H1.2, YB1, and PURα might reflect their repressive action on chromatin acetylation, we also assessed their effect on p53-dependent acetylation of chromatin by p300. Chromatin acetylation was significantly repressed when H1.2, YB1, and PURα were added together (*lane 20*) but not when the three proteins were added individually or in pairs (*lanes 14–19*). Because the C-terminal domain of H1.2 was required for p53 interaction (Fig. 4), we next tested the effect of H1.2 C-terminal deletion on chromatin transcription and acetylation. As expected, deletion of H1.2 C-terminal domain significantly compromised the repressive effects of H1.2, YB1, and PURα on p53-dependent, p300-mediated chromatin acetylation and transcription (Fig. 5*C*).

To validate the conclusions from the *in vitro* studies described above, p53-deficient H1299 cells were transfected with p53 expression vector and luciferase reporter construct (derived from *Bax* gene) along with plasmids expressing H1.2, YB1, and PURα, and luciferase reporter assays were carried out at 48 h after transfection. In agreement with our *in vitro* results, expression of H1.2, YB1, and PURα showed a severe repression of *Bax* reporter gene transcription mediated by p53 (Fig. 5*D*, *lane 9*). However, individual or pairwise expression of the three proteins minimally disrupted p53-induced transcription in all cases (*lanes 3–8*), again indicating their cooperative action in this inhibitory process. Furthermore no significant repression of p53-induced transcription was observed with C terminus-deleted H1.2, YB1, and PURα (*lane 11*). These results are consistent with the results of the *in vitro* analyses (Fig. 5, *B* and *C*) and suggest that H1.2 C-terminal domain is critical for optimal activities of H1.2, YB1, and PURα.

In view of the significant effects of H1.2, YB1, and PURα on p53 transcription, ChIP analysis was also performed in p53-deficient H1299 cells that were transfected with p53. Due to the unavailability of ChIP grade antibodies against H1.2, YB1, and PURα, we expressed GAL4-H1.2, FLAG-YB1, and Xpress-PURα. We checked the recruitment of expressed proteins to the *Bax* p53 response element and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) minimal promoter. As shown in Fig. 5*E*, our results show the significant level of p53 as well as recruitment of H1.2, YB1, and PURα at the response element region of the *Bax* gene. In contrast, similar ChIP analyses on GAPDH promoter did not detect YB1 and PURα but showed the level of H1.2 to be comparable to that observed in the *Bax* gene most likely due to its global localization to alter chromatin structure. These results were reliable because cells transfected with an empty vector did not show any detectable precipitation of DNA fragments. Consistent with ChIP results, RT-PCR analysis also confirmed that H1.2, YB1, and PURα are capable of significantly repressing p53-induced transcription of the *Bax* gene (Fig. 5*F*, *lane 4*). By contrast, YB1 and PURα in the absence of H1.2 showed a modest repressive effect on transcription (*lane 3*) probably resulting from the minimal action of endogenous H1.2.

To further confirm the repressive role of YB1, PURα, and H1.2 on transcription of *Bax* gene, we knocked down expression of H1.2, YB1, and PURα in human U2OS osteosarcoma cells by stably transfecting shRNAs targeting YB1, PURα, or H1.2 (Fig. 5*G*). The cell strain expressing a vector without an shRNA molecule was used as a control (*lane 1*). Our Western analysis confirmed that the cell strain expressing the H1.2 shRNA molecule expressed a much lower level of H1.2 compared with the level in the control cell strain (*lane 2*). Interestingly YB1 and PURα shRNAs repressed expression of YB1 and PURα, but they also had an effect on the level of H1.2 (*lanes 3* and *4*) perhaps because YB1 and PURα could positively regulate H1.2 expression by binding to the CCAAT box present in the H1.2 gene promoter (*lanes 3* and *4*) (48, 49). We then checked the effect of depletion of H1.2, YB1, or PURα on transcription of *Bax* gene with or without DNA damage induced by adriamycin treatment. Albeit to a different extent, the three cell strains showed up-regulation of *Bax* gene transcription in all cases (Fig. 5*H*), strongly suggesting that YB1, PURα, and H1.2 are necessary for optimal repression of *Bax* gene.

DISCUSSION

Previous studies addressing the role of H1 in transcription have focused on identifying its properties in the formation and

FIGURE 5. **Functional characterization of H1.2, PURα, and YB1.** *A*, analysis of recombinant proteins. Recombinant C-terminal tailless H1.2, PURα, and YB1 were purified as described under "Experimental Procedures." The purity of the purified proteins was confirmed by SDS-PAGE and Coomassie staining analysis. *B*, repressive action of H1.2, PURα, and YB1 in p300-mediated, p53-dependent transcription. Chromatin template was transcribed with recombinant H1.2 (50 ng), PURα (100 ng), and/or YB1 (100 ng) as indicated. Final protein concentrations in all reactions were adjusted to be identical by including BSA. *C*, requirement of H1.2 C terminus for transcriptional repression. Transcription assays were performed as in *B* but with mutant H1.2 in which the C-terminal tail was deleted. *D*, effect of H1.2, PURα, and YB1 on p53-dependent transcription *in vivo*. H1299 cells were transiently transfected with *Bax* reporter gene together with vectors that express p53, PURα, YB1, and/or wild type/C-terminal tailless H1.2 as indicated. Data are represented as the means ± S.E. of three independent experiments. *E*, ChIP analysis of *Bax* promoter. H1299 cells were mock-transfected (*lane 2*) or transfected with p53, H1.2, PURα, and YB1 (*lane 3*) and subjected to ChIP analysis with antibodies specific for the indicated proteins. A sample containing 5% total input chromatin was also included for each ChIP assay (*lane 1*). Similar ChIP experiments on the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) minimal promoter region were also included as a control (*lanes 4–6*). *F*, RT-PCR analysis of *Bax* RNA. H1299 cells were transfected with p53, H1.2, PURα, and/or YB1 as indicated. RT-PCR was performed on total RNA isolated from transfected or mock-transfected cells. RT-PCR of actin RNA was used as a loading control. *G*, validation of H1.2, PURα, and YB1 knockdown. Cells were stably transfected with shRNA of H1.2, YB1, or PURα, and expression of targeted proteins was checked by Western blot analysis of cell lysates with anti-H1.2, anti-YB1, and anti-PURα antibodies (*lanes 2–4*). As a control, cells were also transfected with a mock shRNA vector (*lane 1*). *H*, up-regulation of *Bax* gene transcription upon H1.2, PURα, and YB1 knockdown. RNA was extracted from cells transfected with shRNA of H1.2, YB1, or PURα, and relative changes in expression of *Bax* gene were assessed by real time PCR. *Txn*, relative transcription levels; *RNAi*, RNA interference; *ND*, nondetectable; *rH1.2*, recombinant H1.2.

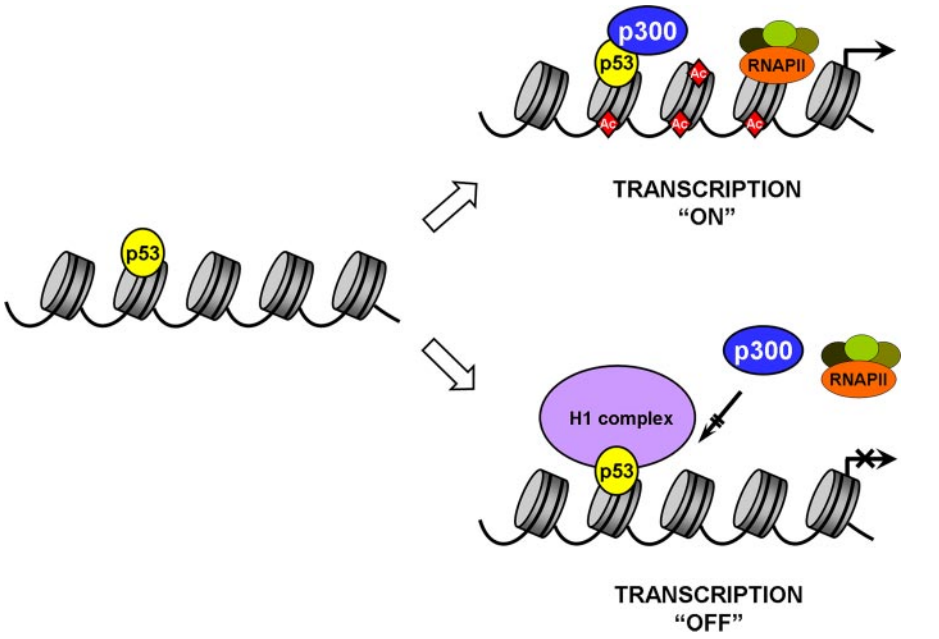


FIGURE 6. **Model for the promoter-selective inhibition of p53-dependent transcription by H1 complex.** We propose that the H1.2 complex binds to p53 and disrupts p53-mediated recruitment of chromatin-regulating factors to the promoter of target genes. This repressive chromatin state will in turn interfere with the formation of functional preinitiation complexes at the promoter to block gene transcription (see "Discussion" for details). *Ac*, acetylation; *RNAPII*, RNA polymerase II.

maintenance of condensed chromatin structure that could globally inhibit transcription initiation (7, 50, 51). However, there have been an increasing number of examples in which H1 plays a more specific role in transcription by differentially acting at the level of individual genes. The original model for this H1 specificity is based on the results obtained from gene knock-out experiments in *Tetrahymena*, fungus, and yeast (15, 52, 53). These studies showed that deletion of H1 gene keeps the organism alive, but specific subsets of genes are differentially regulated. Other studies with higher eukaryotes also showed that linker histones have gene selectivity in their repressive actions as shown in the expression of the *MyoD*, *Xbra*, and *Bmp-4* genes (16, 54). Moreover a similar specificity of H1 has been shown in a recent study that revealed that simultaneous inactivation of three of six H1 subtype genes does not influence global transcription but primarily affects the activity of specific genes (14). These gene-specific effects of H1 might result from its interaction with sequence-specific DNA-binding proteins or specific regulatory factors as has been shown with Msx1, BAF, SirT1, HP1, and DFF40 (16, 55–58).

As a major step toward investigating the effect of H1 on a specific transcription pathway, we sought to determine whether H1 can stably interact with any other proteins with activities potentially critical for its repressive action on transcription. The significant feature of the present study is the purification, identification, and characterization of the H1.2 complex, acting as a repressor of p53-dependent, p300-mediated transcription from chromatin. Our discovery that p300-mediated chromatin acetylation was significantly repressed by the H1.2 complex raises the possibility that it prevents p300 from being recruited to the promoter region by p53. Another possibility is that the H1.2 complex does not affect recruitment of p300 but diminishes accessibility of core histone tails by inducing localized compaction of chromatin. However, given the demonstration of a direct interaction of H1.2 with p53, it is likely that H1.2 acts as an anchoring protein for other regulatory factors that prevent p53-dependent recruitment of p300 (Fig. 6). In all cases, H1.2 itself showed minimal effects on chromatin acetylation and transcription, further confirming that factors co-purified with H1.2 play a key role in the repressive action of H1.2. This finding is somewhat surprising because previous *in vitro* studies showed that H1 itself is capable of repressing chromatin remodeling and transcription (7, 9). This may reflect the use of a higher concentration of H1 in reactions that will result in reorganization of overall chromatin structure. In fact, we also could detect partial repression of transcription at a molar ratio higher than two H1 molecules per nucleosome (data not shown). In this regard, some distinctions need to be made between the repressive effect of the H1.2 complex that we purified and the previously reported effect of H1 as a single structural protein.

Having found that H1.2 engages multiple factors for its repressive action on p53-mediated transcription, our next question was whether H1.2 requires any specific factors to elicit its repressive activity. To investigate this potentially important aspect, we selected PURα and YB1 for functional reconstitution of the entire H1.2 complex. Because YB1 and H1.2 can interact with p53 (Ref. 46 and Fig. 4, *A* and *C*) and PURα can stably associate with p53 (Fig. 4*E*), it is possible that PURα and YB1 can coordinate the repressive action of H1.2 in transcription. Indeed our analysis revealed that H1.2, PURα, and YB1 can closely recapitulate repressive effects of the entire H1.2 complex by blocking p300-mediated chromatin acetylation. These data suggest that the repressive action of the H1.2 complex may be mediated by a subset of factors at least one of which is H1.2.

Given that several other factors associated with H1.2 are also known as a repressor of other activators and genes, it will be interesting to sort out key factors involved in various repressive processes in our future study. For example, our finding that PARP1 is a component of the H1.2 complex implies that PARP1 may participate as a key factor in H1-induced chromatin repression. Because PARP1 can physically interact with H1.2 (Fig. 2) and p53 (59, 60), it probably can function as a repressor by facilitating H1.2 interaction with p53. Our results appear to be contrary to the recent report indicating that H1 and PARP1 exclusively reside in distinct chromatin domains (31). However, this difference could simply be explained by the fact that the previous study was conducted with the mixture of all H1 subtypes, whereas the present study was undertaken with one of the subtypes, H1.2. Thus our results can be interpreted as a

consequence of specific interaction of PARP1 with H1.2 among all subtypes. It is also interesting to note that the H1.2 complex contains several cofactors (*e.g.* CAPERα and nucleolin) that are known to activate transcription, but transcription is still significantly repressed by the H1.2 complex. These results may be due to the use of specific transcription reactions in our studies. For example, previous studies used estrogen receptor as an activator to show coactivator function of CAPERα (37), whereas our studies used p53 to study the effect of the factors. We assume that CAPERα in the H1.2 complex minimally contributes to H1.2 action on p53-dependent transcription, allowing other factors to retain their repressive action in transcription. Therefore it will be interesting to test the functional contribution of the H1.2 complex in transcription induced by various activators in future studies.

Another interesting finding is the purification of free H2A and H2B as components of the H1.2 complex. Although we do not have a clear explanation, the crystallographic structure of the nucleosome indicates that the C-terminal domain of H2A is localized in close proximity to linker DNA where H1 proteins are preferentially localized (38). Thus our results bear an important implication on the possible ability of the C-terminal domain of H2A to interact with H1.2, which will affect the binding of H1.2 to the nucleosome as a structural component. Furthermore in view of the diversity in amino acid sequence and regulation of the synthesis of H1 subtypes (10) as well as the difference in their distribution with respect to particular genes (8, 11), it will be important to check whether individual subtypes may have nonredundant functions in the regulation of specific genes by associating with distinct factors. The ability to purify factors associated with different H1 subtypes and to analyze their function will be most useful to address these questions.

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