Stimulation of DNA Replication from the Polyomavirus Origin by PCAF and GCN5 Acetyltransferases: Acetylation of Large T Antigen

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The PCAF and GCN5 acetyltransferases, but not p300 or CBP, stimulate DNA replication when tethered near the polyomavirus origin. Replication stimulation by PCAF and GCN5 is blocked by mutational inactivation of their acetyltransferase domains but not by deletion of sequences that bind p300 or CBP. Acetylation of histones near the polyomavirus origin assembled into chromatin in vivo is not detectably altered by expression of these acetyltransferases. PCAF and GCN5 interact with polyomavirus large T antigen in vivo, PCAF acetylates large T antigen in vitro, and large T-antigen acetylation in vivo is dependent upon the integrity of the PCAF acetyltransferase domain. These data suggest replication stimulation occurs through recruitment of large T antigen to the origin and acetylation by PCAF or GCN5.

Stimulation of DNA replication by auxiliary sequence elements located in *cis* to origins that function in eukaryotic cells was first observed with murine polyomavirus (Py) (28) and has since been documented for many other viruses. These auxiliary sequence elements bind proteins postulated to recruit and/or activate additional proteins involved directly in replication or to modify origin sequences and chromatin structures or their intranuclear localization (9, 19, 21, 22, 38–40, 48, 60, 63, 64, 72, 99, 100, 106, 108, 113; for reviews, see references 26 and 74). Much can be learned about the control of DNA replication from studying these proteins and their functions.

The Py enhancer PEA1 and PEA3 sites are particularly important for stimulating Py DNA replication (18, 20, 39, 48, 69, 71, 75, 83, 90, 91, 105). Jun, a member of the AP1 (PEA1) complex, recruits Py large T antigen (PyLT) to the origin to stimulate DNA unwinding, particularly at early times after infection when PyLT is limiting (39, 48, 69, 71, 75, 91). The AP1 complex and *ets* family proteins (that bind the PEA3 site) as well as Gal4VP16, NF-κB, E1a, Sp1, and p53, which also can stimulate Py DNA replication (8, 10, 11, 37, 46, 53, 74, 77, 111; reviewed in references 26 and 74), interact with p300/CBP (2, 4–7, 31, 36, 50, 57, 61, 65, 68, 82, 101, 116), PCAF, and GCN5 (15, 67, 107, 110) and other coactivators that acetylate histones and nonhistone proteins involved in transcription, including HMG17, HMGI(Y), E2Fs, p53, c-Jun (109), MyoD, YY1, Tat, $TF_{II}E$, $TF_{II}F$, and $TF_{I}68$ (17, 92, 96). Acetylation regulates these proteins' functions and interactions with other proteins (17, 55, 92, 96, 97).

Proteins directly involved in DNA replication also interact with acetyltransferases, including PyLT, which interacts with p300/CBP (23, 76), MCM2 and ORC1 (which interact with acetyltransferase HBO1 [14, 44]), and MCM3, whose acetylation affects DNA replication (103). Also, acetyltransferases are recruited to double-stranded DNA breaks to facilitate DNA repair (13, 45, 70; for a review see reference 17). However, neither the specific roles for histone acetylation in these processes nor the proteins that catalyze them have been established.

Here we demonstrate that the PCAF and GCN5 acetyltransferases, when tethered near the Py origin via Gal4 DNA binding, stimulate DNA replication in vivo. Our initial hypothesis was that these acetyltransferases modify chromatin structures near the origin, but this was not supported by our experimental evidence. Instead, we observed that PCAF and GCN5 bound PyLT in vivo and that PCAF acetylated PyLT. We propose that PCAF and GCN5 activate replication at the Py origin by helping to recruit and to modify PyLT function by acetylation.

MATERIALS AND METHODS

Plasmids. pBSPyΔE (Fig. 1A) contains Py sequences between nucleotides (nt) 4999 (*Acc*I) and 372 (*Dra*I) at the *Xho*I site of pBluescript (Stratagene), encompassing the Py origin with the viral enhancer (nt 5047 to 5291) replaced by an *Xho*I site. pBSPyGal (Fig. 1A) is a derivative of pBSPyΔE with five Gal4-binding sites from pG5-E4T (16) cloned at the *Xho*I site of pPyXhoI, which contains the polyomavirus A3 genome cloned at the *Eco*RI site of pBR322 with a viral enhancer (nt 5047 to 5291) replaced by an *Xho*I site. pBM129 was described by Hermansen et al. (42). pMKSO11 contains the entire Py genome (with a defective *ori*) cloned in pMK16 (104).

Expression vectors for Gal4 fusion acetyltransferase proteins (Fig. 1B) include the following: pcDNA3GalDB for the Gal4 DNA-binding domain (GalDB) (15); pcDNA3GalhGCN5$_{SF}$ for Gal4 human GCN5$_{SF}$ (GGCN5$_{SF}$) (15); pcDNA3GalhGCN5FTE for an FTE212-214AAA substitution histone acetyltransferase (HAT) mutant of Gal4 human GCN5$_{SF}$ (GGCN5FTE) (15); pcDNA3hGCN5DEY for a DEY254-256AAA substitution HAT mutant of Gal4 human GCN5$_{SF}$ (GGCN5DEY) (15); pcDNA3GGmGCN5FL-flag for Gal4 (2×) mouse GCN5 (GmGCN5) (114); pcDNA3Galp300 for Gal4 p300 (Gp300) (119); pRc/RSVGalCBPFL-flag for Gal4 CBP (GCBP) (102); pCXflag-GalPCAF for Gal4 human PCAF (GPCAF) (56); pCXflag-GalPCAFΔ574-608 for a HAT-truncated mutant of Gal4PCAF (GPCAFΔ574-608) (56); and pCXflag-GalPCAFΔ65-112 for an N-terminal-truncated mutant of Gal4PCAF (GPCAFΔ65-112) (56). PcDNA3GalDB, pcDNA3GalhGCN5$_{SF}$, pcDNA3GalhGCN5FTE, and pcDNA3GalDEY were constructed by transferring Gal4 chimeras from pM2 vectors (15) into pcDNA3 (Invitrogen) by using PCR. pcDNA3GGmGCN5FL-flag containing duplicate GalDBs was constructed by inserting *Eco*RI-digested
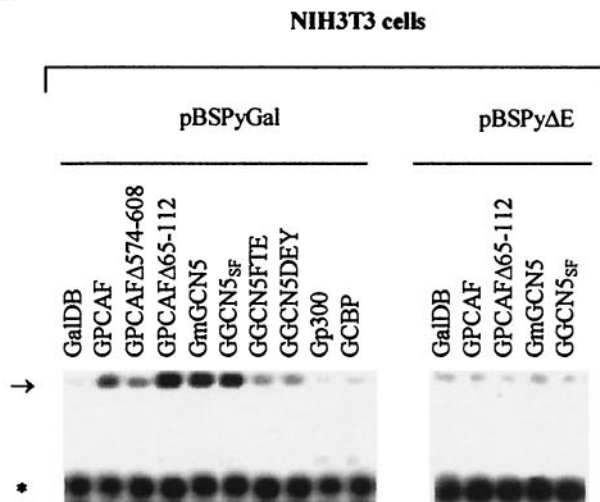
A.

pBSPyGal

| 5XGal4 binding sites | Py *ori*-core |

pBSPyΔE

Py *ori*-core

B.

C.

FOP cells

D.
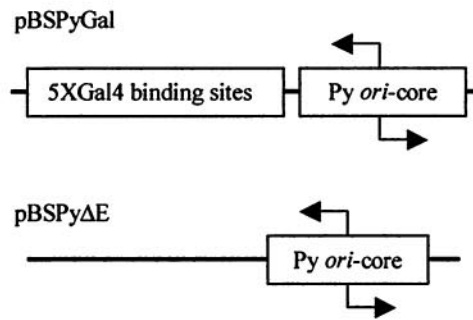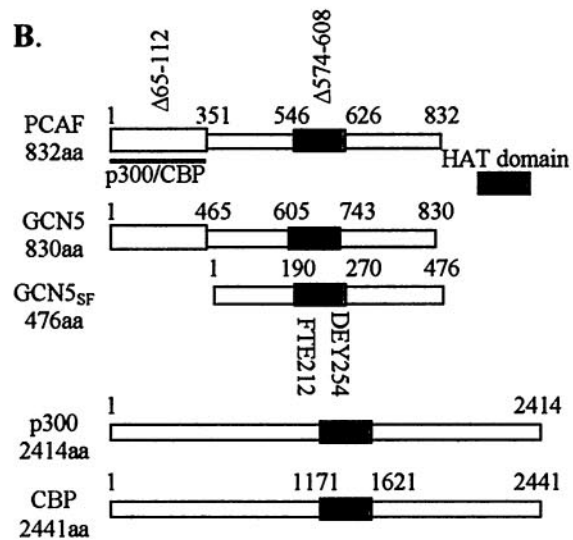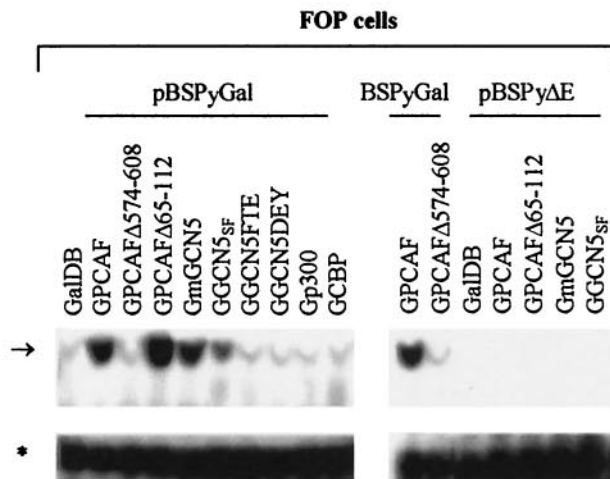
Gal4 Fusion Proteins	Py Ori-Core Stimulation (pBSPyGal)
GalDB	1 ± 0
GPCAF	18 ± 7
GPCAFΔ574-608	2 ± 1
GPCAFΔ65-112	32 ± 11
GmGCN5	17 ± 4
GGCN5$_{SF}$	13 ± 3
GGCN5FTE	1 ± 1
GGCN5DEY	1 ± 0.7
Gp300	0.4 ± 0.6
GCBP	2 ± 0.7

E.

NIH3T3 cells

F.

Gal4 Fusion Proteins	Py Ori-Core Stimulation (pBSPyGal)
GalDB	1 ± 0
GPCAF	19 ± 2
GPCAFΔ574-608	11 ± 1
GPCAFΔ65-112	25 ± 5
GmGCN5	20 ± 7
GGCN5$_{SF}$	21 ± 4
GGCN5FTE	6 ± 2
GGCN5DEY	4 ± 3
Gp300	1 ± 0.3
GCBP	1 ± 0.3

PCR products of the GalDB from pCXflag-GalPCAF by inserting primers 5′-CCGGAATTCATGAAGCTACTGTCTTCTATC-3′ and 5′-CTGTATCGC-CGGAAGAATTCGCCAC-3′ into the *Eco*RI site of pCMVSPORT2mGCN5fl (114). Expression vectors for Gal4PCAF and its deletion mutants were described by Krumm et al. (56).

Cell cultures. NIH 3T3 (mouse fibroblast cell line), FOP (polyomavirus transformed mouse mammary carcinoma cell line), and Cos7 (simian virus 40 [SV40]-transformed Africa green monkey kidney cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM; low glucose) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 4 mM L-glutamine.

Replication assays. NIH 3T3 cells were seeded in 12-well plates (1.5×10^5 cells/well) and were incubated overnight at 37°C. Cells were transiently transfected by using LipofectAMINE PLUS (Invitrogen) with expression plasmids for Gal4 fusion proteins (0.2 µg) and a test plasmid (0.2 µg) pBSPyGal4 containing the Py *ori* core flanked by 5 Gal4-binding sites or pBSPyΔE containing only the Py *ori* core (Fig. 1A). PyLT, which was required for replication, was provided by cotransfection of pMKSO11 (0.01 µg), and the total amount of DNA (0.41 µg) was kept constant by adding vector DNA. After incubating cells with a DNA:LipofectAMINE PLUS mixture for 4 to 5 h in 400 µl of serum-free DMEM, the transfection solution was replaced with 1 ml of DMEM containing 10% FBS.

Similarly, FOP cells (1.5×10^6 cells per well), a cell line that constitutively expresses PyLT (11), were transfected with expression plasmids (0.8 µg) and test plasmid pBSPyGal or pBSPyΔE (0.4 µg) by using LipofectAMINE. After incubating cells with DNA:LipofectAMINE mixture for 6 h in 350 µl of serum-free DMEM, 800 µl of DMEM containing 10% FBS was added. For both types of cells, the medium was changed at 12 h and was incubated for another 24 h. DNAs were isolated by the Hirt procedure (43), digested with RNase A (0.2 µg/µl) and with *Eco*RI and *Hin*dIII to linearize plasmids, and were digested with *Dpn*I in the presence of 200 mM NaCl to distinguish input (methylated) DNA from DNA replicated in animal cells (80, 93). The replicated DpnI-resistant DNA was resolved from DpnI-digested DNA by electrophoresis in agarose gels (0.8%), transferred to a nylon membrane, detected by Southern Blotting with probes generated from the test plasmid, labeled, and visualized with the North2South detection kit (Pierce). Transfection efficiencies were normalized by using input DNA digested by *Dpn*I. For analysis of protein expression, an identical set of transfections was performed, except test plasmids were not included. Cells were lysed with 1% Triton X-100 lysis buffer 36 h after transfection. Proteins in lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and expression of Gal4 fusion proteins was detected by Western blotting by using the anti-GalDB monoclonal antibody sc-510 (Santa Cruz, Inc.).

Transcription assays. NIH 3T3 and FOP cells were transiently transfected as described above with expression plasmids for Gal4 fusion proteins, reporter plasmid pFR-Luc (Stratagene; Fig. 2A), and expression plasmid pRL-CMV (Promega; Fig. 2A) with *Renilla* luciferase as an internal control. At 36 to 48 h after transfection, extracts were prepared by passive lysis and luciferase activities were assayed according to the protocol of the Dual-Luciferase Reporter Assay System (Promega). Luciferase activities were normalized with *Renilla* luciferase activities. All transfections were repeated at least three times. The relative luciferase activities are represented as the means relative to the basal activity of a transfected GalDB (pcDNA3Gal4DB). For analysis of protein expression, a sample of whole-cell extracts was resolved by SDS-PAGE, and expression of Gal4 fusion proteins was detected by Western blotting by using the anti-GalDB monoclonal antibody sc-510 (Santa Cruz, Inc.).

Immunoprecipitation and Western blot analyses. NIH 3T3 cells (approximately 2.5×10^6) were transfected with expression vectors for Gal4 fusion proteins (2.5 µg) and pMKSO11 (2.5 µg) by using LipofectAMINE PLUS in a 100-mm-diameter plate. After 36 h, the cells were extracted with 500 µl of single-detergent lysis buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.02% sodium azide, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1× Complete pro-

tease inhibitors [Roche Diagnostics], 1% Triton X-100) for 1 h at 4°C, and lysates were cleared by centrifugation twice at $13,000 \times g$ for 10 min. A sample (10 µl) was taken for a protein input control, and the remaining supernatants were incubated with 10 µg of rabbit anti-Gal4 polyclonal antibody sc-577 (Santa Cruz, Inc.) or mouse anti-PyLT monoclonal antibody KF4 (78) overnight at 4°C, and the immune complexes were collected by adding 80 µl of protein A-Sepharose beads for sc-577 or protein G-Sepharose beads for KF4. Samples were incubated for 2 h at 4°C, followed by five washes with wash buffer (50 mM Tris-Cl [pH 8.0], 1 mM EDTA, 150 mM NaCl, 1% NP-40, 4 mM NaF, 2 mM Na-Orthovanadate). Following the fifth wash, pelleted beads were suspended in 40 µl of 1× SDS sample buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 4% SDS, 0.1% bromophenol blue, 10% [vol/vol] glycerol), boiled for 10 min, and centrifuged at $13,000 \times g$ for 10 min. Samples of 20 µl of the supernatant were removed for SDS-PAGE. After SDS-PAGE and transfer to polyvinylidene difluoride membrane, the bound T antigen or GalDB fusion proteins were detected with mouse KF4 antibody or rabbit anti-GalDB antibody sc-577 by Western blot analysis. For estimation of Gal4 fusion protein and PyLT expression, 20 µl of the whole-cell extract was resolved by SDS-PAGE and detected by Western blotting by using mouse anti-Gal4DB monoclonal antibodies sc-510 and KF4, respectively, and subsequently horseradish peroxidase-conjugated antibody followed by chemiluminescence measurement.

In vitro acetylation assays. Acetylation reactions were performed with a mixture of 2.8 µg of baculovirus-expressed histidine-tagged PyLT purified by nickel chelate chromatography (62) (or bovine serum albumin [BSA] as a negative control), 0.8 µg of PCAF (Upstate), and 5.0 nmol of [³H]acetyl-coenzyme A (200 mCi/mmol; DuPont-NEN) in 30 µl of HAT assay buffer (50 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 10 mM sodium butyrate, 1.0 mM dithiothreitol, 10% glycerol) at 30°C for 1 h. Reaction mixtures were stopped by addition of 1× SDS sampling buffer, and a 25-µl volume was resolved by SDS-PAGE. The gel was stained with Coomassie blue to estimate the protein quantities, soaked in EN³HANCE solution (DuPont) for 30 min and vacuum dried, and subsequently subjected to autoradiography for 3 weeks. The acetylation reaction was also carried out in 25 µl of HAT assay buffer with unlabeled acetyl-coenzyme A (0.2 mM; Sigma) and PyLT (2.5 µg) as substrates and active PCAF (0.7 µg) or p300 acetyltransferase (0.9 µg or 7 U; Upstate) as a catalyst, and acetylation in half a reaction was detected by Western blotting by using rabbit anti-acetyl lysine polyclonal antibody (Upstate). For filter-binding assays, acetylation reactions were performed with a mixture of 2.5 µg of PyLT (or chicken histones as a positive control), 0.25 µg of active PCAF (Upstate), and 1.25 nmol of [³H]acetyl-coenzyme A (200 mCi/mmol; DuPont-NEN) in 25 µl of HAT assay buffer at 30°C for 1 h. Five microliters of each reaction sample was transferred onto P81 paper (Whatman), washed five times for 5 min per wash with 50 ml of 50 mM Na₂HPO₄ (pH 9.0) and once with 50 ml of acetone, and dried. Each paper was then equilibrated overnight in a scintillation vial containing scintillation fluid and was counted.

Analysis of PyLT acetylation in vivo. NIH 3T3 cells (approximately 5.0×10^6) were transfected with expression vectors for Gal4 fusion proteins (6.0 µg) and pMKSO11 (4.0 µg) by using LipofectAMINE PLUS. After 36 h, acetylation of PyLT was assessed by immunoprecipitation of total cell extracts with rabbit anti-acetyl lysine polyclonal antibody (Upstate) followed by Western blot analysis with mouse KF4 antibody. For estimation of Gal4 fusion protein and PyLT expression, 10 µl of the whole-cell extract was resolved by SDS-PAGE and detected by Western blot analysis by using rabbit anti-GalDB polyclonal antibodies sc-577 and KF4, respectively.

Preparation and analysis of SV40 virus with Py origin. A PCR product with the Py *ori* core flanked by 5× Gal4 binding sites was prepared by using pBSPy-Gal4 as a template with the reverse M13 primer and Ori94 primer (5′-GTG-CAAGGCGCCAGTCCTG). The PCR product was digested with *Eco*RV and inserted into the *Pml*I site of pBM129 (42) to create construct TSPy5 (see Fig.

FIG. 1. Replication of Py *ori* core DNA by acetyltransferases. (A) Structures of test plasmids used to measure replication stimulation. (B) Structures of acetyltransferases fused to GalDBs. Acetyltransferase (HAT) domains are indicated by dark bars. Two HAT substitution mutants of human GCN5 (FTE and DEY), one HAT deletion mutant of PCAF (Δ574-608), and one deletion mutant of PCAF in the p300/CBP interacting domain (Δ65-112) also are indicated. aa, amino acid. (C) Examples of replication assays performed with the test plasmids pBSPyGal and pBSPyΔE in FOP cells. Test plasmids and cotransfected expression vectors are indicated above lanes. The arrow and the asterisk indicate the positions of replicated test DNA and the nonreplicated test DNA (input), respectively. (D) Averages from three independent replication assays (means ± standard deviations). (E) Examples of replication assays performed with the test plasmids pBSPyGal and pBSPyΔE in NIH 3T3 cells. Test plasmids and cotransfected expression vectors are indicated above lanes. The arrow and the asterisk indicate the positions of replicated test DNA (top band) and the nonreplicated test DNA as a reference (lower band), respectively. (F) Averages from three independent transfection assays (means ± standard deviations).

A.

pFR-Luc

5XGal4 binding sites — TATA — Firefly LUC

pRL-CMV

CMV — *Renilla* LUC

B.

Gal4 Fusion Proteins	Relative Activity (pFR-Luc/pRL-CMV)	
	FOP	NIH3T3
Gal4 DNA binding domain	1	1
GPCAF	1	1
GPCAFΔ574-608	1	0.7
GPCAFΔ65-112	1	0.6
GmGCN5	1	2
GGCN5SF	0.5	0.4
GGCN5FTE	0.5	0.5
GGCN5DEY	2	0.6
Gp300	124	121
GCBP	119	210

C.

Western: α GalDB

280 -
120 -
110 -
70 -
20 -

GmGCN5 GPCAF GPCAFΔ65-112 GPCAFΔ574-608 Gp300 GCBP GGCN5SF GGCN5FTE GGCN5DEY GalDB
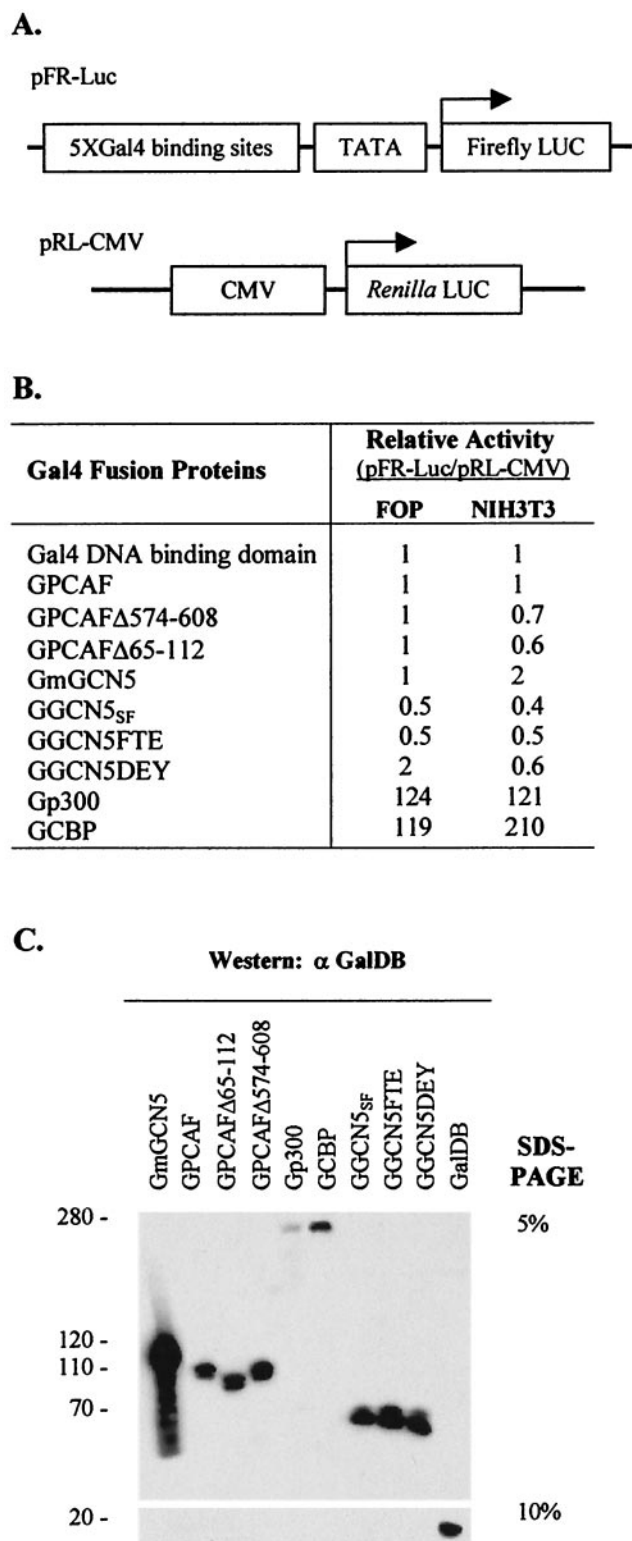
SDS-PAGE

5%

10%

FIG. 2. Transcriptional activation and expression of acetyltransferases. (A) Structure of the test plasmids used to measure transcription stimulation. LUC, luciferase; CMV, cytomegalovirus. (B) Summary of luciferase assays with different acetyltransferases in FOP and NIH 3T3 cells. Each value represents the mean of three independent assays. (C) Protein expression of Gal4 fusion acetyltransferases in NIH 3T3 cells. Molecular sizes (in kilobase pairs) of the proteins are indicated on the left.

6A). The C-to-T point mutation at nt 2151 (i.e., P to S substitution in the viral capsid protein VP1) present in the SV40 tsC219 mutant (24, 25) was introduced into TSPy5 by site-directed mutagenesis. The orientation of the insert was determined by double digestion with *Mlu*I and *Sal*I, and the insert sequence was confirmed by DNA sequencing. To prepare TSPy5 virus, the pBR322 sequences were excised by *Eco*RI digestion and the linearized SV40-TSPy5 DNA was transfected into Cos7 cells by using LipofectAMINE, and the supernatant was harvested after transfected cells were incubated at 37°C for 7 days. Viruses were amplified by further infection of Cos7 cells, and the virus titers were estimated by determining the quantity of purified viral DNA in samples of the infected cells (34). Nuclear extracts containing TSPy5 chromatin were prepared from Cos7 cells infected with the TSPy5 virus, and characterization of TSPy5 chromatin by micrococcal nuclease (MNase) was performed as described by Kingston (54). To ascertain whether nucleosomes occupied the Py *ori* core, TSPy5 minichromosomes were prepared in hypotonic buffers as previously described (34) and were digested with excess restriction endonucleases as described by Hermansen et al. (42) and were analyzed by Southern blotting.

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed essentially as described by Boyd et al. (12) with the following modifications. Cos7 cells were seeded into 100-mm-diameter plates (1.5×10^6 to 2.4×10^6 cells/ plate), and after 12 to 16 h they were infected with TSPy5 virus for 1 h and then transfected with expression plasmids (8 μg) for Gal4 fusion proteins by using LipofectAMINE 2000 (Invitrogen). Aphidicolin (10 μg/ml, prepared as 1-mg/ml concentration of stock solution in dimethylsulfoxide [DMSO] and stored at −20°C; Sigma) was added to the medium at 16 h after transfection to block DNA replication and virion assembly (32). After 24 h of additional incubation, cross-linking solution (11% formaldehyde diluted from 37% stock, 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES, pH 8.0) was added to the culture medium to a final concentration of 1% and was incubated for 30 min at 37°C and then for 24 h at 4°C. As a positive control for the ChIP assays, Cos7 cells were infected with virus and were treated at 24 h after infection with or without trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor (117), at 100 ng/ml (TSA [Sigma] was prepared as a 1-mg/ml concentration of stock solution in DMSO). After 12 h of additional incubation cells were cross-linked as described above, and cross-linking was stopped by the addition 0.55 ml of 2.5 M glycine to a final concentration of 0.125 M for 5 min at room temperature. The cells were scraped and collected by centrifugation at $7,400 \times g$ for 5 min after two washes with ice-cold phosphate-buffered saline. The cells were washed twice with ice-cold phosphate-buffered saline plus 0.5 mM PMSF and resuspended in 120 μl of a solution containing 5 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (pH 8.0), 85 mM KCl, 0.5% NP-40, 0.5 mM PMSF, and 1× complete protease inhibitors for 20 min on ice. The lysate was sonicated four times for 30 s on ice with a Vibra Sonicator and then was digested for 10 min by MNase (0.5 U/μl) at room temperature to reduce the DNA length to between 200 and 1,000 bp. Debris was removed by centrifugation for 10 min at $16,000 \times g$ at 4°C. The supernatant was diluted in 1.2 ml of immunoprecipitation buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 16.7 mM NaCl, 1 mM PMSF, and 1× Complete protease inhibitors). The chromatin solution was precleared with 50 to 80 μl of a 50% protein A-Sepharose (Sigma) slurry containing 0.2 μg of sonicated salmon sperm DNA/μl and 0.5 μg of BSA in TE (10 mM Tris, 1 mM EDTA [pH 8.0], 0.05% sodium azide)/μl for 30 min at 4°C with agitation. Sepharose beads were pelleted at $12,000 \times g$ for 5 min, and 500 μl of supernatant was immunoprecipitated with 5 μg of anti-acetyl histone H3 and H4 polyclonal antibodies (Upstate) each for 12 h at 4°C with rotation. A portion of the supernatant (50 μl) was kept as an input chromatin control. Immune complexes were collected with 60 μl of 50% protein A-Sepharose slurry containing 0.2 μg of sonicated salmon sperm DNA/μl and 0.5 μg of BSA in TE/μl for 1 h at 4°C with rotation. Beads were pelleted by centrifugation and were washed a total of five times with 500 μl of each of following buffers: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl (one washing); 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl (one washing); 0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1) (one washing); and TE (pH 8.0) (two washings). Immune complexes were eluted by two washes with 250 μl of elution buffer (1% SDS, 0.1 M NaHCO₃). NaCl (5 M; 20 μl) was added to the combined eluates, and cross-links, including the input chromatin control, were reversed by incubation at 65°C for at least 4 h. Proteinase K (2 μl of a 10-mg/ml concentration) was added to the eluate and was incubated for 1 h at 45°C. DNA was recovered by phenol-chloroform extraction and ethanol precipitation by using 20 μg of glycogen as a carrier, denatured in 100 μl of denaturation solution (1.5 M NaCl, 0.5 M NaOH), and transferred onto nylon membranes by using a slot blot apparatus. Sequences from immunoprecipitated (bound) and input control (input) DNAs were detected by using a North2South detection kit with

a Py *ori* probe generated from the *Bam*HI-*Mlu*I small fragment of TSPy5 and an SV40 *ori* probe generated from the *Bgl*I-*Ngo*MI small fragment of TSPy5 (see Fig. 6A).

RESULTS

PCAF and GCN5 acetyltransferases tethered near the Py origin stimulate DNA replication. Plasmid pBSPyGal containing the Py *ori* core flanked by Gal4-binding sites in place of the natural enhancer (Fig. 1A) was transfected into FOP or NIH 3T3 cells together with plasmids that express the GalDB, either alone or fused to cellular acetyltransferases, and the replicated pBSPyGal DNA was measured after 48 h. The absence of the natural viral enhancer caused replication of pBSPyGal plasmid to be dependent upon the expression of Gal4 fusion proteins that stimulate DNA replication (Fig. 1C to F) (8, 11, 37). PCAF and GCN5 and their various mutationally altered forms stimulated replication of pBSPyGal to differing degrees (Fig. 1C to F). Compared to GalDB alone, GPCAF and GGCN5$_{SF}$ stimulated replication 18- and 13-fold, respectively, in FOP cells (Fig. 1D) and 19- and 21-fold in NIH 3T3 cells (Fig. 1F). Mutationally altered forms of PCAF and GCN5$_{SF}$ lacking intrinsic acetyltransferase activities (GPCAFΔ574-608, GGCN5FTE, and GGCN5DEY) did not stimulate replication in FOP cells (Fig. 1C and D) and stimulated replication at reduced levels in NIH 3T3 cells (Fig. 1E and F), indicating a requirement for their intrinsic acetyltransferase activities. GP-CAFΔ65-112, a mutant containing a deletion within the p300/CBP-interacting domain, activated replication even better than its parent, and GGCN5$_{SF}$, which does not associate with p300/CBP due to the lack of an N-terminal sequence (114, 115), also activated replication as well as full-length GmGCN5 (Fig. 1C to F), suggesting that the p300 and CBP accessory acetyltransferases are not required to act in concert with PCAF and GCN5 to stimulate replication. This was further supported by the observation that p300 and CBP tethered to the Py origin did not stimulate replication (Fig. 1C to F). A parallel assay with plasmid pBSPyΔE, which lacks Gal4-binding sites, revealed that replication stimulation required tethering the PCAF and GCN5 fusion proteins to the test plasmid (Fig. 1C and E).

Differential stimulation of transcription by PCAF, GCN5, p300, and CBP acetyltransferases. Stimulation of DNA replication by PCAF and GCN5 did not correlate with their capacity to stimulate transcription. For example, Gal4 fusion GCN5 (GmGCN5 and GGCN5$_{SF}$) and PCAF (GPCAF) proteins did not enhance expression of the luciferase gene placed downstream of Gal4 sites (Fig. 2A and B), but Gal4 fusion p300 and CBP proteins significantly stimulated gene expression (Fig. 2B), the latter agreeing with results previously reported by others (102). Western analyses of extracts from transiently transfected NIH 3T3 cells demonstrated that the Gal4 PCAF and GCN5 fusion proteins were expressed equivalently (Fig. 2C). Expression of these proteins was not detected in FOP cells, possibly due to inefficient transfection. These data suggest that activation of transcription and replication by these acetyltransferases differs mechanistically, perhaps as a result of their being present in different multiprotein complexes.

Interaction of acetyltransferases with PyLT. Work by others has indicated that p300 and CBP interact with PyLT (23, 76).

Were PCAF and GCN5 also to interact with PyLT, they might facilitate recruitment of PyLT to the origin (as does Jun when bound to the Py enhancer [39, 48, 69, 75]). To examine this possibility, NIH 3T3 cells were cotransfected with expression plasmids for PyLT and Gal4 fusion proteins and cell lysates were immunoprecipitated with either anti-GalDB antibody or anti-PyLT antibody, separated by SDS-PAGE, and immunoblotted correspondingly with anti-T antisera and anti-GalDB antibody. Anti-GalDB antibody brought down PyLT from cell extracts containing GPCAF, GmGCN5, or GGCN5$_{SF}$ as well as Gp300 and GCBP, but not GalDB alone (Fig. 3A and B). Also, anti-PyLT antibody, but not normal serum immunoglobulin G, coprecipitated GPCAF, GmGCN5, or GGCN5$_{SF}$ but not GalDB (Fig. 3C and D). These results show PyLT stably bound PCAF and GCN5, with the bound fraction of PyLT estimated to be roughly 1 to 4% by comparing band densities of PyLT in internal and coimmunoprecipitate lanes.

Nearly equivalent amounts of PyLT were coprecipitated with mutants of PCAF (GPCAFΔ574-608) or GCN5 (GGCN5FTE and GGCN5DEY) lacking acetyltransferase activities whose expression levels were comparable to those of their parents (Fig. 3A and B). The mutant of PCAF with a deletion in the p300/CBP-interacting domain (GPCAFΔ65-112) also complexed with a similar amount of PyLT. Similarly, GGCN5$_{SF}$ lacking the p300/CBP-interacting domain was associated with PyLT to the same extent as full-length GCN5 (GmGCN5) (Fig. 3B). When anti-PyLT antisera were used for immunoprecipitation, mutants of PCAF with expression levels comparable to those of the parent (GPCAF) were coprecipitated with PyLT at nearly the same level, and expression of PyLT was comparable for these assays (Fig. 3). These results suggest that the mutations in PCAF and GCN5 affect neither their association with PyLT nor, likely, its recruitment to the origin. As mutations abrogating their intrinsic acetyltransferase activities reduce the ability of PCAF or GCN5 to stimulate Py DNA replication, these data indicate that both recruitment of PyLT as well as acetylation are required to stimulate DNA replication.

Acetylation of PyLT by PCAF. Incubation of PCAF with purified PyLT and acetyl-coenzyme A revealed PyLT was a substrate for acetylation (Fig. 4A and B). Based on the data from filter binding assays, acetylation of PyLT was approximately 12% that of purified histones without considering the difference in mass. Approximately 2% of PyLT was acetylated if one [^{3}H]acetyl group was introduced per PyLT molecule (data not shown). Since GCN5 is highly related to PCAF in structure and function (114), it is likely that GCN5 also acetylates PyLT. PyLT appeared to be acetylated very weakly, if at all, by p300 (Fig. 4B).

To assess acetylation of PyLT in vivo, extracts from NIH 3T3 cells expressing PyLT and Gal4 fusion PCAF were immunoprecipitated by using rabbit anti-acetyl lysine antibody and were analyzed by Western blotting by using anti-PyLT antibody KF4. PyLT was immunoprecipitated by the anti-acetyl lysine antibody from extracts of cells expressing GalPCAF and GPCAFΔ65-112 (Fig. 5), both of which stimulate DNA replication. By contrast, no PyLT was detected in the immunoprecipitates of cells expressing GPCAFΔ574-608, the mutant of GPCAF lacking HAT activity. This result suggests that PyLT is
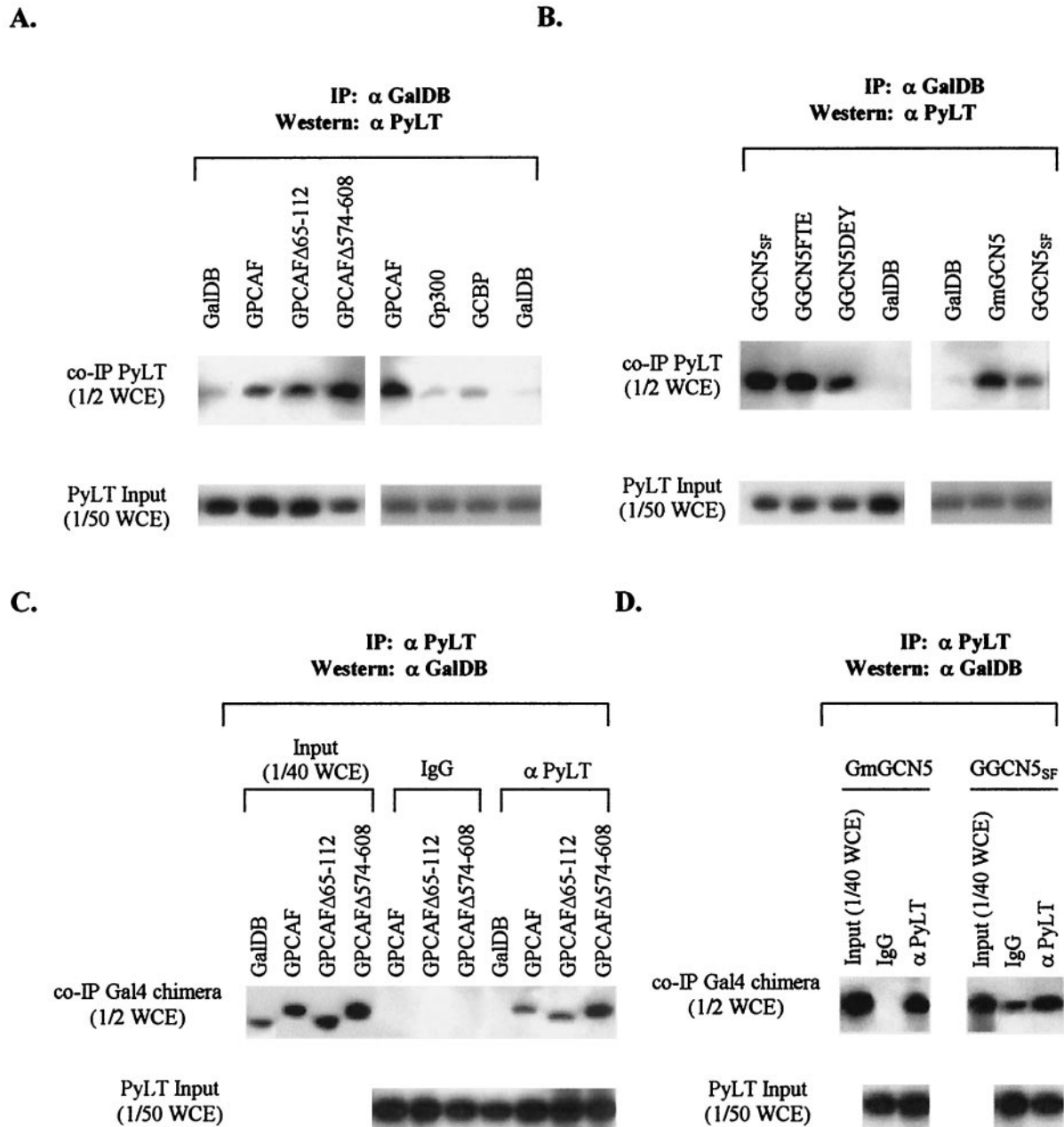
FIG. 3. Coimmunoprecipitation of acetyltransferases and PyLT. (A) Analysis of PyLT coimmunoprecipitated with Gal4 fusion PCAF. Cell extracts from NIH 3T3 cells transiently cotransfected with expression vector for PyLT and an expression vector for Gal4 fusion acetyltransferase were precipitated with rabbit polyclonal anti-GalDB antibody. One half of the immune complexes (top band), equivalent to one half of whole-cell extracts (WCE), and one fiftieth of WCE for measurement of input PyLT (lower band) were analyzed by Western blots. (B) Analysis of PyLT coimmunoprecipitated with Gal4 fusion GCN5 as described for panel A. (C and D) Analysis of acetyltransferases coimmunoprecipitated with PyLT. Cell extracts from NIH 3T3 cells transiently cotransfected with expression vector for PyLT and an expression vector for Gal4 fusion acetyltransferase were incubated overnight at 4°C with mouse monoclonal anti-PyLT antibody and mouse normal serum immunoglobulin G (IgG). One half of the immune complexes, equivalent to one half of WCE, and one fortieth of WCE for measurement of input Gal4 fusion acetyltransferases were analyzed by Western blotting by using rabbit polyclonal anti-GalDB antibody (top lane) and one fiftieth of WCE for measurement of input PyLT analyzed by Western blotting by using anti-PyLT antisera (lower lane). Gal4 fusion acetyltransferases transfected and antibodies (anti-PyLT and normal IgG) used for immunoprecipitation are indicated above each lane. α PyLT, anti-PyLT antibody; α GalDB, anti-GalDB antibody; IP, immunoprecipitation.

acetylated in vivo and that the acetyltransferase activity of PCAF is required.

Py origin chromatin acetylation is not detectably changed by PCAF and GCN5. While plasmids with bacterial DNA back-

bones transfected into eukaryotic cells can be assembled into chromatin, a significant portion is assembled into nonnucleosomal structures or has an atypical nucleosomal structure (3, 51, 52, 88) and is unlikely to be representative of chromatin
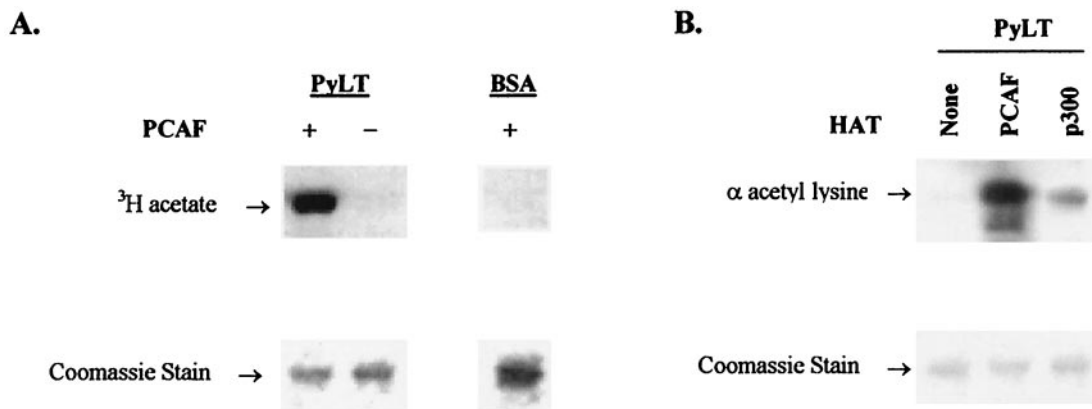
FIG. 4. In vitro acetylation of PyLT by PCAF. (A) PyLT incubated with (+) or without (−) PCAF with [³H]acetyl-coenzyme A as described in Materials and Methods. The right-hand panel shows BSA incubated with PCAF as a control. Reaction products were separated by SDS-PAGE and stained with Coomassie blue, and [³H]acetate reaction products were visualized by fluorography. (B) PyLT incubated with acetyl-coenzyme A and PCAF or p300 detected by Western blotting by using rabbit anti-acetyl-lysine polyclonal antibody (α acetyl lysine). Upper panel, Western blot with anti-α-acetyl-lysine antibody; lower panel, Coomassie-stained gel.

structures in vivo. In contrast, the structure of the SV40 minichromosome is nearly indistinguishable from that of cellular chromatin (27, 73). Consequently, to study the chromatin structures assembled near the Py origin in vivo we utilized an SV40 virus whose genome contained the Py origin sequences (TSPy5; Fig. 6A). MNase digestion of extracts of TSPy5 virus-infected Cos7 cells indicated that the TSPy5 genome was as-sembled into chromatin, and restriction enzyme analysis of TSPy5 minichromosomes revealed that the Py *ori* core region was occupied by nucleosomes (data not shown). To inhibit extensive DNA replication and SV40 virion assembly that might disrupt histone modifications caused by Gal4 fusion proteins expressed in the Cos7 cells (49), aphidicolin was added to the cells at 16 h after infection.

To validate ChIP assays as a means to study TSPy5 chromatin acetylation, we analyzed whether TSA treatment of Cos7 cells infected with TSPy5 viruses changed the acetylation status of TSPy5 chromatin. HDAC inhibitors, such as TSA, cause hyperacetylation of core histones (especially H4) in cellular chromatin (98, 118) and SV40 minichromosomes (1). ChIP assays with probes for Py *ori* core and SV40 *ori* demonstrated that the acetylation of H4 was increased by 2.6-fold, and acetylation of H3 slightly increased in TSPy5 minichromosomes extracted from TSA-treated cells compared to that of minichromosomes from untreated cells (Fig. 6B and C).

By contrast, introduction of Gal4 fusion PCAF or GCN5 (and also GGCN5$_{SF}$ and GmGCN5) into the Cos7 cells infected by TSPy5 virus caused no detectable changes of acetylation relative to GalDB protein alone, as detected by a probe for Py *ori* core sequences (Fig. 6D and E). Neither PCAF nor GCN5 had any effect on the chromatin structure near the SV40 origin (data not shown). Expression of Gal4 fusion proteins in Cos7 cells was detected by Western analysis (data not shown). Thus, it appears that stimulation of DNA replication by PCAF and GCN5 is not accompanied by detectable changes in histone acetylation around the Py *ori* core.
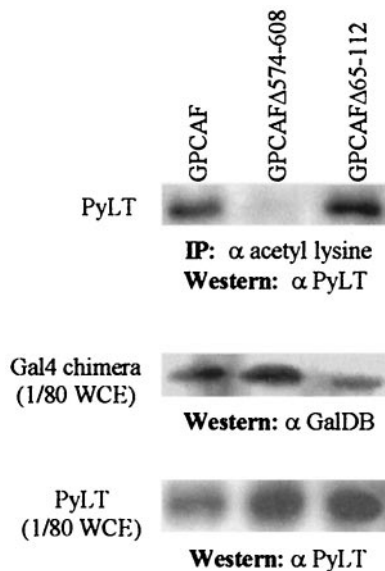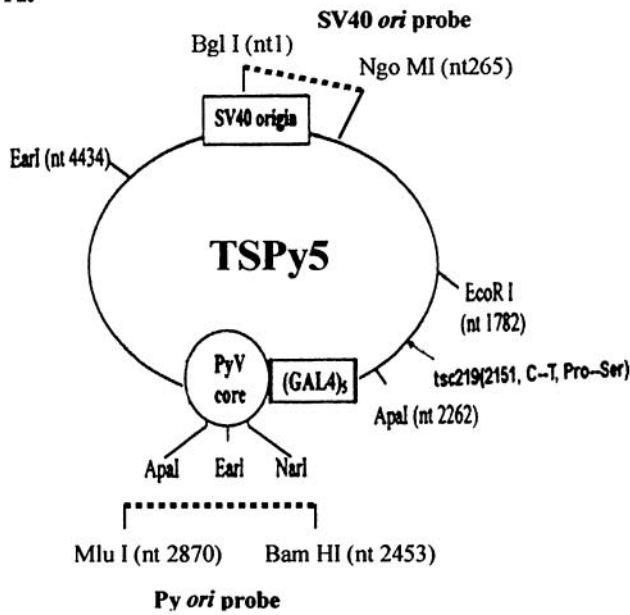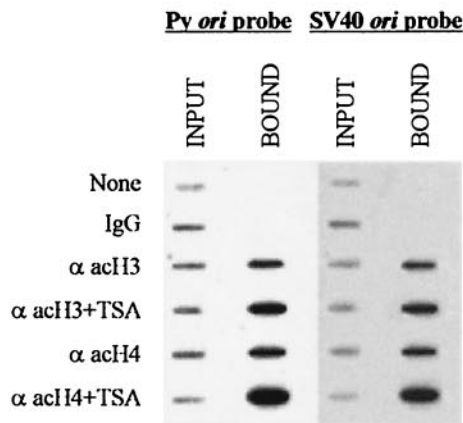

FIG. 5. Analysis of PyLT acetylation in vivo. Cell extracts from NIH 3T3 cells transiently cotransfected with expression vector for PyLT and an expression vector for Gal4 fusion PCAF were immuno-precipitated with rabbit polyclonal anti-acetyl-lysine antibody (α acetyl lysine). The immunoprecipitates (upper panel) were analyzed for PyLT by Western blotting by using anti-PyLT antibody (α PyLT). Expression of Gal4 fusion PCAF and its mutants (middle panel) and expression of PyLT transfected (bottom panel) also were analyzed in one eightieth of whole-cell extract (WCE) by Western blotting by using anti-GalDB (α GalDB) and anti-PyLT (α PyLT) antibodies. Gal4 fusion PCAF and its mutants transfected are indicated above the lanes. IP, immunoprecipitation.
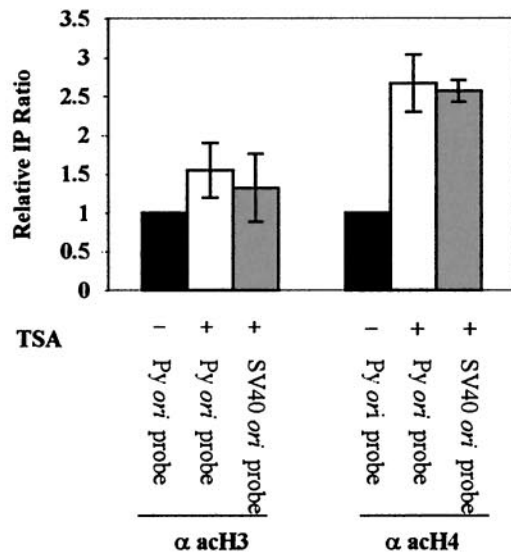
DISCUSSION

PCAF and GCN5 acetyltransferases interact, either directly or through cofactors, with a large variety of cellular proteins, including nuclear steroid receptors, c-Jun, c-Fos, c-Myb, p53, YY1, NF-κB, Stat1, Stat2, Ets-1, HIF1, GATA1, MyoD, Smad proteins, E2F-1, and members of RNA polymerase II holoen-zyme (33, 92, 96), some of which stimulate Py origin-dependent DNA replication when tethered in *cis*. Evidence presented
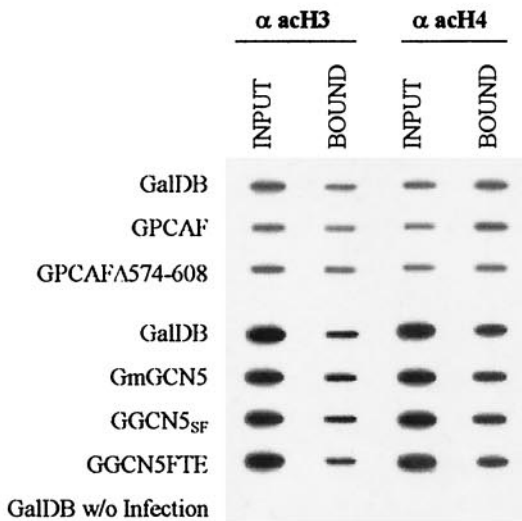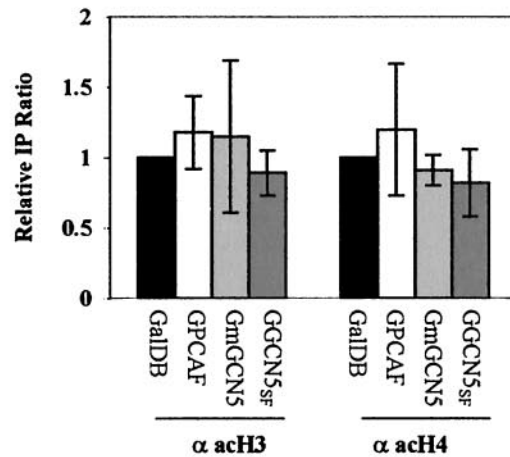
A.

B.

C.

D.

E.

here indicates the PCAF and GCN5 acetyltransferases bind to and acetylate PyLT. Inactivation of their intrinsic acetylation activities greatly reduces the stimulation of DNA replication. By contrast, p300 and CBP acetyltransferases bind PyLT but acetylate it to a much lower extent, if at all, and do not detectably stimulate DNA replication. These data implicate acetylation of PyLT as being important for replication in vivo.

As tethering of PCAF and GCN5 to the origin by using Gal4-binding sites is required to stimulate replication, structural features of PyLT associated with prereplication complex at the origin are likely to be important. There may be parallels between these events and those occurring at origins in cellular chromosomes: the recent finding that MCM2 and ORC1 interact with the HAT HBO1 (14, 44) and that acetylation of MCM3 by a novel acetyltransferase affects DNA replication (103) suggest that acetyltransferases also have direct roles in DNA replication of higher eukaryotes.

Mammalian PCAF and GCN5 are elements of several distinct multiprotein HAT complexes (17, 92, 96), including those recruited to double-stranded DNA breaks that facilitate DNA repair (13, 45, 70). PCAF and GCN5, in the NIH 3T3 and FOP cells used here, did not activate expression of Luc genes when tethered upstream; equivocal transcriptional activation by PCAF and GCN5 also has been reported in other studies (56, 58, 86, 95). Perhaps, as with transcription activation, distinct complexes are involved in replication stimulation.

For the Py natural enhancer, AP-1 complex and *ets* family protein interactions with p300/CBP (4, 6, 7, 50, 116) might recruit PCAF and GCN5 to the origin region (Fig. 7). Although p300/CBP is required for certain CREB-dependent transcriptional responses (33, 79, 89, 94) and thereby assists in the activation of viral transcription by the enhancer, others have noted that CREB did not stimulate DNA replication when bound near the Py *ori* core (75). Our data indicating p300/CBP is not directly involved in replication stimulation can be explained by the lack of acetylation of PyLT by p300/CBP, or alternatively by their being inhibited by PyLT, as has been suggested for activation of transcription (23). Viruses with mutationally altered PyLT incapable of binding p300/CBP are restricted in forming tumors in newborn mice, leading to the suggestion these interactions are essential for virus replication and spread (23). Dissecting the roles of these complexes should provide new information about the control of eukaryotic replication and transcription.

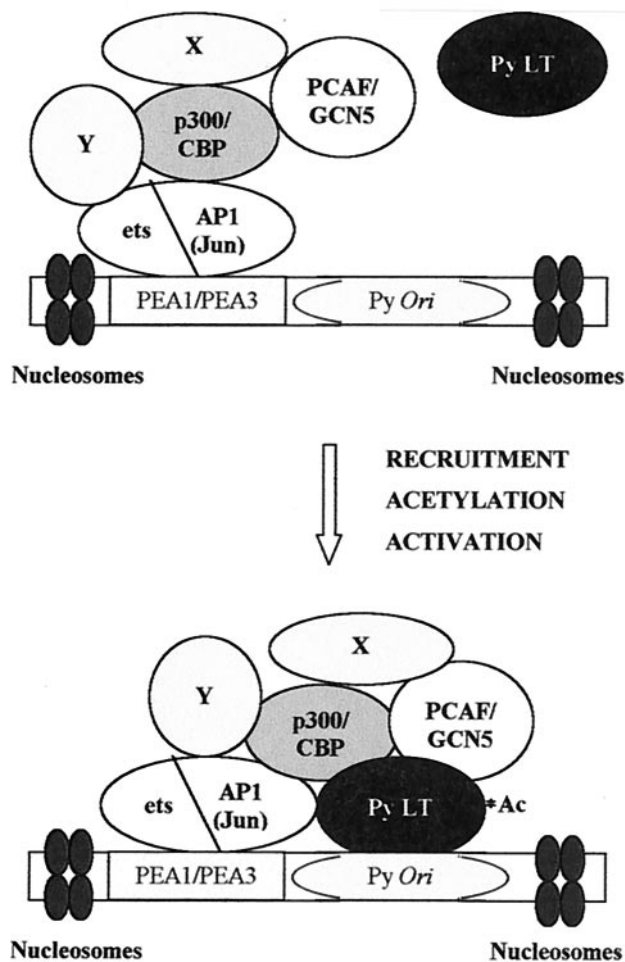Phosphorylation of proteins involved in DNA replication

FIG. 7. Model for Py enhancer-origin complexes containing accessory proteins PCAF, GCN5, and PyLT. See the text for details.

provides a very important means to regulate initiation, and this has been extensively dissected for SV40 and Py DNA replication (85, 112). Analogously, acetylation of PyLT might affect its DNA binding and association/disassociation with itself or other proteins in the replisome. Low pH enhances the binding of PyLT to the origin in vitro (81), and substitution of Asn-286 for the negatively charged Asp-286 within a positively charged

FIG. 6. Analysis of acetylation of histones around Py *ori* core. (A) Schematic representation of TSPy5 with location and orientation of the Py *ori* core and adjacent Gal4-binding sites. Fragments used to generate the Py *ori* probe and SV40 *ori* probe for ChIP assays are indicated by broken lines. (B) Example of ChIP assays from TSA-treated or untreated cells. TSPy5 chromatin fragments immunoprecipitated with anti-acetylated H3 and H4, and DNA input (INPUT) and DNA immunoprecipitated (BOUND) analyzed by Py *ori* and SV40 *ori* probes as indicated above the lanes. Antibodies used for ChIP assays and TSA treatment are indicated to the left to the corresponding rows. (C) Average from three independent experiments represented in panel B. Immunoprecipitation ratio for each Gal4 chimera protein was represented as the ratio of bound DNA intensity to input DNA intensity quantified from slot blots. Relative immunoprecipitation ratio was measured by comparing each immunoprecipitation ratio relative to that (set as 1) for cells without TSA treatment. (D) Example of ChIP assays with acetyltransferases using Py *ori* probe. TSPy5 minichromosome fragments for ChIP assays from Cos7 cells infected with TSPy5 virus and transfected with expression vectors of Gal4 fusion acetyltransferases. DNA input (INPUT) and coprecipitated chromatin DNA with anti-acetyl H3 or H4 antibody (BOUND) analyzed by slot blot using Py *ori* probe are indicated above the lanes, and Gal4 fusion proteins are indicated at the left. (E) Average from three independent experiments represented in panel D. Relative immunoprecipitation ratio was measured by comparing each immunoprecipitation ratio relative to that (set as 1) for GalDB. IgG, immunoglobulin G.

region of the PyLT DNA-binding domain affects association with the origin and DNA replication (104). Were acetylation to neutralize the positively charged Lys residues in the DNA-binding domain of PyLT, this might affect its binding to the origin.

As PyLT helicase activity is required for movement of the replisome, acetyltransferases associated with PyLT might also mediate chromatin remodeling to facilitate elongation. Hyperacetylation of histones, or removal of histone tails, the primary targets for acetylation, facilitate elongation of SV40 DNA replication (1, 87). Acetylation of PyLT also could affect its association with cellular proteins, such as pRB or p53, that are important for inducing G_1-to-S cell cycle progression and DNA replication (29, 30, 59, 84). These and other possibilities can be better understood once the extent to which PCAF (and GCN5) acetylate PyLT in vivo and the sites that are acetylated are known.

In *Saccharomyces cerevisiae*, nucleosome positioning mediated by ORC (origin recognition complex) affects replication initiation (66) and SV40 large T antigen promotes the formation of an origin free of nucleosomes and competent for replication (35, 47). Normal SV40 minichromosomes are more highly acetylated than cellular chromatin, with H4 occurring in mono-, di-, and triacetylated forms and H3 occurring in a diacetylated form (1). The sites for these particular hyperacetylations remain undetermined, although acetylation sites of core histones in vertebrate chromatin include K5 and K9 of H2A, K5, K12, K15, and K20 of H2B, K9, K14, K18, and K23 of H3, and K5, K8, K12, and K16 of H4 (92). Our not being able to detect changes in the acetylation of histones near the Py origin sequences in SV40 minichromosomes might be due to high levels of normal acetylation. It is still possible that specific sites in these histones are targeted by PCAF and GCN5 and thus could be detected by ChIP assays more refined than were conducted here.

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