THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 28, Issue of July 12, pp. 25026–25031, 2002

Printed in U.S.A.

The Oncoprotein Set/TAF-1β, an Inhibitor of Histone Acetyltransferase, Inhibits Active Demethylation of DNA, Integrating DNA Methylation and Transcriptional Silencing*

Received for publication, March 7, 2002, and in revised form, April 25, 2002
Published, JBC Papers in Press, April 26, 2002, DOI 10.1074/jbc.M202256200

Nadia Cervoni‡, Nancy Detich‡, Sang-beom Seo§, Debabrata Chakravarti§, and Moshe Szyf‡¶

From the ‡Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec H3G 1Y6, Canada and the §Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Histone hypoacetylation and DNA hypermethylation are hallmarks of gene silencing. Although a role for DNA methylation in regulating histone acetylation has been established, it is not clear how and whether epigenetic histone markings influence DNA modifications in transcriptional silencing. We have previously shown that induction of histone acetylation by trichostatin A promotes demethylation of ectopically methylated DNA (Cervoni, N., and Szyf, M. (2001) *J. Biol. Chem.* 276, 40778–40787). The oncoprotein Set/TAF-Iβ is a subunit of the recently identified inhibitor of acetyltransferases complex that inhibits histone acetylation by binding to and masking histone acetyltransferase targets (Seo, S. B., McNamara, P., Heo, S., Turner, A., Lane, W. S., and Chakravarti, D. (2001) *Cell* 104, 119–130). We show here that the overexpression of Set/TAF-Iβ, whose expression is up-regulated in multiple tumor tissues, inhibits demethylation of ectopically methylated DNA resulting in gene silencing. Overexpression of a mutant Set/TAF-Iβ that does not inhibit histone acetylation is defective in inhibiting DNA demethylation. Taken together, these results are consistent with a novel regulatory role for Set/TAF-Iβ, integrating epigenetic states of histones and DNA in gene regulation and provide a new mechanism that can explain how hypermethylation of specific regions might come about by inhibition of demethylation in cancer cells.

The DNA methylation pattern is tightly correlated with chromatin structure in that transcriptionally active chromatin domains are hypomethylated, whereas inactive regions are hypermethylated (3). However, the mechanisms defining the relationship between histone hypoacetylation and DNA hypermethylation are not clear. While it is well accepted that DNA methylation can promote chromatin deacetylation and inactivation (4), a number of studies suggest that chromatin status can also alter the pattern of DNA methylation. Genome wide demethylation was shown to be induced by *n*-butyrate, an inhibitor of histone acetylation (5). Tichostatin A (TSA),[1] an inhibitor of histone deacetylase, was shown to induce selective loss of DNA methylation in *Neurospora* (6), and very recently disruption of histone methylation was shown to eliminate DNA methylation in *Neurospora* (7). We have recently shown that TSA can induce demethylation of ectopically methylated genes (1).

Hypermethylation of CpG islands has attracted considerable attention as a mechanism responsible for gene silencing during tumor suppression. It is clear that methylation of tumor suppressor genes cannot be explained by the increase in the general level of DNA methyltransferase activity observed in cancer cells. Significant effort has been therefore directed toward identification of factors that recruit DNA methyltransferases to specific promoters. In accordance with this hypothesis, a recent publication demonstrated that the oncogenic transcription factor PML-RAR fusion protein recruits DNMT1 to the retinoic acid βII promoter, resulting in methylation and silencing of this promoter (8).

An alternative potential mechanism of bringing about DNA hypermethylation is inhibition of demethylase activity. This possibility was not previously explored, since it has long been believed that the DNA methylation pattern is controlled exclusively by DNA methyltransferases and that the DNA methylation reaction is irreversible. Nevertheless, we have recently extracted DNA demethylase activity from human lung cancer cell line A549 (9) and have shown that a human embryonal kidney cell line HEK293 possesses active demethylase activity (1).

The DNA methylation state of a gene in a living cell represents a steady state, and it is hard to determine whether it is a consequence of increased DNA methylation or reduced demethylation. Moreover, it is hard to determine whether demethylation of a gene in a proliferating cell is passive, resulting from inhibition of DNA methylation during DNA synthesis or an active true removal of a methyl group. We have therefore recently developed a transient transfection system that enables studying demethylase activity in cancer cells. A reporter CMV-GFP construct is methylated *in vitro* by a CpG methyltransferase m*Sss*I and introduced into human transformed HEK293 cells. The plasmid is extracted 96 h after transfection, and its state of methylation is determined by either methylation sensitive restriction enzyme analysis or bisulfite mapping. Since we have demonstrated that this plasmid does not replicate in HEK cells (1), any change in DNA methylation must result from active demethylation. We have also shown that these constructs are not *de novo* methylated during the transient transfection period, and therefore the transient transfection assay measures exclusively active demethylation in the cell. This system could be utilized to measure the effects of specific

* This work was supported by the National Cancer Institute of Canada. Work in D. C.'s laboratory was supported by National Institutes of Health Grant RO1 DK57079. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed at the above address: Dept. of Pharmacology and Therapeutics, McGill University, 3655 Drummond St., Montreal, Quebec H3G 1Y6, Canada. Tel.: 514-398-7107; Fax: 514-398-6690; E-mail: mszyf@pharma.mcgill.ca.

[1] The abbreviations used are: TSA, trichostatin A; GFP, green fluorescent protein; INHAT, inhibitor of acetyltransferases; CHIP, chromatin immunoprecipitation; CMV, cytomegalovirus; HAT, histone acetyltransferase; GST, glutathione *S*-transferase.

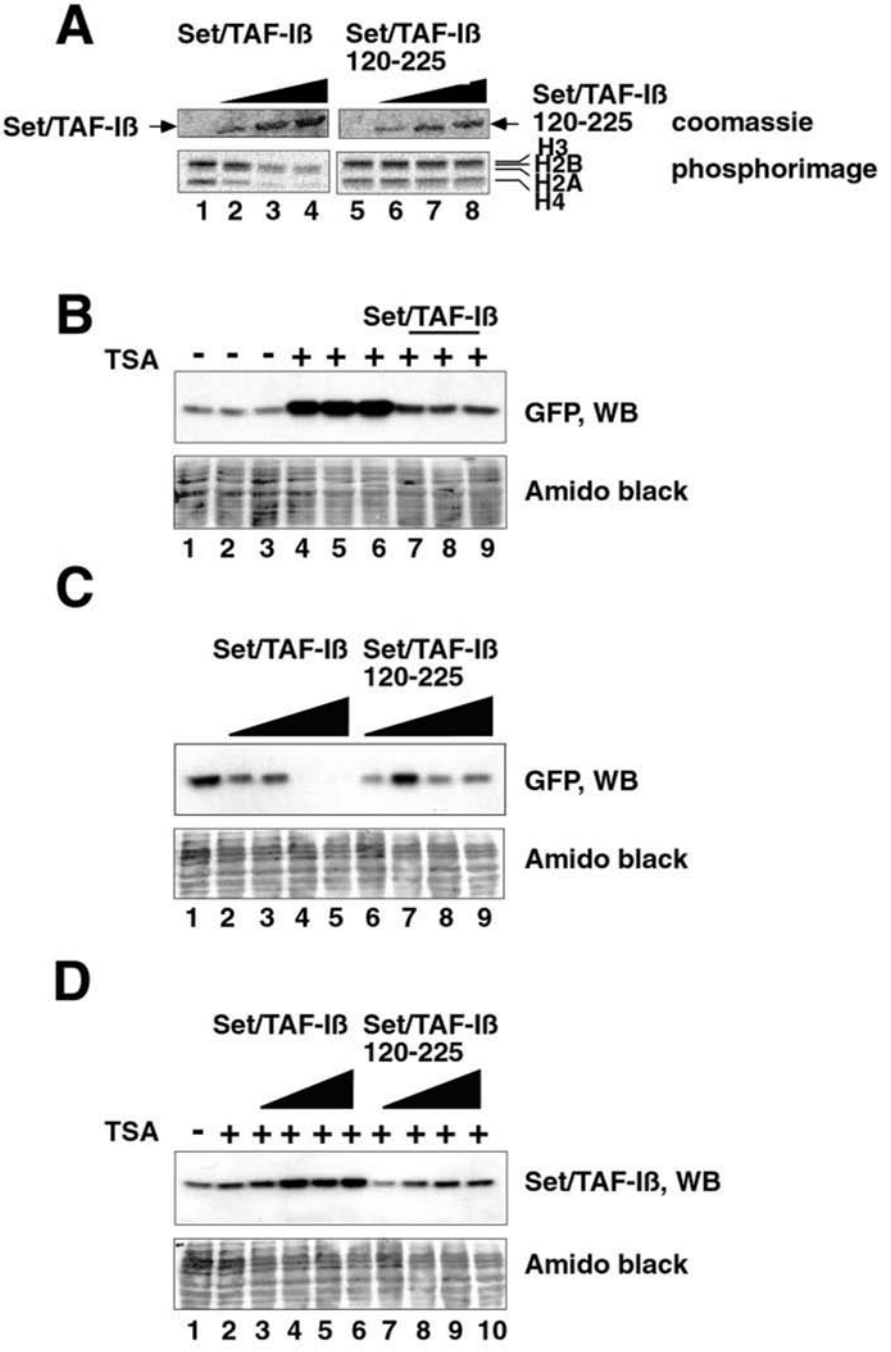

FIG. 1. *A*, Set/TAF-Iβ inhibits histone acetylation. HAT assays were performed with increasing concentrations of GST-Set/TAF-Iβ (*lanes 2–4*) and GST-Set/TAF-Iβ 120–225 (*lanes 6–8*), respectively. In *lanes 1* and *5*, p300 was incubated with histones only. The *upper panels* show Coomassie staining of both proteins used, and positions of individual histones are shown by phosphorimaging of acetylated histones (*lower panels*). *B* and *C*, Set/TAF-Iβ decreases acetylation-induced increase of GFP expression. *B*, total cell extracts from HEK293 cells transfected with either CMV-GFP plasmid alone (*lanes 1–6*) or CMV-GFP and Set/TAF-Iβ (*lanes 7–9*) in the absence (*lanes 1–3*) or presence of 0.3 μM TSA (*lanes 4–9*) were prepared using standard protocols and resolved on a 12.5% SDS-polyacrylamide gel in triplicates. *C*, total cell extracts from HEK cells transfected with increasing concentrations of Set/TAF-Iβ (0.1, 0.5, 1.0, and 2.0 μg) (*lanes 2–5*) or Set/TAF-Iβ 120–225 mutant (0.1, 0.5, 1.0, and 2.0 μg) (*lanes 7–9*), in the absence (*lane 1*) or presence of 0.3 μM TSA (*lanes 2–9*), were prepared using standard protocols and resolved on a 12.5% SDS-polyacrylamide gel. *D*, total cell extracts from HEK cells transfected with increasing concentrations of Set/TAF-Iβ (0.1, 0.5, 1.0, and 2.0 μg) (*lanes 3–6*) or Set/TAF-Iβ 120–225 mutant (0.1, 0.5, 1.0, and 2.0 μg) (*lanes 7–10*), in the absence (*lane 1*) or presence of 0.3 μM TSA (*lanes 2–10*), were prepared using standard protocols and resolved on a 12.5% SDS-polyacrylamide gel. All blots were transferred to PDVF membranes. *C,* GFP protein was detected using rabbit polyclonal IgG (Santa Cruz, sc-8334) at 1:500 dilution, followed by peroxidase-conjugated anti-rabbit IgG (Sigma) at 1:5000. *D*, Set/TAF-Iβ protein was detected using mouse monoclonal IgG 1:400 dilution, followed by peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.) at 1:20,000. Signal was detected by using enhanced chemiluminescence detection kit (Amersham Biosciences). To show equal loading of the protein, membranes were stained with Amido Black (*lower panels*).

proteins on active demethylation of specific sequences in living cells. Using this system we have recently shown that histone acetylation stimulates active demethylation of ectopically methylated reporter constructs.

Although we have shown that TSA treatment could potentially stimulate demethylation, this effect is not universal, and it is clear that many methylated genes are neither demethylated nor activated following TSA treatment (10). We therefore reasoned that proteins that inhibit acetylation of histones in response to TSA might also inhibit DNA demethylation. Similar proteins might also inhibit corrective demethylation of spuriously methylated genes and lead to hypermethylation of certain sequences explaining the progressive methylation of tumor suppressor genes in cancer. Recently, experiments performed by Seo *et al.* (2) led to the discovery of a novel human cellular complex which inhibits the histone acetyltransferase (HAT) activity of transcriptional coactivators p300/CBP and PCAF. This complex called INHAT (inhibitor of acetyltransferases) comprised of the myeloid leukemia-associated oncoprotein SET/TAF-Iβ, the template-activating factor TAF-I∂, and the nuclear phosphoprotein pp32 masks the effect of histone acetyltransferases by binding to histones, thereby inhibiting transcription and serving as a novel mechanism of transcriptional regulation (2). INHAT subunits were shown to individually inhibit histone acetylation. Since INHATs influence the state of histone acetylation (2), and since histone acetylation affects chromatin structure (11, 12), we tested the hypothesis that INHATs could directly or indirectly modulate DNA methylation patterns by inhibiting DNA methylation and thus play a role in integrating the status of histone acetylation, DNA methylation, and transcriptional silencing. Since Set/TAF-Iβ's association with leukemia suggests a fundamental cellular function perhaps related to neoplastic progression, we focused primarily on the Set/TAF-Iβ oncoprotein in this study.

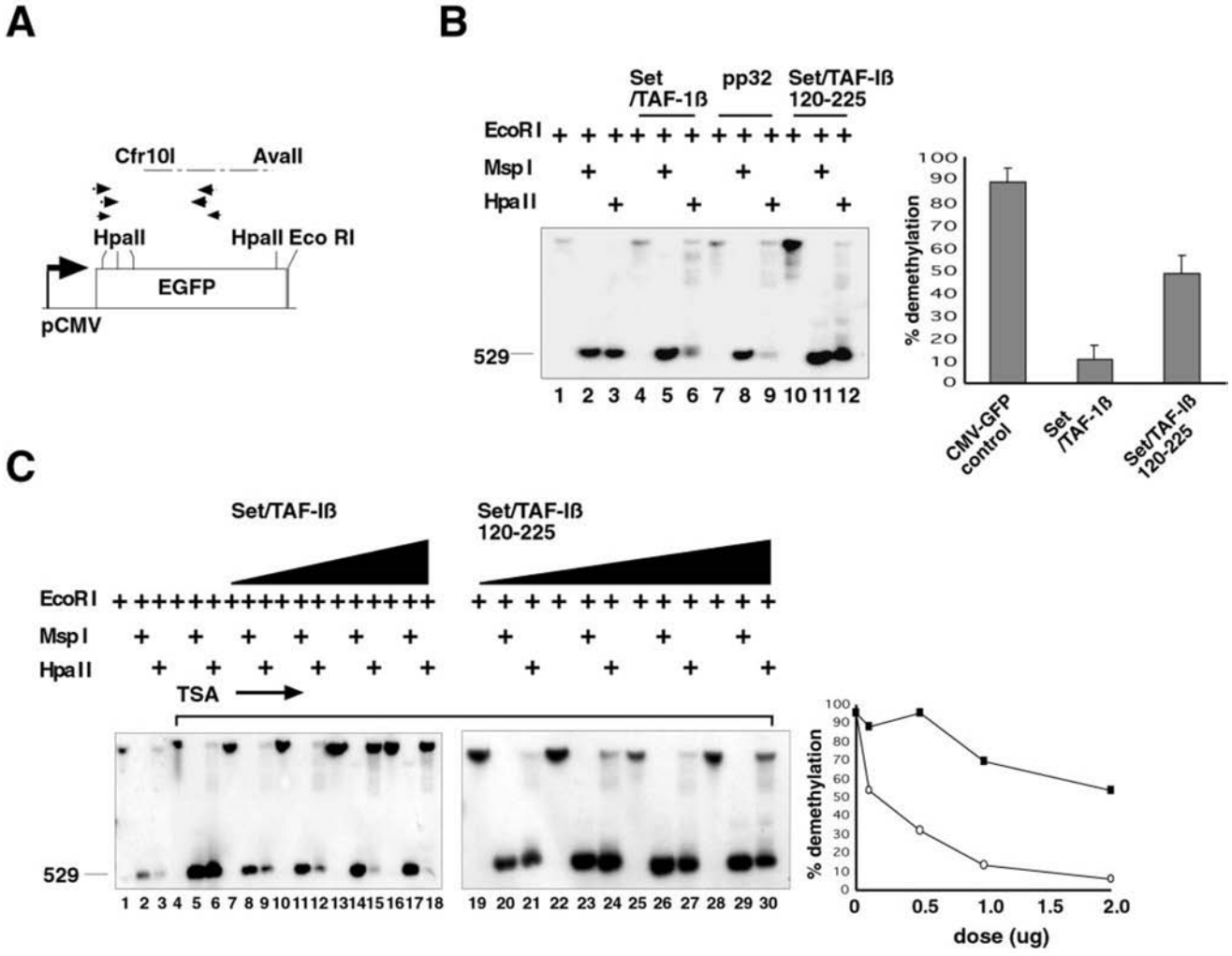

FIG. 2. **INHAT subunits inhibit demethylation of GFP.** *A*, a physical map of the CMV-GFP region analyzed is shown. *B*, *in vitro* methylated CMV-GFP plasmid was transfected alone (*lanes 1–3*) or cotransfected with either: 2 μg of Set/TAF-Iβ (*lanes 4–6*), pp32 (*lanes 7–9*), or Set/TAF-Iβ 120–225 mutant (*lanes 10–12*) plasmids and treated with a final concentration of 0.3 μM TSA. The results of three independent experiments were quantified by densitometry, and the percent demethylation for each sample was determined by calculating the ratio of *Hpa*II/*Msp*I-digested 529-bp fragment. *C, in vitro* methylated CMV-GFP plasmid was transfected alone (*lanes 1–6*) or with either increasing concentrations of Set/TAF-Iβ (0.1, 0.5, 1.0, and 2.0 μg) (*lanes 7–18*) or Set/TAF-Iβ 120–225 mutant (0.1, 0.5, 1.0, and 2.0 μg) (*lanes 19–30*), in the absence (*lanes 1–3*) or presence of 0.3 μM TSA (*lanes 4–30*). Results were quantified as in *B* and are shown in the graph. ■, Set/TAF-Iβ 120–225 mutant; ○, Set/TAF-Iβ. For both *B* and *C*, 10 μg of isolated DNA was digested with 50 units of *Eco*RI (*B*, *lanes 1*, *4*, *7*, and *10*; *C*, *lanes 1*, *4*, *7*, *10*, *13*, *16*, *19*, *22*, *25*, and *28*) alone or *Eco*RI followed by either digestion with 20 units of *Msp*I (*B*, *lanes 2*, *5*, *8*, and *11*; *C*, *lanes 2*, *5*, *8*, *11*, *14*, *17*, *20*, *23*, *26*, and *29*) or *Hpa*II restriction enzymes (*B, lanes 3*, *6*, *9*, and *12*; *C*, *lanes 3*, *6*, *9*, *12*, *15*, *18*, *21*, *24*, *27*, and *30*), fractionated on a 1.7% agarose gel, and was then subjected to Southern blot transfer and hybridization with a ^{32}P-labeled GFP fragment (see map of probe in *A*, indicated by a *dashed line* flanked by restriction sites *Cfr*10I and *Ava*II).

MATERIALS AND METHODS

Cell Culture—HEK293 cells were plated at a density of 8×10^4/well in a six-well tissue culture dish and transiently transfected with 80 ng of methylated CMV-GFP plasmid DNA alone or cotransfected with up to a maximum of 2 μg of INHAT plasmid DNA (2) per well, using the calcium phosphate precipitation method as described previously (13). Transfections were performed in five individual wells and were repeated three times using different cultures of HEK293 cells.

In Vitro Methylation of Substrates—CMV-GFP plasmid was methylated *in vitro* by incubating 10 μg of plasmid DNA with 20 units of *Sss*I CpG DNA methyltransferase (14) (New England Biolabs Inc.) in a buffer recommended by the manufacturer containing 160 μM *S*-adenosylmethionine, at 37 °C for 2 h. After repeating this procedure three times, full protection from *Hpa*II digestion was observed.

Histone Acetylation Assays—The assay was performed essentially as described previously (2) utilizing increasing concentrations of wild type Set/TAF-Iβ and mutant Set/TAF-Iβ proteins. In short, ~1 pmol of baculovirus-expressed and purified p300 was incubated with (Fig. 1*A*, *lanes 2–4* and *6–8*) or without (Fig. 1, *lanes 1* and *5*) increasing concentrations of purified wild type or mutant GST-Set/TAF-Iβ as indicated in the legend to Fig. 1 for 5 min at 40 °C before the addition of ~1 μg of purified core histones and [^{14}C]acetyl-CoA (1000 pmol/μl, Sigma) and incubation continued for an additional 30 min. HAT assays were performed in the presence of 10 mM sodium butyrate. Reaction products were separated by SDS-PAGE and analyzed by a phosphorimager (2). GST alone had no effect in the assay system.

Bisulfite Mapping—Bisulfite mapping was performed as described previously with minor modifications (15). 5 μg of sodium bisulfite-treated DNA samples were subjected to PCR amplification using the first set of primers described below. PCR products were used as templates for subsequent PCR reactions utilizing nested primers. The PCR products of the second reaction were then subcloned using the Invitrogen TA cloning kit (we followed the manufacturer's protocol), and the clones were sequenced using the T7 sequencing kit (Amersham Biosciences; we followed the manufacturer's protocol, procedure C). The primers used for the enhanced green fluorescent protein (pEGFP-1) (CLONTECH) (GenBank™ accession number U55761) were: GFP5′, 1,5′-gttattatggtgagtaaggg-3′; GFP5′ (nested), 5′-ggggtggtgtttattttgg-3′; GFP3′, 1,5′-tataactattataatatactcca-3′; GFP3′ (nested), 5′-cttataccccaaaatattacc-3′.

Chromatin Immunoprecipitation (CHIP) Assay—CHIP assays (16) were performed by following the Upstate Biotechnology CHIP assay kit protocol (catalog number 17-295). HEK293 cells were transfected with 80 ng of *in vitro* CMV-GFP plasmid, using the calcium phosphate method (see above). A final concentration of 0.3 μM TSA was added or

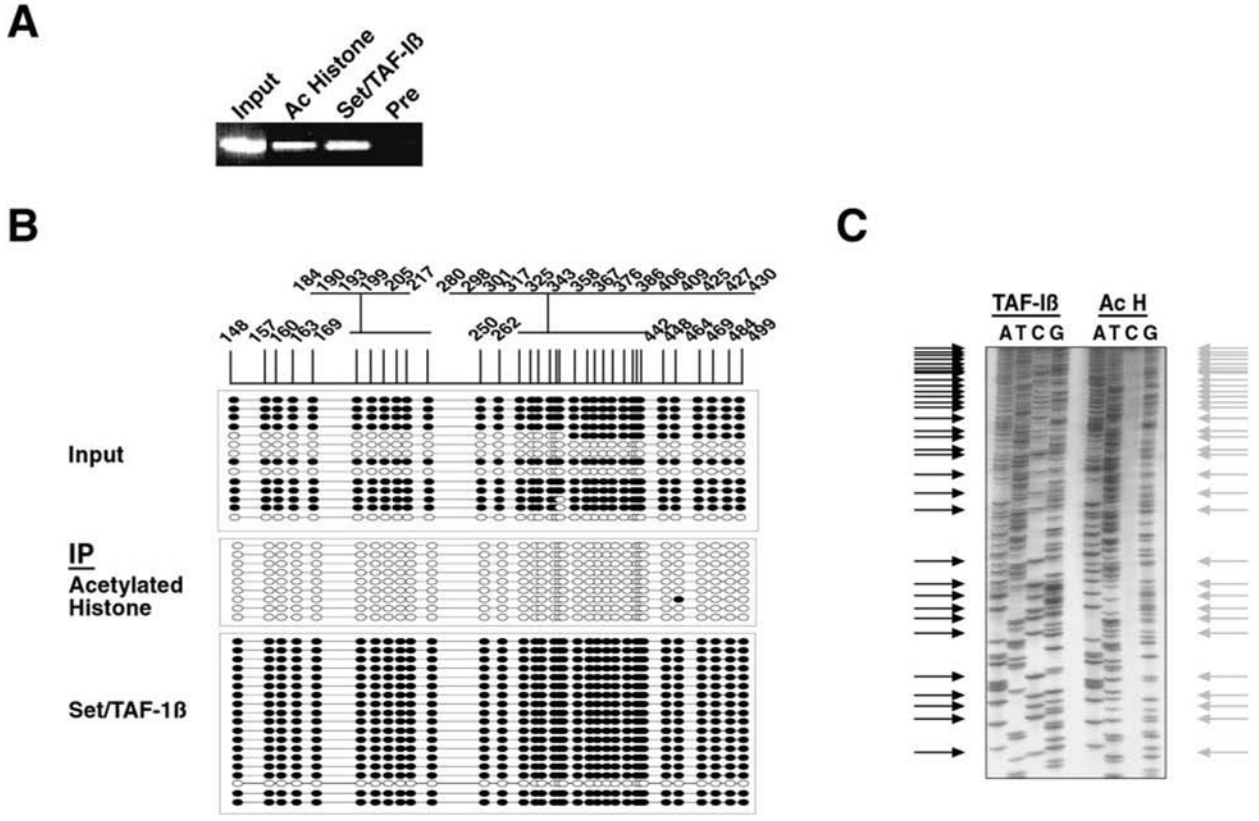

FIG. 3. **Bisulfite mapping and CHIP analysis of the association of transfected plasmids with Set/TAF-Iβ.** *A*, HEK293 cells were cotransfected with *in vitro* methylated GFP plasmid and 1 μg of Set/TAF-Iβ plasmid, treated with a final concentration of 0.3 μM TSA, formaldehyde cross-linked after 96 h, and subjected to a chromatin immunoprecipitation assay using antibodies against acetylated histone H3 (*Ac Histone*), Set/TAF-Iβ, or rabbit preimmune serum (*Pre*). *Input* denotes 10% of total DNA prior to immunoprecipitation. The GFP sequence was amplified from purified DNA by PCR. Primers (indicated in Fig. 2*A* as *solid arrows*) were diluted to 50 μM and are described under "Materials and Methods." A representative PCR is shown. *B*, immunoprecipitated DNA was subjected to bisulfite mapping analysis. *Hatched arrows* (displayed in Fig. 2*A*) indicate the location of both the outside and nested primers used to amplify bisulfited DNA. Each *line* within the *boxes* represents an independent clone. A *filled circle* represents a methylated CG dinucleotide, and an *empty circle* represents a demethylated CG dinucleotide. Clones from TSA-treated DNA not immunoprecipitated (*Input*), or immunoprecipitated with anti-acetylated H3 antibody or anti-Set/TAF-Iβ, are presented. *C*, representative sequencing gels of immunoprecipitated clones using either anti-acetylated H3 (*Ac H*) or anti-Set/TAF-Iβ (*TAF-Iβ*) antibodies. *Black arrows* point to methylated CGs from clones pulled down with anti-Set/TAF-Iβ antibody, and *gray arrows* point to demethylated CGs (converted to Ts) from clones pulled down with anti-acetylated histone H3 antibody

not added to fresh medium 24 h after transfection. Formaldehyde was added to the culture media at a final concentration of 1%, 96 h post-transfection and incubated at 37 °C for 10 min. Chromatin was immunoprecipitated using either an anti-acetylated histone H3 antibody (Upstate Biotechnology) as recommended by the manufacturer or the anti-Set/TAF-Iβ antibody (2) or rabbit preimmune serum, as a control. One-tenth of the lysate was kept to quantitate the amount of DNA present in different samples before immunoprecipitation (*input*). PCR reactions on DNA purified from non-immunoprecipitated samples (*input*) and immunoprecipitated samples (*Ac H*, *Set/TAF-Iβ*, and *Pre*) were repeated exhaustively using varying amounts of template to ensure that results were within the linear range of the PCR. The following primers were used to amplify the GFP gene: GFP 5′, 5′-caagggcgaggagctgtt-3′; GFP 3′, 5′-cggccatgatatagacgttg3′.

Western Blot Analysis—Total cell extracts were prepared using standard protocols and resolved on SDS-polyacrylamide gel electrophoresis (12.5%). After transferring to polyvinylidene difluoride membrane (Amersham Biosciences) and blocking the non-specific binding with 5% skim milk, GFP protein was detected using rabbit polyclonal IgG (Santa Cruz, sc-8334) at 1:500 dilution, followed by peroxidase-conjugated anti-rabbit IgG (Sigma) at 1:5000, and an enhanced chemiluminescence detection kit (Amersham Biosciences). Set/TAF-Iβ protein was detected as described previously (17).

Multiple Tumor Blot Analysis—The matched tumor/normal expression assay membrane was hybridized using a ^{32}P-labeled Set/TAF-Iβ cDNA, according to the CLONTECH Laboratories Inc. Matched Tumor/Normal Expression Array User Manual (catalog number 7840-1). To obtain final expression values, each hybridization signal was quantified by densitometry and normalized against signal obtained by hybridization with a cDNA probe for ubiquitin.

RESULTS

INHAT Set/TAF-1β Inhibits Expression of a Cotransfected Methylated CMV-GFP Reporter Gene in a Dose-dependent Manner—We utilized a previously described transient transfection-based assay system to study active demethylation of ectopically methylated DNA and the different parameters that might affect it. In prior studies we have shown that *in vitro* methylated CMV-GFP reporter plasmid is actively demethylated 72–96 h following transfection into human embryonal kidney HEK293 cells and that this demethylation is dramatically enhanced when histone hyperacetylation is induced pharmacologically with TSA (1). If Set/TAF-Iβ functions by inhibiting histone acetylation, and also functionally interacts with methylated CMV-GFP, then overexpression of Set/TAF-Iβ should inhibit expression of CMV-GFP. To test this, Set/TAF-Iβ plasmid was cotranfected into human embryonic kidney (HEK293) cells, with *in vitro* methylated CMV-GFP plasmid, in the presence or absence of TSA (72 h), and harvested 96 h post-transfection. Cell extracts were prepared and subjected to Western blot analysis using antibodies directed against GFP protein (Fig. 1, *B* and *C*) or against the amino-terminal region of the Set/TAF-Iβ oncoprotein (which only recognizes the endogenous and wild type Set/TAF1β construct) (Fig. 1*D*). GFP expression is significantly increased in the presence of TSA compared with no TSA treatment (Fig. 1*B*) (1); and in agreement with previous

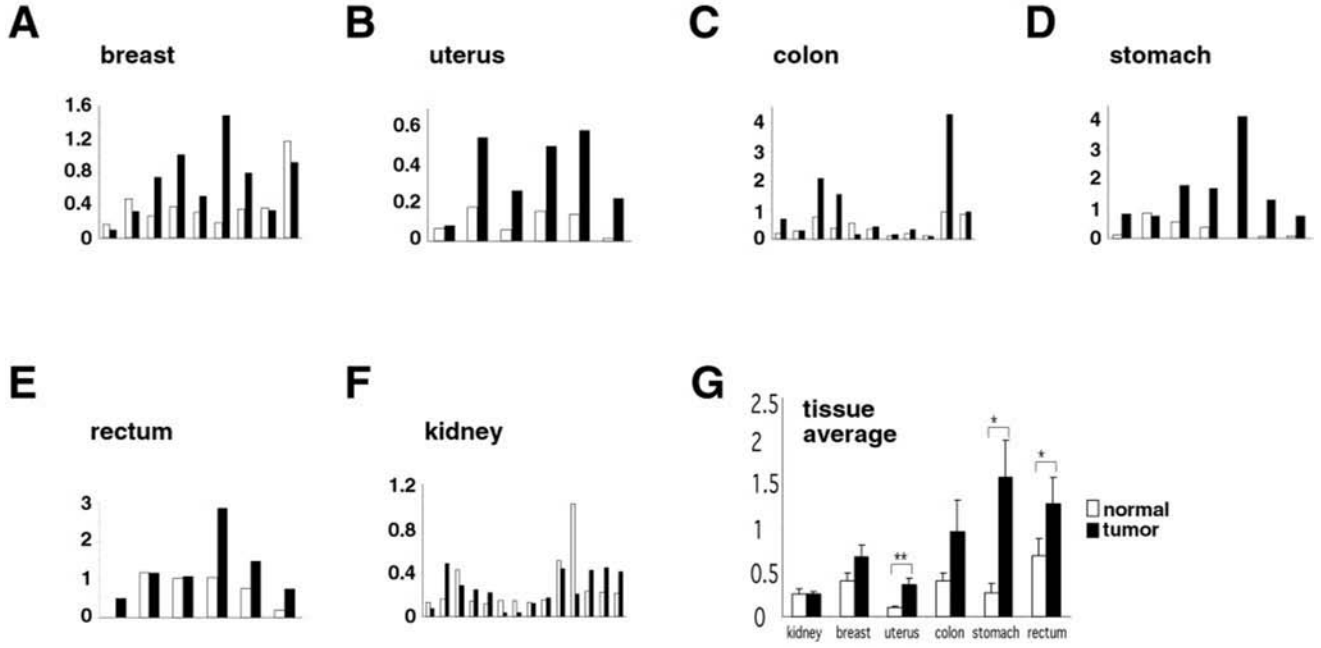

FIG. 4. **Set/TAF-Iβ oncogene is up-regulated in tumor tissues.** The matched tumor/normal expression assay membrane was hybridized using a ^{32}P-labeled Set/TAF-Iβ cDNA. Histograms represent values analyzed by densitometry from Set/TAF-Iβ signal normalized against ubiquitin signal. *Black bars* represent values obtained from tumor tissues, and *white bars* represent values obtained from normal tissues. The following tissues were analyzed: breast (*A*), uterus (*B*), colon (*C*), stomach (*D*), rectum (*E*), kidney (*F*). *G*, the tissue average of Set/TAF-Iβ message was calculated and found to be significantly greater in uterus, stomach, and rectum (**, $p < 0.005$; *, $p < 0.05$) when compared with Set/TAF-Iβ signal from normal tissues. Colon ($p = 0.053$) and breast ($p = 0.063$) were nearly significant.

results (2), Fig. 1*A* shows that while the wild type Set/TAF-Iβ inhibits p300-mediated histone acetylation in a dose-dependent manner, the mutant Set/TAF-Iβ 120–225, which lacks the previously determined HAT inhibitory domain (INHAT domain) and histone binding domain, fails to inhibit histone acetylation (Fig. 1*A* and Ref. 2). Cotransfection with Set/TAF-Iβ (Fig. 1, *B* and *C*), but not the mutant Set/TAF-Iβ 120–225 plasmid (Fig. 1*C*), attenuated GFP expression to a level that is only slightly above the basal value obtained in the absence of TSA (Fig. 1*B*), in a dose-dependent manner (Fig. 1, *C* and *D*), establishing a role for the INHAT activity of Set/TAF-Iβ in regulating GFP expression.

INHAT Subunits Inhibit Demethylation of Ectopically Methylated CMV-GFP Transiently Transfected into HEK293 Cells in a Dose-dependent Manner—Since different subunits of the INHAT complex inhibited expression of CMV-GFP, we determined their effect on DNA demethylation induced by TSA (1). DNA was isolated from HEK293 cells cotransfected with methylated GFP DNA and Set/TAF-Iβ, pp32, or Set/TAF-Iβ 120–225 mutant and was treated with 0.3 μM TSA for 72 h. A map of the analyzed GFP gene is presented in Fig. 2*A*. DNA was first linearized with the *Eco*RI restriction enzyme, followed by digestion with *Msp*I (which cleaves the sequence CCGG) or *Hpa*II (which cleaves the sequence CCGG only when it is not methylated). The results of this experiment demonstrate that histone deacetylase inhibitor (TSA) treatment alone results in nearly complete demethylation (90%) as indicated by the detection of a fully demethylated *Hpa*II-digested fragment (529 kb) as shown previously (Fig. 2*B*, *lane 3* and *graph bar 1*) (1). Remarkably, addition of individual INHAT subunits Set/TAF-Iβ and pp32, which block histone acetylation, inhibit demethylation of the reporter gene, as indicated by the 9-fold reduction in the relative abundance of the *Hpa*II-digested fragment with Set/TAF1β (Fig. 2*B*, compare *lane 3* with *lanes 6* and *9*, and *graph bar 2 versus bar 1*). The Set/TAF-Iβ 120–225 mutant, which does not inhibit histone acetylation, had a minimal effect (2-fold) on blocking DNA demethylation (Fig. 2*B*, compare *lane 12* with *lane 6*, and *graph bar 3*). There is a dose-response relationship between demethylation and inhibition of histone acetylation. The relative abundance of the fully demethylated 0.529-kb *Hpa*II fragment was reduced from 95% (with TSA alone) to nearly undetectable levels (6%) with increasing doses of Set/TAF-Iβ (Fig. 2*C*, *lanes 6*, *9*, *12*, *15*, *18*, and *graph open circles*), but not with the Set/TAF-Iβ 120–225 mutant (Fig. 2*C*, *lanes 21*, *24*, *27*, *30*, and *graph closed squares*). In summary, these experiments demonstrate that INHAT subunits inhibit demethylation of ectopically methylated DNA in a dose-dependent manner.

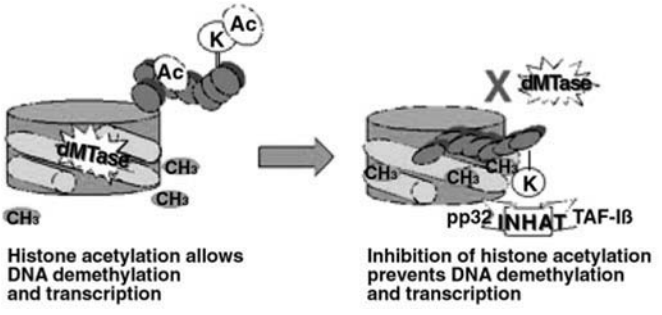

FIG. 5. **Model illustrating how endogenous proteins such as demethylase can access and demethylate DNA within the nucleosome when histone tails are acetylated.** INHATs' ability to mask histones and therefore prevent histone acetylation inhibit the association between demethylase and the nucleosome-wrapped DNA. The resulting phenotype is hypermethylated DNA.

INHAT Subunit Set/TAF-Iβ Inhibits Demethylation by Directly Interacting with the Chromatin Associated with It—Set/TAF-Iβ might inhibit demethylation of CMV-GFP DNA indirectly by inducing other activities required for demethylation or they might act directly on CMV-GFP by binding to its chromatin and preventing its acetylation and, as a consequence, its demethylation. If Set/TAF-Iβ acts on histones associated with the target DNA, then CMV-GFP DNA associated with Set/TAF-Iβ

should remain hypermethylated, whereas DNAs that escaped Set/TAF-Iβ binding should be associated with acetylated histones and hypomethylated (1). To address this issue we used the CHIP assay with anti-acetyl-histone H3 and anti-Set/TAF-Iβ antibodies on cells cotransfected with methylated GFP and 1 μg of Set/TAF-Iβ (a concentration of Set/TAF-Iβ that results in partial inhibition of demethylation as shown in Fig. 2*C*) and treated with TSA (Fig. 3). The results presented in Fig. 3*A* show that the CMV-GFP gene sequence was immunoprecipitated with both the anti-acetylhistone H3 and anti-Set/TAF-Iβ antibodies but not with preimmune rabbit serum. GFP plasmid DNAs isolated from both acetylhistone H3 and Set/TAF-Iβ-immunoprecipitated samples were then subjected to bisulfite mapping to determine their respective methylation patterns (Fig. 3*B*). GFP DNA isolated before immunoprecipitation (*Input*) was partially demethylated as anticipated (Fig. 2*C*). All DNAs immunoprecipitated with acetylhistone H3 antibody displayed almost complete demethylation (Fig. 3, *B* and *C*). Remarkably, however, DNAs immunoprecipitated with anti-Set/TAF-Iβ antibodies appeared completely hypermethylated except for one demethylated clone. This result shows that Set/TAF-Iβ associates with methylated transfected CMV-GFP chromatin and facilitates the maintenance of the methylated status.

Set/TAF-1β Is Overexpressed in Multiple Tumors—To test the hypothesis that the level of expression of INHATs might vary under pathological conditions where aberrations in chromatin acetylation and DNA methylation are commonly observed, we used a cDNA expression array (Atlas^{TM}) containing mRNA from various tumors and their respective non-tumor tissues (Fig. 4). The membrane was hybridized with full-length ^{32}P-labeled Set/TAF-Iβ oncoprotein cDNA and quantified by densitometry. From the multiple samples assayed for each tissue type, an increased expression of Set/TAF-Iβ in certain tumor tissues relative to normal tissues was observed (Fig. 4, *A–F*). The tissue averages from uterus, colon, stomach, and rectum displayed a 2-fold or greater increase in Set/TAF-Iβ expression in tumor tissues compared with normal tissues (Fig. 4*G*).

DISCUSSION

This paper demonstrates that a chromatin-modifying protein can determine the state of methylation of certain ectopically methylated sequences by inhibiting DNA demethylation. There are several possibilities by which INHAT subunits may regulate DNA methylation. First Set/TAF-Iβ, by virtue of its ability to inhibit histone acetylation, maintains target DNAs in hypoacetylated form. Since histone hyperacetylation is necessary for DNA demethylation of ectopically methylated DNA (1), Set/TAF-Iβ-targeted DNAs remain hypermethylated. Second, the histone binding and HAT inhibitory functions of Set/TAF-Iβ are not necessary for its role in the maintenance of DNA hypermethylation. However, the greatly reduced ability of the Set/TAF-Iβ mutant (defective in histone binding and INHAT functions) to protect methylated DNA as compared with the wild type Set/TAF-Iβ eliminates this possibility. Third, INHAT functions by directly recognizing methylated DNAs and protecting them from being targeted by DNA demethylases, which is supported by the fact that the Set/TAF-Iβ mutant still retains a minor ability to inhibit demethylation. Finally INHAT may function by both binding to methylated DNAs and preventing histone acetylation. While the last two remain formal possibilities and are testable, based on our results we suggest a model depicted in Fig. 5 directly linking HAT-inhibitory property, INHAT, in the establishment of an inverse relationship between histone acetylation and DNA methylation in gene silencing. Based on our previous results and the results shown here, we propose that the balance of demethylase and INHATs in cells may therefore determine the final DNA methylation pattern. Histone hypoacetylation due to an elevation in the cellular concentration of factors such as INHATs (Fig. 4*G*) may prevent access of demethylases to chromatin, therefore preserving the existing ectopic hypermethylation.

This paper identifies for the first time a potential mechanism of inhibition of active demethylation and further supports our previous hypothesis that cancer cells bear a level of demethylase activity that demethylates ectopically methylated sequences. Further extensive experiments are obviously required to test whether such a mechanism participates in precipitating the hypermethylated state of specific tumor suppressor genes. An obvious question that has to be addressed is how could inhibitors of demethylation cause hypermethylation of normally unmethylated genes such as tumor suppressors. We would like to speculate that spurious methylation events do occur during normal replication and that they are normally removed from transcribed genes that are associated with hyperacetylated histones by demethylase(s). Interaction of proteins such as Set/TAF-Iβ with histones associated with a gene lead to inhibition of acetylation, and as a consequence corrective demethylation is inhibited. This can lead to progressive hypermethylation of a gene.

In summary, this paper demonstrates that a protein that inhibits histone acetylation also blocks demethylation as well as expression of ectopically methylated DNA. This is to our knowledge the first example of a class of proteins shown to affect a methylation pattern by inhibiting demethylation. These data redirect our understanding of how DNA methylation patterns are generated, maintained, and pathologically altered in cancer and provide novel potential targets for regulation of aberrant methylation patterns, such as those found in tumors or transformed cells.

While our experiments suggest that Set/TAF-Iβ could potentially affect DNA methylation states by inhibiting demethylation, further experiments will be necessary to demonstrate that hypermethylation of specific tumor suppressor genes involves interaction with proteins like Set/TAF-Iβ. We are currently searching for endogenous genes targeted by INHATs, to test our model and confirm the results of our transient system. Other important questions that have to be resolved are what are the mechanisms responsible for up-regulation of Set/TAF-Iβ during tumorigenesis and what may target Set/TAF-Iβ to certain genes and not others? Nevertheless, our data illustrates some of the first principles integrating the process of demethylation, histone hypoacetylation, and DNA hypermethylation in the establishment of silent chromatin loci.

REFERENCES

1. Cervoni, N., and Szyf, M. (2001) *J. Biol. Chem.* **276,** 40778–40787
2. Seo, S. B., McNamara, P., Heo, S., Turner, A., Lane, W. S., and Chakravarti, D. (2001) *Cell* **104,** 119–130
3. Razin, A., and Cedar, H. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 2725–2728
4. Razin, A. (1998) *EMBO J.* **17,** 4905–4908
5. Szyf, M., Kaplan, F., Mann, V., Giloh, H., Kedar, E., and Razin, A. (1985) *J. Biol. Chem.* **260,** 8653–8656
6. Selker, E. U. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9430–9435
7. Tamaru, H., and Selker, E. U. (2001) *Nature* **414,** 277–283
8. Di Croce, L., Raker, V. A., Corsaro, M., Fazi, F., Fanelli, M., Faretta, M., Fuks, F., Lo Coco, F., Kouzarides, T., Nervi, C., Minucci, S., and Pelicci, P. G. (2002) *Science* **295,** 1079–1082
9. Ramchandani, S., Bhattacharya, S. K., Cervoni, N., and Szyf, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 6107–6112
10. Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G., and Baylin, S. B. (1999) *Nat. Genet.* **21,** 103–107
11. Chen, H., Tini, M., and Evans, R. M. (2001) *Curr. Opin. Cell Biol.* **13,** 218–224
12. Jenuwein, T., and Allis, C. D. (2001) *Science* **293,** 1074–1080
13. Rouleau, J., Tanigawa, G., and Szyf, M. (1992) *J. Biol. Chem.* **267,** 7368–7377
14. Nur, I., Szyf, M., Razin, A., Glaser, G., Rottem, S., and Razin, S. (1985) *J. Bacteriol.* **164,** 19–24
15. Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. (1994) *Nucleic Acids Res.* **22,** 2990–2997
16. Crane-Robinson, C., Myers, F. A., Hebbes, T. R., Clayton, A. L., and Thorne, A. W. (1999) *Methods Enzymol.* **304,** 533–547
17. Nagata, K., Saito, S., Okuwaki, M., Kawase, H., Furuya, A., Kusano, A., Hanai, N., Okuda, A., and Kikuchi, A. (1998) *Exp. Cell Res.* **240,** 274–281