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Simian Virus 40 Small Tumor Antigen Inhibits Dephosphorylation of Protein Kinase A-Phosphorylated CREB and Regulates CREB Transcriptional Stimulation

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We report that the small tumor (small-t) antigen of simian virus 40 (SV40) forms complexes with nuclear protein phosphatase 2A (PP2A) and regulates the phosphorylation and transcriptional transactivation function of the cyclic AMP (cAMP)-regulatory element binding protein (CREB). PP2A coimmunoprecipitated with small t from nuclear extracts from HepG2 cells expressing small t or from rat liver nuclear extracts to which recombinant small t was added. Protein phosphatase 1 was not detected in small-t immunoprecipitates. In HepG2 cells expressing small t, dibutyryl-cAMP (Bt_2cAMP) stimulated the phosphorylation of CREB 65-fold, whereas CREB phosphorylation was stimulated only 5- to 8-fold by Bt_2cAMP in cells not expressing small t. Small t also inhibited the dephosphorylation of cAMP-dependent protein kinase (PKA)-phosphorylated CREB in rat liver nuclear extracts. In cells expressing small t, Bt_2cAMP-stimulated transcription from the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter was enhanced over the level of transcription from the PEPCK promoter in cells not expressing small t. Small t also enhanced Bt_2cAMP-stimulated transcription from a Gal4-responsive promoter in cells expressing a chimeric protein containing the Gal4 DNA-binding domain linked to the CREB transactivation domain. However, small t did not stimulate transcription either from a 5′ deletion mutant of the PEPCK promoter that is not able to bind CREB or from the Gal4-responsive promoter in the absence of the Gal4-CREB protein. These data suggest that small t enhances Bt_2cAMP-stimulated gene transcription by inhibiting the dephosphorylation of PKA-phosphorylated CREB by nuclear PP2A. These findings support previous observations that nuclear PP2A is the primary phosphatase that dephosphorylates PKA-phosphorylated CREB.

The simian virus 40 (SV40) small tumor (small-t) antigen is required for the efficient transformation of growth-arrested cells and for the maintenance of the transformed phenotype (4, 10, 15, 29, 35, 38, 41, 42). The mechanism(s) by which small t enhances the transformation of resting cells has not been resolved but may be related to its ability to stimulate the transcription of certain genes (27), disrupt actin cable networks (18, 21), and decrease sensitivity to DNA synthesis inhibitors (36). Recent evidence suggests that small t may regulate these biochemical processes by forming complexes with the cellular, serine- and threonine-specific protein phosphatase 2A (PP2A) (9, 37, 43, 50).

PP2A is the only cellular protein known to interact with small t (6, 30, 47). It is composed of a 32-kDa catalytic (C) subunit and A and B regulatory subunits of 61 and 55 kDa, respectively (11). Small t binds heterodimeric PP2A, which is composed of C and A subunits (50), and the free A subunit but does not associate with the free C subunit or purified, heterotrimeric PP2A (ABC). Small t alters the ability of PP2A to dephosphorylate substrate proteins. In particular, small t inhibits the PP2A-mediated dephosphorylation of myosin light chain, myelin basic protein (50), mitogen-activated protein kinase 2, extracellular-signal-regulated protein kinase 2, mitogen-activated protein/extracellular-signal-regulated protein kinase (43), and specific phosphorylation sites in the tumor-suppressor protein, p53, and in SV40 large T (37). While these findings suggest that small t may promote cell transformation by regulating PP2A activity, there is no direct evidence that the interaction of small t with PP2A plays a role in cell transformation or transcriptional stimulation.

Elevated intracellular cyclic AMP (cAMP) levels stimulate the transcription of certain genes through the phosphorylation of the cAMP-regulatory element binding protein (CREB) on a specific serine residue (serine 119 of human placental CREB-327 [25] or serine 133 of rat PC-12 cell CREB-341 [17]) by the cAMP-dependent protein kinase (PKA) (49). The rapid stimulation of transcription by cAMP is generally followed by a decline in the rate of transcription to basal levels. Recent reports indicate that the decrease in the rate of transcription is due, in part, to the dephosphorylation of PKA-phosphorylated CREB (P-CREB) (20, 46). Hagiwara et al. (20) have tested the ability of PP1 and PP2A catalytic subunits purified from skeletal muscle cytosolic extracts to dephosphorylate P-CREB and found PP1 to be more efficient at dephosphorylating P-CREB than PP2A. The PP1 catalytic subunit also inhibited the expression of reporter proteins from a cAMP-responsive promoter when introduced into cells either by microinjection or through the use of a PP1 expression vector. These results suggested that PP1 could dephosphorylate P-CREB and regulate the expression of cAMP-responsive genes. However, Wadzinski et al. (46) have shown that P-CREB phosphatase

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activity in nuclear extracts copurifies with PP2A and is completely resolved from PP1 on Mono Q, amino-hexyl Sepharose, and heparin agarose columns. Furthermore, nuclear PP2A was more efficient at dephosphorylating P-CREB than nuclear PP1. The data presented by Wadzinski et al. (46) identified nuclear PP2A as the primary phosphatase that dephosphorylates P-CREB. On the basis of these findings we hypothesized that SV40 small t might regulate cAMP-stimulated gene transcription by altering the dephosphorylation of P-CREB by PP2A.

In this article we demonstrate that small t binds to nuclear PP2A but not to PP1. Small t enhances cAMP-stimulated phosphorylation of CREB in cells and protects P-CREB from dephosphorylation in rat liver nuclear extracts. The ability of small t to enhance the phosphorylation of CREB and protect P-CREB from dephosphorylation directly correlates with its ability to enhance cAMP-stimulated transcription from the phosphoenolpyruvate carboxykinase (PEPCK) promoter. These results support the hypothesis that small t inhibits the dephosphorylation of P-CREB by nuclear PP2A, thereby enhancing cAMP-stimulated gene transcription. These are the first data to demonstrate that small t can regulate the phosphorylation and activity of a specific transcription factor and suggest that one mechanism by which small t may participate in cell transformation is by regulating the activity of transcription factors involved in the expression of genes that control cell growth. These results support our previous observations showing that the cAMP-stimulated transcription is attenuated by the dephosphorylation of P-CREB and that PP2A is the primary P-CREB phosphatase.

MATERIALS AND METHODS

Materials. Mouse monoclonal antibodies to SV40 small t (Ab-3) were purchased from Oncogene Science (Uniondale, N.Y.). Recombinant CREB protein, polyclonal antisera to CREB, and PP1 and PP2A catalytic subunits were prepared as previously described (46). The eukaryotic small-t expression vector, pSRtcDNA, was generously provided by Lauren Sompayrac (University of Colorado, Boulder). An empty expression vector, pSRα, was prepared by removing the small-t open reading frame from pSRtcDNA with *Xho*I and then religating the plasmid. A plasmid with an enhancerless thymidine kinase (TK) promoter linked to four copies of the Gal4 regulatory sequence driving expression of a luciferase reporter gene (pGal4TKLuc) was provided by James Hoeffler (University of Colorado School of Medicine, Denver). $^{32}P_i$ and [γ-^{32}P]ATP were purchased from ICN (Irvine, Calif.). Okadaic acid was obtained from Upstate Biotechnology, Inc. (Lake Placid, N.Y.). Vanadyl ribonucleosides, ATP, GTP, CTP, and RNase-free DNase were purchased from Life Technologies, Inc. (Gaithersburg, Md.). All other materials were obtained from Sigma Chemical Co. (St. Louis, Mo.). Rat liver nuclear extracts were prepared as previously described (24, 46), and nuclear extracts from HepG2 and H4IIE cells were prepared by the method of Dignam et al. (13).

Cell transfections and luciferase assays. HepG2 (human hepatoma) cells and H4IIE (rat hepatoma) cells were grown in Dulbecco's modified Eagle's medium F12 containing 5% fetal bovine serum and 5% calf serum. Plates of HepG2 cells, grown to approximately 80% confluency, were transfected with the indicated plasmids by calcium phosphate-DNA coprecipitation as described by Park et al. (31). H4IIE cells were transfected in suspension by calcium phosphate-DNA coprecipitation as described by Magnuson et al. (28).

Luciferase assays were performed on a Monolight 2010 luminometer by using the Enhanced Luciferase Assay kit (Analytical Luminescence Laboratories, San Diego, Calif.) according to the supplier's directions. Transfection efficiencies were normalized by cotransfecting the cells with a plasmid containing a chimeric enhancerless SV40 promoter β-galactosidase gene, and β-galactosidase activities were measured in cell lysates as previously described (31). All experiments were repeated three times, and consistent results were obtained in all cases.

Western blot (immunoblot) analysis of immunoprecipitated small t, PP2A, and PP1. Mouse monoclonal small-t antibodies were linked to protein A-Sepharose beads by first incubating the beads with rabbit anti-mouse antibodies. The beads were then washed three times with 1-ml portions of phosphate-buffered saline (PBS) and incubated with the small-t antibodies. The antibodies were covalently cross-linked to the beads with dimethyl pimelimidate.

Immunoprecipitations were performed by incubating 50 μl (packed volume) of the cross-linked antibody-protein A-Sepharose beads with 50 μl of nuclear extract from rat liver tissue or from HepG2 or H4IIE cells for 1 h at 4°C. The beads were recovered by centrifugation and washed five times with 1-ml volumes of PBS.

Nuclear extract proteins and immunoprecipitated material were resolved on 10% polyacrylamide–sodium dodecyl sulfate (SDS) gels and transferred to nitrocellulose. The nitrocellulose blots were blocked with PBS containing 5% dry milk and then treated with antibodies to small t or antiserum to PP1 or PP2A. Blots treated with small-t antibodies were subsequently treated with rabbit anti-mouse immunoglobulin G and then with protein A-alkaline phosphatase conjugate. Blots treated with PP1 or PP2A antiserum were treated with just the protein A-alkaline phosphatase conjugate. After the blots were washed, specific immune complexes were visualized with bromo-chloro-indolyl-phosphate and nitroblue tetrazolium.

Preparation of recombinant small t. A plasmid for the expression of small t in bacteria was prepared by ligating a 1,169-bp *Hin*dIII-*Hin*dIII fragment from the SV40 genome containing the small-t open reading frame into pRSET A (Invitrogen, San Diego, Calif.). The plasmid was transformed into *Escherichia coli* BL21/DE3/pLysS. The bacteria were grown in 1-liter volumes of Luria-Bertani broth containing 50 μg of ampicillin per ml to an optical density at 600 nm of 0.5 to 0.8. Small-t synthesis was initiated by adding isopropyl-β-D-thiogalactopyranoside to the cultures to a final concentration of 0.5 mM. After 3 h, the cells were harvested by centrifugation and lysed in 20 mM Tris-HCl, pH 8.0, containing 6 M guanidine-HCl. Insoluble material was removed from the lysates by centrifugation at 15,000 × *g* for 15 min. The supernatant was applied to a 1-ml bed volume column of ProBond Ni^{2+}-charged Sepharose resin (Invitrogen) equilibrated in 20 mM Tris-HCl, pH 8.0, containing 6 M guanidine-HCl. The column was washed with 50 ml of 20 mM Tris-HCl, pH 8.0, containing 8 M urea (wash buffer). The column was then eluted with 20 ml of wash buffer at pH 6.0 and then with 20 ml of wash buffer at pH 4.0. The bulk of the small t was eluted in the pH 4.0 wash.

Small-t-containing fractions were pooled, and small t was renatured in the presence of Zn^{2+} (50) by being dialyzed against 20 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol, 100 μM $ZnCl_2$, and 10% glycerol. The polyhistidine leader sequence on the recombinant protein was removed by overnight treatment with enterokinase (Invitrogen) according to the supplier's directions. Monomeric small t was separated from aggregated small-t protein by chromatography over a DEAE-cellulose column (6, 50) equilibrated with 20 mM

Tris-HCl (pH 8.0)–1 mM dithiothreitol–10% glycerol. Small-t protein eluted in the flowthrough fractions from the DEAE-cellulose column was shown to be monomeric by analytical size exclusion chromatography, in which the protein migrated with a molecular weight of approximately 20,000. Purified small t was stored in 20 mM Tris-HCl (pH 8.0)–1 mM dithiothreitol–10% glycerol at −20°C.

Tryptic phosphopeptide mapping of CREB from $^{32}P_i$-labeled cells. HepG2 cells were grown to 80% confluency in Dulbecco's modified Eagle's medium F12 with 5% fetal bovine serum and 5% calf serum. Some cells were transfected with the small-t expression vector pSRtcDNA by calcium phosphate-DNA coprecipitation as described above. Plates of cells (2.5×10^6 per plate) were washed with PBS, and the medium was replaced with 5 ml of phosphate-free Dulbecco's modified Eagle's medium F12 containing 1% bovine serum albumin and 1 mCi of $^{32}P_i$. The plates were incubated for 4 h at 37°C and then treated with dibutyryl-cAMP (Bt_2cAMP) and/or okadaic acid for the indicated times.

At each time point the medium was removed from the cells, which were washed once with cold PBS. One milliliter of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 8.0, containing 250 mM NaCl and 0.1% Nonidet P-40 was added to each plate, which was then rocked gently for 30 min at 4°C. The lysed material was transferred to a microcentrifuge tube, and spun at 15,000 × *g* for 15 min. The supernatants were transferred to fresh tubes. After the supernatants were normalized for protein concentration, CREB was recovered from the supernatants by immunoprecipitation with CREB-specific antibodies covalently linked to protein A-Sepharose beads as described above.

Immunoprecipitated material was resolved on 10% polyacrylamide–SDS gels, and the 45-kDa CREB was excised. The identity of the CREB band and the relative amounts of CREB recovered at each time point were determined by Western blotting. The CREB protein was eluted from the gels, and equal amounts of CREB were subjected to tryptic phosphopeptide mapping as described by Kazlauskas and Cooper (22).

Protein phosphatase assays. Protein phosphatase activity in nuclear extracts was determined essentially as described by Wadzinski et al. (46). The effect of recombinant small t on CREB dephosphorylation in nuclear extracts was measured by first preincubating approximately 45 μg of rat liver nuclear extract protein with the indicated amounts of small t for 30 min on ice. The reactions were then initiated by adding 25,000 cpm of [^{32}P]phosphate-labeled CREB (4,400 cpm/pmol), and the mixtures were incubated for 10 min at 37°C. Release of $^{32}P_i$ was measured as previously described (46). Under standard reaction conditions $^{32}P_i$ release was linear for 20 to 25 min. Experiments were repeated at least three times, and consistent results were obtained in all assays.

Nuclear run-on transcription assays. H4IIE cells were transfected with either the empty vector pSRα or the small-t expression vector pSRtcDNA by calcium phosphate-DNA coprecipitation as described above. Eighteen hours after transfection, the cells were treated with 0.5 mM Bt_2cAMP for 30 min. The cells were harvested, their nuclei were isolated, and nuclear run-on transcription assays were performed as described by Granner et al. (19). PEPCK RNA was hybridized to a 600-bp *Bgl*II-*Hin*dIII fragment of the PEPCK coding region excised from plasmid pBH1.2 (51). Levels of PEPCK transcription were corrected for differences in β-actin gene transcription by hybridizing RNA to a 1,150-bp fragment of the rat β-actin gene excised from plasmid pSK-actin.

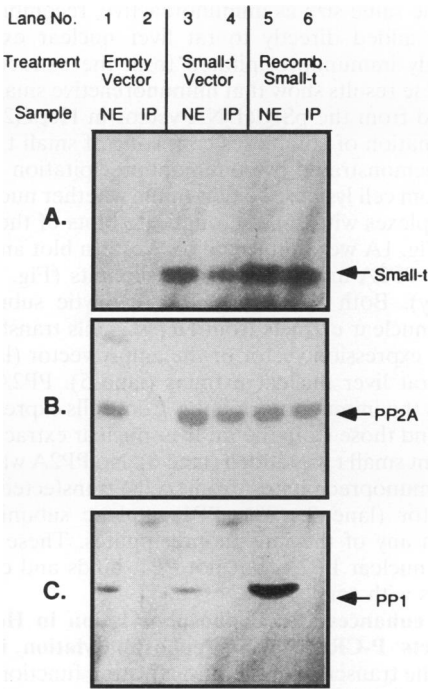

FIG. 1. Small t binds to nuclear PP2A. HepG2 cells were transfected with either an empty vector, pSRα, or the small-t expression vector pSRtcDNA by calcium phosphate-DNA coprecipitation as described in Materials and Methods. For lanes 5 and 6 recombinant small-t protein was added directly to rat liver nuclear extracts. Nuclear extracts from the transfected cells and rat liver tissue were then immunoprecipitated with mouse monoclonal anti-small-t antibodies covalently linked to protein A-Sepharose beads. Nuclear extract (NE) and immunoprecipitates (IP) were resolved in triplicate on 10% polyacrylamide–SDS gels and transferred to nitrocellulose. Individual blots were then subjected to Western blot analysis with antibodies to small-t (A) or antiserum to the PP2A (B) or the PP1 (C) catalytic subunit as indicated.

RESULTS

Small t binds to nuclear PP2A. The cAMP signaling pathway stimulates the transcription of target genes through the phosphorylation of CREB by PKA (19, 30). Evidence reported by Wadzinski et al. (46) indicates that PP2A dephosphorylates P-CREB and inhibits CREB transcriptional activity. Since small t binds to PP2A and generally inhibits PP2A activity (50), we hypothesized that small-t might influence cAMP-stimulated gene transcription by altering PP2A-mediated P-CREB dephosphorylation. To address this hypothesis, we first determined if small t could be expressed in HepG2 cells and if the expressed protein would associate with nuclear PP2A. HepG2 cells were transfected with either a small-t expression vector, pSRtcDNA, in which small-t expression is directed by the strong, constitutive SRα promoter (human immunodeficiency virus long terminal repeat linked to an enhancerless SV40 promoter [44]) or an empty vector, pSRα. Nuclear extracts were prepared from these cells, and immunoprecipitates of the nuclear extracts were prepared with small-t-specific antibodies linked to Sepharose beads. Both nuclear extracts and immunoprecipitates were subjected to Western blot analysis using antibodies to small t. As shown in Fig. 1A, lanes 1 to 4, a 20-kDa small-t band was detected only in nuclear extracts and immunoprecipitates from cells transfected with the pSRtcDNA vector. The small-t protein expressed in HepG2

cells was the same size as immunoreactive, recombinant small t that was added directly to rat liver nuclear extracts and subsequently immunoprecipitated from the extracts (lanes 5 and 6). These results show that immunoreactive small-t protein is expressed from the pSRtcDNA vector in HepG2 cells.

The formation of complexes consisting of small t and PP2A has been demonstrated by coimmunoprecipitation of the two proteins from cell lysates. To determine whether nuclear PP2A forms complexes with small t, duplicate blots of the materials shown in Fig. 1A were subjected to Western blot analysis with antisera to PP2A and PP1 catalytic subunits (Fig. 1B and C, respectively). Both PP2A and PP1 catalytic subunits were present in nuclear extracts from HepG2 cells transfected with the small-t expression vector or the empty vector (lanes 1 and 3) and in rat liver nuclear extracts (lane 5). PP2A was also detected in the immunoprecipitates from cells expressing small t (lane 4) and those from the rat liver nuclear extracts to which recombinant small t was added (lane 6). No PP2A was detected in the immunoprecipitates from cells transfected with the empty vector (lane 2). The PP1 catalytic subunit was not detected in any of the immunoprecipitates. These data indicated that nuclear PP2A, but not PP1, binds and coimmunoprecipitates with small t.

Small t enhances CREB phosphorylation in HepG2 cells and protects P-CREB from dephosphorylation in nuclear extracts. The transcriptional transactivation function of CREB is stimulated through the phosphorylation of a single serine residue (Ser-119 of human placental CREB [25] or Ser-133 of bovine brain CREB [17]) by the PKA (49). Dephosphorylation of this serine by nuclear PP2A decreases the transcriptional activity of CREB (46), suggesting that CREB may be a target for regulation by small t. The effect of small t on the phosphorylation of the CREB PKA site in response to Bt_2cAMP in living cells was determined by tryptic phosphopeptide mapping of CREB recovered from $^{32}P_i$-labeled HepG2 cells. In control cells transfected with the empty vector pSRα and treated with Bt_2cAMP (Fig. 2, top row), phosphorylation of CREB at the PKA site was stimulated five- to eightfold over basal levels (time zero) in 30 min. At 2 h of Bt_2cAMP treatment CREB phosphorylation dropped to two- to threefold over basal levels, and no phosphorylation was detected on maps of CREB recovered from cells exposed to Bt_2cAMP for 12 h. However, when HepG2 cells were transfected with the small-t expression vector and subsequently treated with Bt_2cAMP, phosphorylation of CREB at the PKA site was stimulated more than CREB phosphorylation in cells treated with Bt_2cAMP alone (Fig. 2, middle row). At 30 min CREB phosphorylation in small-t-expressing cells was approximately 65-fold higher than basal levels and remained at this elevated level for at least 2 h. CREB phosphorylation declined at 12 h but was still 15- to 20-fold higher than basal levels and significantly higher than the levels of CREB phosphorylation in control cells at 30 min. The basal level of CREB phosphorylation in small-t-expressing cells was approximately two- to threefold higher than that in control cells. This modest increase in CREB phosphorylation in untreated, small-t-expressing cells discounts the direct activation of PKA or other protein kinases by small t. Rather, this response may be due to the phosphorylation of CREB by an active fraction of PKA in unstimulated cells or another protein kinase and its protection by small-t inhibition of nuclear PP2A.

Similar results were obtained with cells treated with Bt_2cAMP and the protein phosphatase inhibitor okadaic acid (OA) (Fig. 2, bottom row). Phosphorylation of CREB in these cells was also stimulated over 60-fold following 30 min of treatment with Bt_2cAMP and remained elevated at the 2-h time point. CREB phosphorylation declined at 12 h but was higher than either the 30-min level or the 12-h level in control cells. It is interesting that transfection of cells with the small-t expression vector, in which only 5 to 10% of the cells are transfected, results in an increase in CREB phosphorylation similar to the increase after treatment of the entire cell population with okadaic acid. While the exact reason for this response is not known, the intracellular and nuclear levels of okadaic acid achieved under these conditions may be insufficient to completely inhibit P-CREB phosphatase activity. Higher levels of okadaic acid rapidly killed the cells.

The phosphorylation of several other peptides was also enhanced by small t and okadaic acid. However, the stoichiometry of phosphorylation of these sites is not as great as that noted for the PKA site, and the relevance of these sites with regard to CREB function is unknown. These data demonstrate that small t stimulates CREB phosphorylation and inhibits P-CREB dephosphorylation in Bt_2cAMP-treated cells.

Small t also protected P-CREB from dephosphorylation in cell-free reactions with nuclear extracts as the source of protein phosphatases. We have previously shown that P-CREB is rapidly dephosphorylated by nuclear PP2A when added to rat liver nuclear extracts (46). However, the data in Fig. 3 show that P-CREB dephosphorylation was inhibited if nuclear extracts were preincubated with recombinant small-t protein. The inhibition of P-CREB dephosphorylation by small t was proportional to the concentration of small t in the reaction mixtures, from 10 to 600 nM, with a 50% inhibitory concentration of approximately 80 nM. Maximal inhibition was observed at approximately 600 nM small t, at which concentration P-CREB dephosphorylation was inhibited by approximately 50%. This level of inhibition is consistent with the 40 to 70% inhibition of purified, heterodimeric PP2A activity reported by Yang et al. (50). However, the levels of small t required to inhibit P-CREB dephosphorylation in our experiments were significantly higher than those reported by Yang et al. (50) for the inhibition of dephosphorylation of myelin basic protein or myosin light chain. The difference between our results and the data reported by Yang et al. (50) may reflect differences in the ability of small t to interact with nuclear PP2A in our experiments versus the purified, heterodimeric PP2A used by Yang et al. (50). The differences might also reflect the presence of nonfunctional small-t protein in our preparations, requiring the addition of more total protein to achieve the same effective concentrations of functional small t. The failure of small t to completely inhibit P-CREB dephosphorylation may be due to an inability of small t to associate with and inhibit all forms of PP2A present in nuclear extracts. Concentrations of small t higher than 600 nM were less effective at inhibiting P-CREB dephosphorylation. The reason for this response is not known but may involve the formation of nonfunctional aggregates of small-t protein that effectively reduce the concentration of functional small t in the reaction mixtures. The inhibition of P-CREB dephosphorylation was also dependent on the time of preincubation of small t with nuclear extracts (data not shown). Inhibition increased linearly for 10 to 15 min and reached maximal levels between 20 and 30 min. Recombinant small t also partially inhibited the dephosphorylation of phosphorylase *a* in nuclear extracts by 20 to 25% (data not shown), indicating that both PP1 and PP2A in nuclear extracts are active with respect to this substrate.

Small t enhances transcriptional stimulation by cAMP and CREB. Small t enhanced the phosphorylation of CREB in response to Bt_2cAMP in HepG2 cells and inhibited the dephosphorylation of P-CREB in rat liver nuclear extracts. To determine whether the effect of small t on CREB phosphorylation also modulated CREB transcriptional activity, HepG2

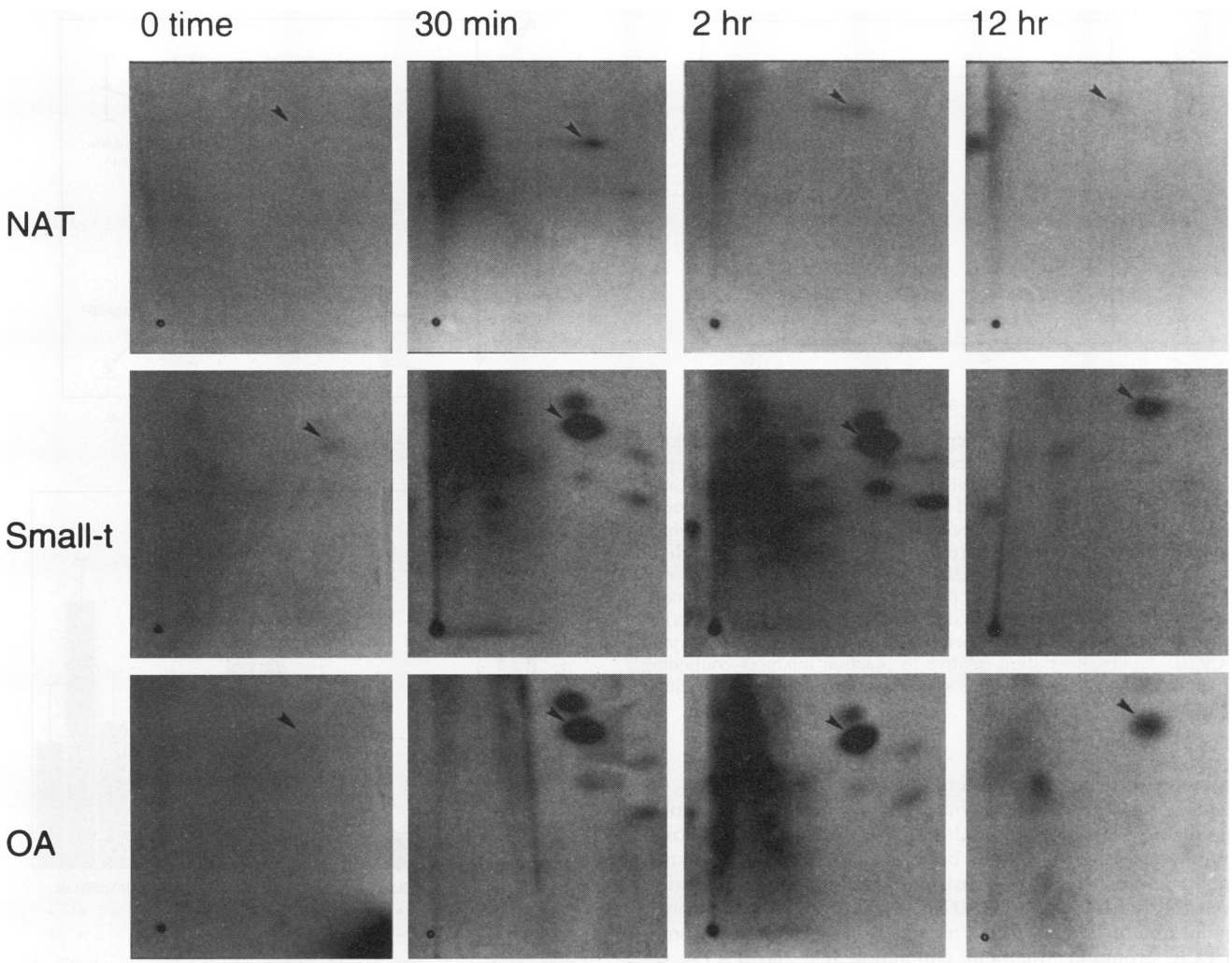

FIG. 2. Bt_2cAMP-stimulated phosphorylation of the CREB PKA-site is enhanced by small-t in HepG2 cells. HepG2 cells were grown on plates with 5 ml of phosphate-free Dulbecco's modified Eagle's medium F12 containing 1% bovine serum albumin and 1 mCi of $^{32}P_i$ for 4 h. The cells were then treated with 0.5 mM Bt_2cAMP for the indicated times. Control cells transfected with the empty vector pSRα received no additional treatment (NAT), while other cells also received 1 μM okadaic acid (OA) or were previously transfected with the small-t expression vector pSRtcDNA. At the times indicated the cells were lysed, and CREB was recovered from the lysates by immunoprecipitation with CREB-specific antisera. CREB was resolved on a 10% polyacrylamide–SDS gel, and the ^{32}P-labeled band was excised from the gel. CREB was subjected to tryptic phosphopeptide mapping as described in Materials and Methods. Peptides were separated on cellulose thin-layer plates by electrophoresis at pH 1.9 from left to right (anode on the right), followed by chromatography from bottom to top. The origin where peptides were spotted is indicated (o). The PKA phosphorylation site is indicated (arrowheads).

cells were transfected with a plasmid containing a cAMP-responsive 5′ deletion mutant of the PEPCK promoter (−109 to +73) linked to the luciferase reporter gene. This 5′ deletion mutant of the PEPCK promoter contains the TATA box and the proximal cAMP-regulatory element (CRE-1 [TTACG TCA; −91 to −84]) that confers transcriptional activation by CREB in cells (26, 34) and in cell-free transcription assays (24, 32). The cells were also transfected with either the small-t expression vector pSRtcDNA or the empty vector pSRα. The effect of Bt_2cAMP on luciferase expression in the cells is shown in Fig. 4A. In cells not expressing small t the addition of Bt_2cAMP stimulated luciferase expression from the PEPCK promoter 9- to 10-fold in 4 h, after which luciferase expression declined. However, when cells expressing small t (verified by Western blot analysis [data not shown]) were treated with Bt_2cAMP, luciferase expression was stimulated almost 14-fold in 4 h and continued to increase to approximately 17-fold at 8 h. These data indicate that small t can enhance Bt_2cAMP-stimulated transcription from the PEPCK promoter and prevent the attenuation of transcription from stimulated levels. In addition, zero-time levels of luciferase expression were approximately threefold higher in small-t-expressing cells than that in cells transfected with the empty vector pSRα. This increase in luciferase expression prior to the activation of PKA is consistent with the threefold increase in the basal phosphorylation of CREB at the PKA site in cells expressing small t (Fig. 2). As described above, this response is probably due to the phosphorylation of CREB by an active fraction of PKA or another protein kinase and its protection by small-t inhibition of nuclear PP2A.

The ability of small t to enhance basal and Bt_2cAMP-stimulated transcription was observed with either the PEPCK

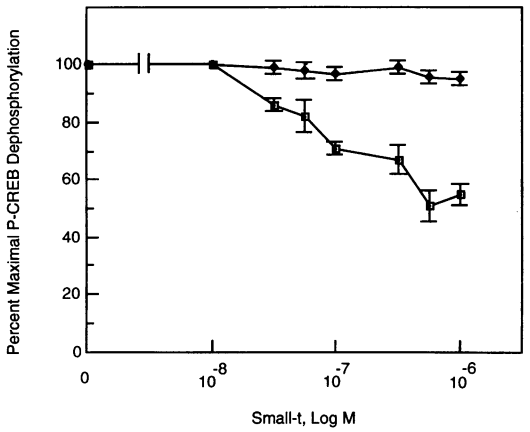

FIG. 3. Small t inhibits the dephosphorylation of PKA-phosphorylated CREB in rat liver nuclear extracts. Protein phosphatase reaction mixtures containing approximately 45 μg of rat liver nuclear protein were incubated with the indicated concentrations of recombinant small t (□) for 30 min on ice. Control reaction mixtures (◆) contained equal concentrations of protein prepared from *E. coli* transformed with an empty pRSET A vector. The reactions were initiated by the addition of approximately 25,000 cpm of ^{32}P-labeled CREB (4,400 cpm/pmol). Release of $^{32}P_i$ was measured as described in Materials and Methods. Levels of phosphatase activity are shown as the percent of maximal P-CREB dephosphorylation relative to reaction mixtures containing no small t. The data are averages and standard deviations for three experiments.

promoter fragment containing the CRE-1 and TATA sequences (−109 to +73) or the full-length PEPCK promoter (−490 to +73) (Fig. 4B). Although levels of transcriptional stimulation varied markedly between individual experiments, small t and/or Bt_2cAMP consistently stimulated transcription from the full-length and −109 PEPCK promoters. However, small t and/or Bt_2cAMP had no effect on transcription from a PEPCK promoter fragment containing just the TATA sequence (−68 to +73). Likewise, small t had no effect on transcription from the enhancerless SV40 promoter which was used in these experiments as an internal control. These results indicate that CRE-1 is required for the stimulation of transcription from the PEPCK promoter by small t and Bt_2cAMP and discounts an effect of small t on the core transcription machinery. Since CREB has been shown to bind and regulate transcription through CRE-1 (31, 34, 46), the data suggest that the effects of small t and Bt_2cAMP on transcription are mediated by CREB.

To demonstrate that the stimulation of luciferase expression from the PEPCK promoter by small t reflects changes in the rate of transcription, we measured the effect of small t on the rate of Bt_2cAMP-stimulated transcription of the endogenous PEPCK gene in H4IIE cells by nuclear run-on transcription analysis. H4IIE (rat hepatoma) cells were selected for nuclear run-on assays since the endogenous PEPCK gene in HepG2 cells is poorly expressed. H4IIE cells were transfected with either the small-t expression vector pSRtcDNA or the empty vector pSRα. The cells were then treated with Bt_2cAMP for 30 min, and PEPCK gene transcription was measured by nuclear run-on transcription analysis. In cells not expressing small t, Bt_2cAMP stimulated PEPCK gene transcription 5- to 6-fold, whereas PEPCK gene transcription was stimulated more than 25-fold by Bt_2cAMP in cells expressing small t (Fig. 5). Small t also stimulated the basal level of PEPCK gene transcription, an observation consistent with the ability of small t to enhance

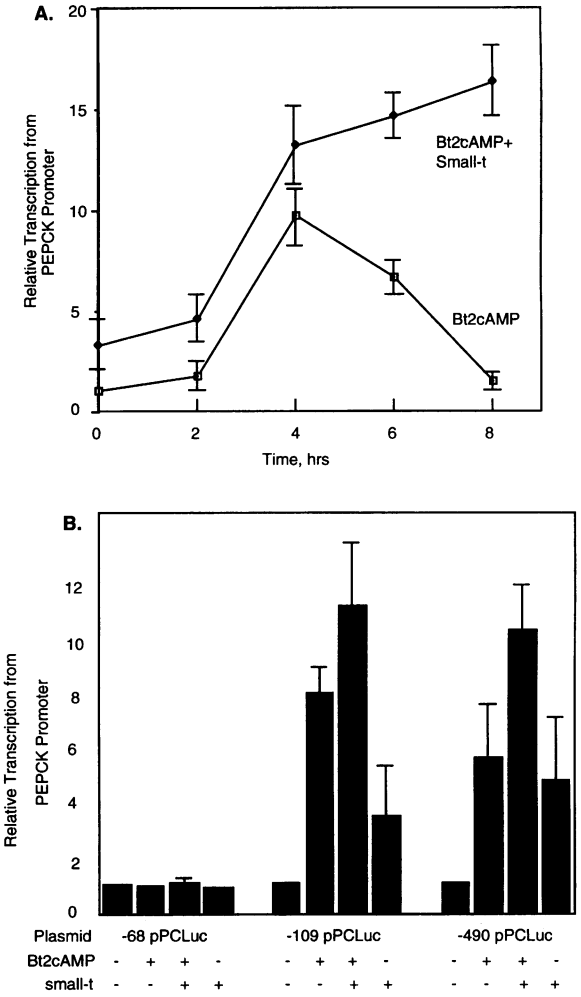

FIG. 4. Small t enhances Bt_2cAMP-stimulated transcription from PEPCK promoter fragments containing a CREB-binding site. (A) HepG2 cells were transfected with a plasmid containing a portion of the PEPCK promoter (−109 to +73) linked to a luciferase reporter gene (−109pPCLuc) by calcium phosphate-DNA coprecipitation. Control cells (□) were cotransfected with the empty vector pSRα, and small-t-expressing cells (◆) were cotransfected with pSRtcDNA. The cells were then treated with 0.5 mM Bt_2cAMP for the times indicated. (B) HepG2 cells were transfected with plasmids containing one of the following regions of the PEPCK promoter linked to a luciferase reporter gene: −68 to +73 (−68pPCLuc), −109 to +73 (−109pPCLuc), and −490 to +73 (−490pPCLuc). The cells were cotransfected with pSRα (not expressing [−] small t) or pSRtcDNA (expressing [+] small t) and treated with Bt_2cAMP for 4 h as indicated. Luciferase was measured in cell lysates as described in Materials and Methods. Levels of transcription were corrected for transfection efficiencies by measuring β-galactosidase expression from a cotransfected plasmid containing the Rous sarcoma virus long terminal repeat linked to the β-galactosidase reporter gene. Levels of transcription in panel A are shown relative to zero-time levels in control cells. Transcription levels in panel B are shown relative to levels in cells not expressing small t and not treated with Bt_2cAMP for each PEPCK-luciferase plasmid. The data are averages and standard deviations for three experiments.

basal levels of luciferase expression from the PEPCK promoter in transient transfection experiments (Fig. 4). These results indicate that the effects of small t on luciferase expression from the PEPCK promoter shown in Fig. 4 are due, at least in part,

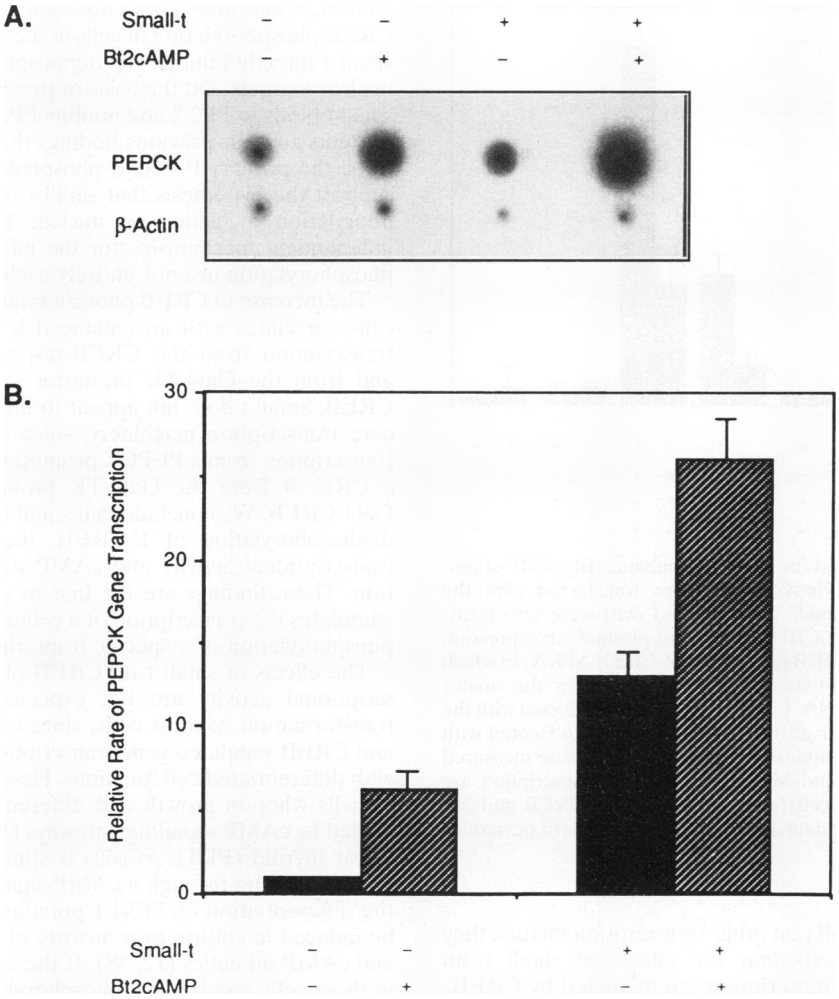

FIG. 5. Small t enhances Bt_2cAMP-stimulated PEPCK gene transcription in H4IIE cells. H4IIE cells were transfected with either the empty vector pSRα or the small-t expression vector pSRtcDNA as indicated. The cells were subsequently treated with 0.5 mM Bt_2cAMP for 30 min as indicated. Nuclei were isolated from the cells, and nuclear run-on transcription assays were performed as described in Materials and Methods. (A) Autoradiograph of ^{32}P-labeled RNA hybridized to PEPCK and actin probes linked to nitrocellulose filters. (B) Levels of PEPCK gene transcription were corrected for differences in transcription of the rat β-actin gene and are shown relative to transcription levels in cells not treated with Bt_2cAMP and not expressing small t. The data are averages and standard deviations for three experiments.

to changes in the rate of transcription from the PEPCK promoter. These findings also indicate that the effect of small t on cAMP-stimulated gene transcription is not limited to HepG2 cells.

Further evidence that CREB mediates the stimulatory effect of small t on cAMP-regulated transcription is shown in Fig. 6. HepG2 cells were transfected with a plasmid containing an enhancerless TK promoter linked to four copies of the Gal4 regulatory sequence driving expression of a luciferase reporter gene (pGal4TKLuc). These cells were also transfected with the small-t expression vector pSRtcDNA or the empty vector pSRα, and some cells were also transfected with a plasmid (pSVGal4-CREB) for the expression of a chimeric protein containing the Gal4 DNA-binding domain linked to the transactivation region (amino acids 1 to 261) of PC-12 cell CREB-341 (33) (Fig. 6). Expression of small t and Gal4-CREB was verified by Western blot analysis (data not shown). Previous studies have shown that phosphorylation of the CREB PKA site in the Gal4-CREB protein in response to cAMP or overexpression of PKA stimulates transcription from a Gal4-responsive promoter (30). Since cellular proteins in HepG2 cells do not bind the yeast Gal4 sequence, effects of small t on the transcriptional activity of the CREB transactivation domain could be specifically monitored in this system. Bt_2cAMP and/or small t had no effect on transcription from the Gal4-TK promoter in the absence of Gal4-CREB protein (Fig. 6). However, in cells expressing Gal4-CREB, Bt_2cAMP stimulated transcription 4-fold, and in cells expressing both Gal4-CREB and small t, addition of Bt_2cAMP resulted in a 9- to 10-fold stimulation of transcription. These results show that Gal4-CREB protein is required for transcriptional stimulation by either Bt_2cAMP or small-t and suggest that small t has no effect on the activity of the core transcription machinery. Furthermore, the effects of Bt_2cAMP and small t on transcription are mediated through phosphorylation of the CREB PKA phosphorylation site. As also shown in Fig. 6, Bt_2cAMP and small t did not stimulate transcription from the Gal4-responsive promoter in cells expressing a Gal4-CREB protein (Gal4-CREB ΔPKA) in which serine 133 (the PKA phosphorylation site) was mutated to an alanine. While these results do not

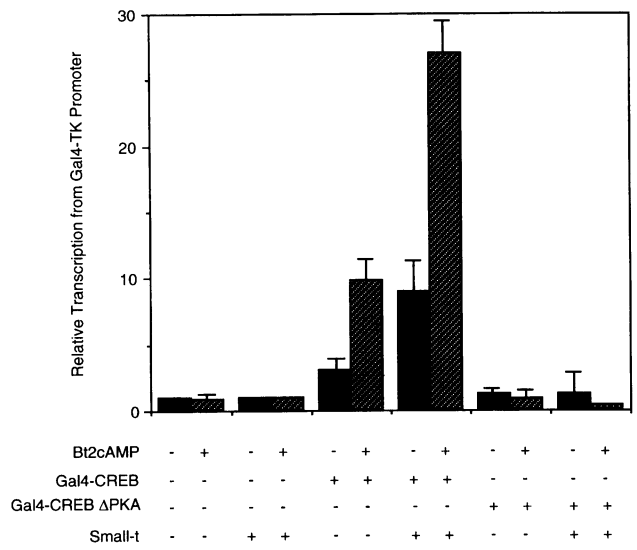

FIG. 6. CREB is required for small t to enhance Bt_2cAMP-stimulated gene transcription. HepG2 cells were transfected with the pGal4TKLuc reporter plasmid. The indicated cells were also transfected with a wild-type Gal4-CREB expression plasmid, an expression vector for a mutant Gal4-CREB protein (Gal4-CREB ΔPKA) in which the PKA site serine was mutated to an alanine, and/or the small-t expression plasmid pSRtcDNA. Control cells were transfected with the empty (−) expression vector pSRα. The cells were then treated with 0.5 mM Bt_2cAMP for 4 h as indicated. Luciferase levels were measured as described in Materials and Methods. Levels of transcription are shown relative to levels in cells not expressing Gal4-CREB and not treated with Bt_2cAMP. The data are averages and standard deviations for three experiments.

preclude an effect of small t on other transcription factors, they do support the hypothesis that the effects of small t on cAMP-stimulated gene transcription are mediated by CREB.

Small t also raised basal levels of transcription in the presence of Gal4-CREB, an effect similar to its stimulation of basal transcription from the PEPCK promoter in transient transfection (Fig. 4) and nuclear run-on (Fig. 5) experiments and its ability to enhance basal CREB phosphorylation (Fig. 2). Gal4-CREB by itself also stimulated transcription from the Gal4-TK promoter in the absence of Bt_2cAMP and small t, an effect consistent with previous reports showing that CREB functions as a basal transcriptional enhancer as well as a hormone-regulated factor (24, 40, 46).

DISCUSSION

Small t participates in viral replication and cell transformation in part by regulating the transcription of cellular genes, but the mechanism(s) by which it regulates transcription has not been defined. Small t binds specifically to cellular PP2A and generally inhibits PP2A activity, suggesting that small t might regulate gene transcription and other cellular functions by interacting with PP2A. Recently, we showed (46) that nuclear PP2A dephosphorylates PKA-phosphorylated CREB, thereby reversing or attenuating the stimulation of gene transcription by the cAMP-signaling pathway. In this article we demonstrate that small t binds to nuclear PP2A, resulting in the enhanced and prolonged phosphorylation of CREB in response to cAMP. The increase in CREB phosphorylation with small t appears to be due to a decrease in the rate of P-CREB dephosphorylation since phosphopeptide mapping data show that small t does not significantly increase the rate of CREB phosphorylation in cells in the absence of Bt_2cAMP and small t directly inhibits the dephosphorylation of P-CREB in nuclear extracts. On the basis of previous studies showing that small t binds to PP2A and inhibits PP2A activity towards other proteins and our previous findings that nuclear PP2A appears to be the primary P-CREB phosphatase, our current findings support the hypothesis that small t inhibits P-CREB dephosphorylation by acting on nuclear PP2A. However, PP2A-independent mechanisms for the effect of small t on CREB phosphorylation are not entirely excluded by our data.

The increase in CREB phosphorylation in small-t-expressing cells correlates with an enhanced level of cAMP-stimulated transcription from the CREB-responsive PEPCK promoter and from the Gal4-TK promoter in the presence of Gal4-CREB. Small t does not appear to influence the activity of the core transcription machinery, since small t did not regulate transcription from a PEPCK promoter deletion mutant lacking a CRE or from the Gal4-TK promoter in the absence of Gal4-CREB. We conclude that small t inhibits PP2A-mediated dephosphorylation of P-CREB, thereby enhancing CREB transcriptional activity and cAMP-stimulated gene transcription. These findings are the first to demonstrate that small t stimulates the transcription of a cellular gene by regulating the phosphorylation of a specific transcription factor.

The effects of small t on CREB phosphorylation and transcriptional activity are not expected to contribute to the transformation of most cells, since cAMP-signaling pathways and CREB-regulated gene transcription are usually associated with differentiated cell functions. However, there are examples of cells wherein growth and differentiation are directly controlled by cAMP signaling pathways (14). For example, growth of rat thyroid (FRTL 5) cells is stimulated by thyroid-stimulating hormone through a cAMP-signaling system (8, 45), and the differentiation of 3T3-L1 preadipocytes to adipocytes can be induced in culture by a mixture of insulin, dexamethasone, and cAMP mimetics (12, 48). If the cAMP-signaling pathways in these cells regulate the phosphorylation and transcriptional activity of CREB, then small t presumably would potentiate the effects of thyroid-stimulating hormone on FRTL 5 cell proliferation and the effects of cAMP mimetics on 3T3-L1 preadipocyte differentiation. Future studies with these cells should further define the role of small t in regulating CREB transcriptional activity and clarify the relationship of these events to cell growth and differentiation.

In other cells small t could participate in transformation by regulating the phosphorylation and activity of other transcription factors associated with growth control. SV40 large T is an obvious target since it is expressed with small t in SV40-infected cells and stimulates transcription of numerous viral and cellular genes (3, 7, 23, 52) and small t inhibits the dephosphorylation of specific sites in large T (37). Small t also inhibits the dephosphorylation of specific sites in the tumor suppressor protein p53 by PP2A (37). The effects of phosphorylation on the transcriptional activities of large T and p53 are unclear, and it is therefore possible that small t could play a significant role in controlling the transcriptional functions of these proteins. c-Jun/AP1 is another potential target for small t, since specific phosphorylation sites in the c-Jun DNA transactivation region are dephosphorylated by PP2A (5) and introduction of PP2A into cells has been shown to potentiate transcription from c-Jun/AP-1-responsive promoters (1). Small t could also regulate c-Jun and other transcription factors like c-Myc (2, 39) and $p62^{TCF}$ (16) through its ability to stimulate mitogen-activated protein kinase activity (43). These findings suggest that small t could regulate the transcriptional activity

of several cellular transcription factors that control cell proliferation and differentiation. The ability of small t to regulate gene transcription by modulating transcription factor phosphorylation would be an important mechanism by which small t participates in transformation. The existence of these processes and their contribution to cell transformation remain to be resolved.

Our data confirm and extend previous observations that nuclear PP2A is the primary protein phosphatase that dephosphorylates P-CREB (46). We previously demonstrated that P-CREB is dephosphorylated by an okadaic acid-sensitive protein phosphatase in cells and nuclear extracts (46). Nuclear PP2A was identified as the primary P-CREB phosphatase since both enzymes copurified from nuclear extracts over several chromatographic matrices and inhibitor peptide 2 was unable to inhibit P-CREB phosphatase activity in nuclear extracts. Furthermore, nuclear PP2A was more efficient at dephosphorylating P-CREB than nuclear PP1. Our current findings show that small t inhibits CREB dephosphorylation and enhances CREB transcriptional activity in living cells and in nuclear extracts. While our findings do not preclude PP2A-independent mechanisms by which small t regulates CREB phosphorylation or effects of small t on other transcription factors, they do support the hypothesis that nuclear PP2A is the primary P-CREB phosphatase.

In this article we have shown that small t can regulate and enhance cAMP stimulation of gene transcription by inhibiting the dephosphorylation of P-CREB by PP2A. These are the first data to demonstrate that small t controls the transcription of a cellular gene by regulating the phosphorylation of a specific transcription factor. It will be interesting to see if the effect of small t on CREB-regulated transcription plays a role in cell growth control, particularly in cells like FRTL 5 or 3T3-L1 preadipocytes, wherein cAMP signaling plays a central role in cell growth and differentiation. Furthermore, it will be important to determine if small t regulates the phosphorylation and activity of other transcription factors, as this may be a general mechanism by which small t contributes to cell transformation. In addition to defining the role of small t in transformation, such studies should also provide insights into the function of other transcription factors, like large T and p53, that modulate cell growth and differentiation.

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