

# Inhibition of Anchorage-Independent Cell Growth, Adhesion, and Cyclin D1 Gene Expression by a Dominant Negative Mutant of a Tyrosine Phosphatase

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**PTP-S4/TC48 protein tyrosine phosphatase is localized in the nuclear and cytoplasmic membranes. To investigate the role of PTP-S4 in cell growth, adhesion, and transformation, normal and a catalytically inactive mutant form of this phosphatase were expressed in polyoma virus-transformed F111 fibroblast cell line, PyF. Expression of mutant PTP-S4 in PyF cells resulted in strong inhibition of anchorage-independent growth in soft agar but had no significant effect on growth in liquid culture. Tumor formation in nude mice was also reduced by mutant PTP-S4. Expression of normal PTP-S4 in PyF cells significantly increased anchorage-independent cell growth and tumor formation in nude mice. Overexpression of catalytically inactive mutant of PTP-S2/TC45 (a splice variant of PTP-S4 that is nuclear) did not inhibit anchorage-independent growth of PyF cells. Mutant PTP-S4-expressing cells were inhibited in adhesion and spreading on tissue culture plates compared to control cells. Expression of mutant PTP-S4 in PyF cells reduced the levels of cyclin D1 and cyclin A mRNA, whereas cyclin D2 mRNA level was not affected significantly. Expression of antisense cyclin D1 strongly inhibited anchorage-independent growth. Inhibition of anchorage-independent growth by mutant PTP-S4 was overcome to a large extent by coexpression of cyclin D1. These results suggest that mutant PTP-S4 inhibits anchorage-independent growth and adhesion of polyoma virus-transformed cells by interfering with the normal function of PTP-S4 upstream of cyclin D1 gene expression.** © 2001 Academic Press

**Key Words:** tyrosine phosphatase; anchorage-independent cell growth; adhesion; cyclin D1; tumorigenicity; dominant negative mutant.

## INTRODUCTION

The role of protein tyrosine phosphatases (PTPs) has been implicated in several biological phenomena such

as cell growth, transformation, adhesion, motility, and cytoskeletal architecture [1–3]. PTPs such as PRL-1 [4] and PTP $\alpha$  [5] are positive regulators of cell transformation, whereas PTP-1B [6] and PTP-MEG [7] inhibit cell transformation. PTP-1B also affects cell spreading, cytoskeletal architecture, and formation of focal adhesion complexes [8–10]. Overexpression of a catalytically inactive mutant of PTP-1B in mouse L cells results in reduced adhesion and spreading on fibronectin, and almost complete absence of focal adhesions and stress fibers [8]. These effects are mediated by c-Src tyrosine kinase which is regulated positively by PTP-1B [8, 9]. In apparent contrast to these effects, expression of PTP-1B in 3Y1 fibroblasts results in reduced cell spreading and inhibition of adhesion-dependent MAP kinase activation [10]. In addition, other PTPs which affect adhesion and/or motility are PTP-PEST and PTP36 [11, 12].

PTP-S/TCPTP is a ubiquitously expressed non-receptor-type protein tyrosine phosphatase [13–16]. In rat cells there are four alternatively spliced isoforms—PTP-S1, PTP-S2, PTP-S3, and PTP-S4 [17]. PTP-S2/TC45 and PTP-S4/TC48 are major forms which are expressed widely in rat, mouse, and human cells. PTP-S2 localizes to the nucleus and has DNA-binding ability, whereas PTP-S4 does not bind to DNA and localizes to the endoplasmic reticulum and nuclear membrane [18–20]. *In vitro*, PTP-S2 and PTP-S4 show different substrate specificity [20]. Overexpression of PTP-S2 in HeLa cells results in increased cell growth due to shortening of G1 phase which may be mediated by increased c-Myc protein level [21, 22]. However, in p53-positive cell lines such as MCF-7 and A549, PTP-S2 overexpression induces apoptosis [23]. Overexpression of PTP-S4 in p53-positive cells results in much lower levels of apoptosis compared to that obtained with PTP-S2.

Mitogenic stimulation of T lymphocytes, F111 fibroblasts, NIH 3T3 cells, and HeLa cells results in an increase in PTP-S mRNA level [24–26]. In these studies individual isoforms were not analyzed. During liver regeneration after partial hepatectomy, PTP-S4 as

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well as PTP-S2 mRNA levels increased over 10-fold after 6 h and declined thereafter [20]. These observations indicate that PTP-S4 may have a role related to cell proliferation. Expression of a truncated form of TC48, from which C-terminal 11-kDa fragment has been removed, causes a change in morphology and loss of anchorage-independent growth of *v-fms*-transformed rat fibroblasts [27]. But the full-length TC48 had no effect. Overexpression of the truncated form of TC48 in BHK cells results in cytokinetic failure and asynchronous nuclear division leading to the formation of multinucleate cells [28]. The full-length TC48 had no effect in these cells. PTP-S knockout mice show defect in bone marrow hematopoiesis and poor T- and B-cell proliferative responses [29]. These mice are deficient in all splice variants of PTP-S; therefore the contribution of individual isoforms to the observed phenotype of knockout mice is not known. Substrate-trapping mutants generated by replacing aspartate 182 with alanine (D182A) identified p52Shc and epidermal growth factor receptor (EGFR) as specific substrates of TC45 [30]. The complex formation of both TC45 and TC45D182A with TC45 substrates inhibits EGF-dependent activation of PI3-kinase and PKB/Akt [31]. However TC48D182A complexes only with EGFR and this complex formation does not inhibit EGF-dependent activation of PI3-kinase and PKB/Akt [31]. PTP-S associates with the receptor for hepatocyte growth factor (HGF) upon activation by the HGF [32].

This study was undertaken to explore the possibility of a role of PTP-S4 in cell growth, adhesion, and transformation. A catalytically inactive mutant PTP-S4 construct (mS4) was prepared by replacing cysteine 216 with serine. Wild-type and mutant PTP-S4 constructs were expressed in PyF cells and the effects on cell growth, transformation, and adhesion were studied. We found that expression of mutant PTP-S4 inhibited anchorage-independent growth and adhesion, whereas overexpression of wild-type PTP-S4 increased anchorage-independent growth and cell adhesion. Hence PTP-S4 is a positive regulator of anchorage-independent growth and cell adhesion. In addition we have analyzed the role of cyclin D1 and cyclin A in mediating the observed effects of mutant and normal PTP-S4 on anchorage-independent growth.

## MATERIALS AND METHODS

**Expression plasmids.** PTP-S4 and PTP-S2 cDNAs cloned in eukaryotic expression vector pCB6 have been described previously [20]. In order to construct a vector expressing catalytically inactive mutant of PTP-S4, cysteine at position 216 was mutated to serine using a polymerase chain reaction (PCR)-based approach utilizing an upstream unique *NcoI* site. Two primers were designed to amplify the 3' half of PTP-S4 by PCR (mutagenic 5' primer SK7, 5'-GGAATTC-CATGGCCTGCAGTGATCCATTCCAGTGCA-3', and 3' primer RR13, 5'-CGGGATCCTGTTTATAGGACATTTAACTGAAA-3'). The PCR amplicon was doubly digested with *EcoRI/BamHI* and the re-

sulting 621-bp fragment was cloned in pUC9. The mutation was confirmed by sequencing. The fragment was subcloned in pBS(KS) and this plasmid could be maintained only in the presence of 0.2% glucose. The 5' 612 bp of PTP-S4 was obtained by *HindIII/NcoI* digestion and ligated upstream of the 3' fragment in pBS(KS). Cloning was confirmed by restriction digestion patterns and also by automated sequencing. The full-length mutant construct (1233 bp) thus obtained was released by *BamHI* and cloned into the expression vector pCB6 at the compatible *Bg/II* site. Sense and antisense clones were differentiated by their *PstI* digestion patterns and finally clone DNS4×X5 was sequenced using primer SKM1 (5'-CAGCGT-GAAGCTCTTATCTGAA-3') designed 200 bp upstream of the targeted site. The mutant PTP-S2 construct was prepared using a similar strategy.

Cyclin D1, cyclin D1 antisense, and cyclin A antisense cDNAs were cloned using reverse transcription and PCR. Specific primers were designed for amplifying the complete coding region of the known rat cyclinD1 cDNA: forward, 5'-CGGAATTCGCGCATGGAACAC-CAGCTC-3'; and reverse, 5'-CGGAATTCAGATGTCCACATC-TCGGACG-3'. A 910-bp product was amplified from total RNA of PyF cells. The resulting cDNA was cloned using a pMOS Blue blunt end cloning kit as per manufacturer's instructions (Amersham Pharmacia, Biotech). The clone was sequenced by automated sequencing and digested by *EcoRI* and cloned into the *EcoRI* site of mammalian expression vector pCB6. The sense/antisense orientation was determined by *PstI* digestion. *PstI*-digested products of 730 and 189 bp are unique to sense and antisense orientations, respectively. Since the sequence of rat cyclin A was not known, primers designed from conserved regions of mouse and human cyclin A were used to clone a partial cyclin A cDNA from rat cells. Forward primer 5'-CGGAATTCGAGCCGCGATGCCGGCA-3' and reverse primer 5'-ACA-CAAACCTCTGCTACTTCTGG-3' was used to amplify an 823-bp cDNA from PyF cells. The resulting cDNA was cloned in pMOS Blue and sequenced. This rat cyclin A cDNA (GenBank Accession No. 367448) showed over 90% amino acid sequence identity with amino acids 10–269 of mouse cyclin A. The cloned fragment was released by *EcoRI/XbaI* double digestion and ligated into the *EcoRI/XbaI* site of vector pCB6. The antisense orientation of this rat cyclin A cDNA was confirmed by *PvuII* digestion.

**Transfections, immunofluorescence, and Western blotting.** Cos-1 or MCF-7 cells were grown as monolayers on coverslips or petri dishes to about 80% confluence and transfected using Lipofectamine reagent (Life Technologies, Inc.) according to manufacturer's instructions. Cells were then processed for immunofluorescence as described earlier using anti-PTP-S monoclonal antibody G11, which recognizes the rat PTP-S2 and PTP-S4 proteins but not human homologs in MCF-7 cells or monkey homolog in Cos-1 cells [18–20]. PyF cells obtained from Dr. Thomas Benjamin [33] were grown as monolayers in 35-mm petridishes to about 40% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and transfected using Lipofectamine reagent and indicated plasmids. Selection for obtaining pools of stably expressing clones was carried out using 100  $\mu$ g/ml G418 (GIBCO BRL). Cells were maintained in G418 for the duration of the experiments. Western blotting was done using whole cell lysates as described previously [20]. Cyclin D1 and Cdk 2 antibodies were obtained from Santa Cruz Biotechnology, Inc.

**Immunoprecipitation and phosphatase activity assay.** Cos-1 cells were grown as monolayers in 35-mm dishes and transfected with PTP-S4 (500 ng), mS4 (500 ng), or both PTP-S4 and mS4 in equal (1:1) stoichiometry (500 ng each). Total amount of DNA was equalized using pCB6 plasmid DNA and 1  $\mu$ g pCB6 plasmid was used as control for transfection. After 48 h of transfection cells were washed with PBS, harvested by scraping, and extracted in 2X IP buffer (1X IP buffer contains 1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.2, 5 mM EDTA, 0.5% sodium deoxycholate, 1 mM PMSF, and 2  $\mu$ g/ml each of leupeptin, soybean trypsin inhibitor, and aprotinin).

Protein extracts were then immunoprecipitated with 1:1 diluted anti-PTP-S monoclonal antibody G11 for 2 h. Immunoprecipitates on protein A agarose beads (Life Technologies, Inc) were washed six times with 1X IP buffer. The beads were then resuspended in 150  $\mu$ l phosphatase assay buffer (described in Ref. [20]). Fifty microliters of each IP extract was added to 25  $\mu$ l of 3X SDS sample buffer and the samples were boiled for 5 min. These samples were then run on 10% SDS-polyacrylamide gels and subjected to Western blotting with PTP-S antibody. The remaining 100  $\mu$ l of each IP extract was used for activity assay as described previously [20], using 25,000–50,000 cpm of labeled poly (Glu<sup>4</sup>, Tyr<sup>1</sup>) per assay. Phosphatase activity contributed by pCB6 transfection alone was deducted as background and percentage dephosphorylation for each immunoprecipitate was calculated taking the activity of wild-type PTP-S4 or PTP-S2 protein as 100%.

**Growth in liquid culture and soft agar assay.** Cells were trypsinized and enumerated by hemocytometer (Neubauer Instruments); 10<sup>5</sup> cells in 2 ml of DMEM, containing 10% FBS, were plated in 35-mm dishes. After 48 h the dishes were washed with PBS and trypsinized. Medium was added to a final volume of 2 ml and cells were counted. For soft agar assay 2X DMEM containing 20% FBS was mixed 1:1 with 1% agar (Sigma, cell culture tested) and 5 ml of this 0.5% agar was poured in 60-mm petri dishes as the agar base. The required number of cells (generally 50,000 cells) was suspended in 0.33% top agar by mixing 0.5 ml of cells in 10% FBS with 0.5 ml of 2X DMEM supplemented with 20% FBS, and 0.5 ml of 1% agar, and pouring on top of the solidified agar base. The colonies were allowed to grow for the required duration (5–7 days) after which they were stained with 0.1% methylene blue in 50% ethanol. Plates were destained by washing three times with water and last with 25% ethanol. Quantitation was done by scanning the whole plate and by using GeneTools software (SynGene, UK). All plates of a given experiment were scanned under identical conditions and then analyzed for number of colonies of various sizes. Colonies larger than 5 and 10 pixels were counted for data in Tables 1, 3, 4, and Table 5, respectively.

**Tumor formation in nude mice.** Cells were enumerated by hemocytometric counting after washing three times with PBS and 10<sup>6</sup> cells in PBS were injected subcutaneously at a single site in 1-week-old nude mice. Tumor measurements were taken 2 and 3 weeks postinoculation by measuring the tumors in two orthogonal axes. Tumor volume was calculated by the formula  $V = L \times W^2/2$ , where  $V$  = tumor volume,  $L$  = tumor length, and  $W$  = tumor width [34].

**Cell adhesion assay.** Cell adhesion assay was carried out as described [11]. Cells were trypsinized and counted and  $5 \times 10^4$  or  $1 \times 10^5$  cells in 2 ml medium were plated in uncoated 35-mm tissue culture dishes. Random fields were photographed after 2–4 h at 100 $\times$  magnification using an inverted phase-contrast microscope. Unspread cells were described as phase-bright and punctual, whereas spread cells were not phase-bright with extensive visible membrane protrusions. The two kinds of cells were distinguishable enough so that two independent counts of the same field gave the same result  $\pm$  2%. At least 300 cells were counted for each data point and the experiment was repeated three times.

**Reverse transcription (RT) and PCR.** RNA was prepared using Trizol reagent (Life Technologies, Inc.) and reverse transcription was done with random hexamers using a kit from Life Technologies as per the manufacturers' instructions. In order to determine the expression of exogenously expressed PTP-S4 or mutant PTP-S4, 1  $\mu$ l of the RT product was used as template in subsequent PCR with downstream primer M3 (5'-GATGCAACTTAATTTTATTAGGACAA-3') and forward primer M4 (5'-GAAGAGAGCAGTGAGAGTATTCT-3'). The following conditions were used for PCR: denaturation, 94°C for 30 s; annealing, 64°C for 30 s; and extension, 72°C for 60 s. After 35 cycles, a final extension was done at 72°C for 7 min. The amplified product (10  $\mu$ l) was run on a 1.5% agarose gel with pBR322HinfI markers at 5 V/cm.

In order to determine the relative mRNA levels of cyclin D1, cyclin D2, and cyclin A these mRNAs were coamplified with the mRNA for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as control for desired number of cycles using 1  $\mu$ l of RT product derived from 100 ng of total RNA as described previously [20]. The cycling conditions were 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a final step of extension for 7 min at 72°C. GAPDH was amplified for 23 cycles by adding primers later in the PCR reactions. Cyclin D1, cyclin D2, and cyclin A were amplified for 38, 40, and 28 cycles, respectively. The PCR products were analyzed by agarose gel electrophoresis and the bands were quantitated using GeneTools software from SynGene. The primers for GAPDH have been described previously [20]. The sequence of primers for cyclins were as follows:

Cyclin D1	
Forward	5'-CACAGATGTGAAGTTCATTTCCAA-3'
Reverse	5'-ACGTCGGTGGGTGTGCAAGCCA-3'
Cyclin D2	
Forward	5'-GCTCGCCACCTTCCACTCTTCTC-3'
Reverse	5'-TGAAATAGGAACACTGCGGGAGG-3'
Cyclin A	
Forward	5'-GGTAAATGTAAACCTAAAGTGGGT-3'
Reverse	5'-ACACAAACTCTGCTACTTCTGG-3'

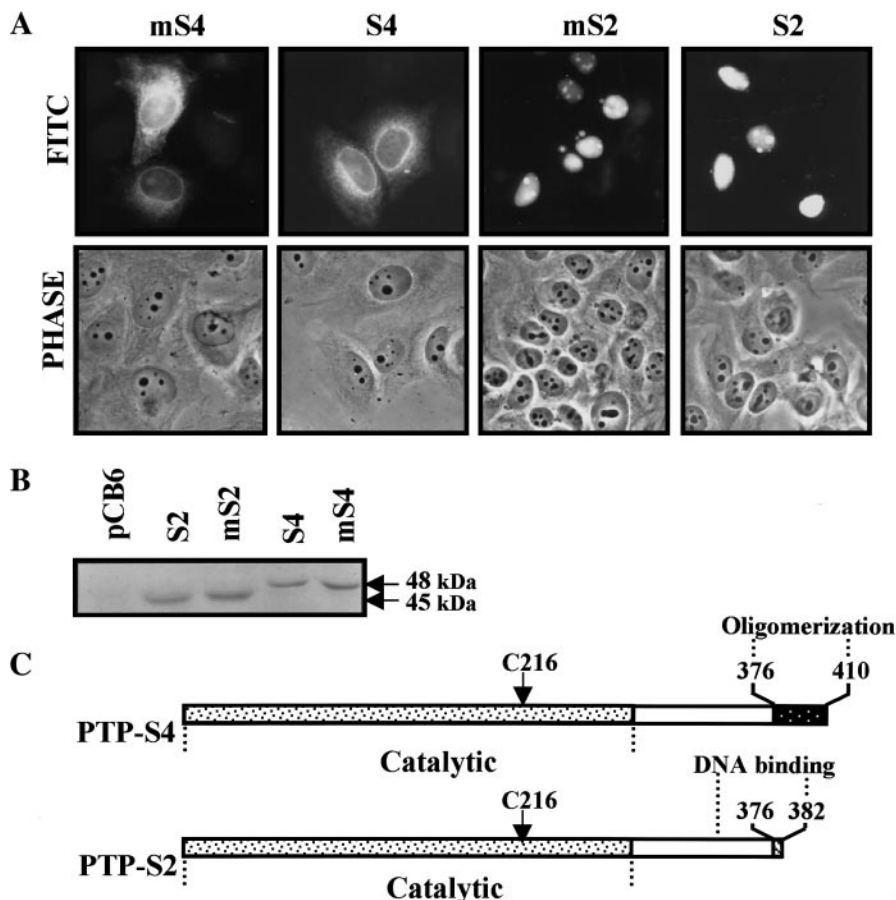
## RESULTS

### Characterization of Mutant PTP-S4 and Mutant PTP-S2

Wild-type PTP-S4 localizes to cytoplasmic and nuclear membranes. In the mutant PTP-S4 construct (mS4), the catalytic site cysteine was replaced with serine (C216S) by PCR mutagenesis. The mutated PTP-S4 cDNA was cloned in pCB6 vector in which CMV promoter drives the expression. The MCF-7 and Cos-1 cells grown on coverslips were transfected with expression plasmids and cells were stained with anti-PTP-S monoclonal antibody G11, which recognizes rat PTP-S4 and PTP-S2 proteins but not the endogenous PTP-S2 or PTP-S4 proteins in human or monkey cells [18, 20]. The mutation in PTP-S4 did not alter its subcellular localization to cytoplasmic and nuclear membranes in MCF-7 cells (Fig. 1A) or in Cos-1 cells (not shown). The mobility of the mutant PTP-S4 protein is of the expected size of 48 kDa and is identical to that of the wild-type protein as determined by Western blotting of transfected Cos-1 cells (Fig. 1B). Since PTP-S2 is very similar in structure to PTP-S4 (Fig. 1C), we have used a catalytically inactive mutant of PTP-S2 (C216S) as an additional control in some of our experiments. The mutant PTP-S2 protein (mS2) showed nuclear localization with prominent staining of nucleoli in MCF-7 cells (Fig. 1A) and also in Cos-1 cells (not shown) as seen with normal PTP-S2 protein. The mobility of mutant and normal PTP-S2 as indicated by SDS-PAGE was identical (Fig. 1B).

### Inhibition of PTP-S4 Activity by Mutant PTP-S4

Since wild-type PTP-S4 protein is a tetramer [20], it is possible that mutant PTP-S4 inhibits wild-type pro-



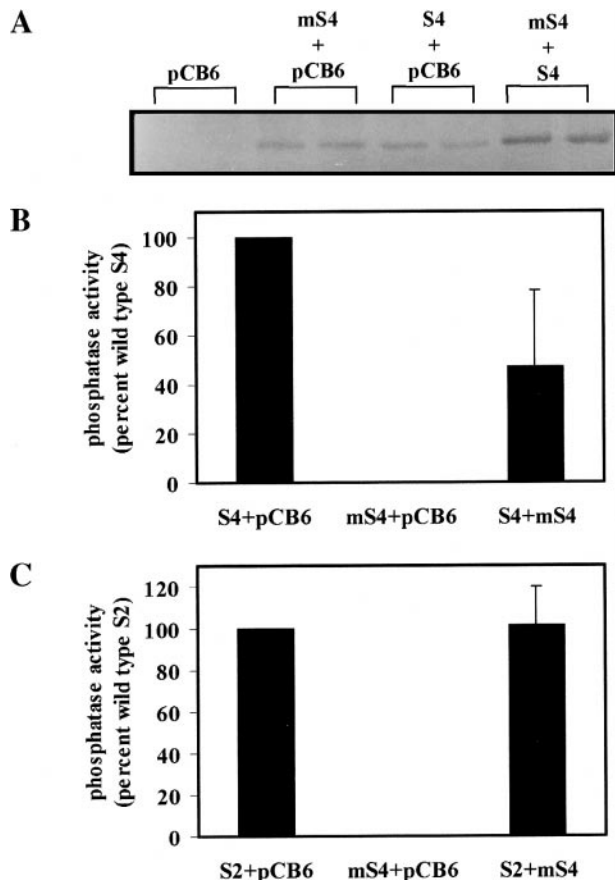
**FIG. 1.** Characterization of mutant PTP-S4 and mutant PTP-S2 constructs. (A) Subcellular localization of mutant proteins. MCF-7 cells were transiently transfected with mutant PTP-S4, wild-type PTP-S4, mutant PTP-S2, and wild-type PTP-S2. Cells were fixed, permeabilized, and stained with anti-PTP-S antibody, G11. Anti-mouse FITC was used as secondary antibody. (B) Western blot analysis of mutant proteins. Cos-1 cells were transiently transfected with indicated constructs and whole-cell lysates were prepared after 48 h. Equal amounts of lysates were run on 8% SDS-polyacrylamide gel and Western blotting was done using G11 and anti-mouse alkaline phosphatase antibodies. pCB6 plasmid-transfected cells were used as controls. (C) Schematic presentation of PTP-S4 and PTP-S2 proteins.

tein function in a dominant negative manner. To test this possibility we transfected Cos-1 cells using 500 ng of PTP-S4 with 500 ng of control plasmid, 500 ng of mS4 with 500 ng of control plasmid, and both PTP-S4 and mS4 in equal stoichiometry (500 ng each). After 48 h total protein was extracted and immunoprecipitated using G11 monoclonal antibody. Resulting immunoprecipitate was split such that one-third was used for determining PTP-S4 amounts in Western blots and the remaining two-thirds was used for protein tyrosine phosphatase activity assay using labeled poly (Glu<sup>4</sup>, Tyr<sup>1</sup>) as substrate. Mutant PTP-S4 protein in the immunoprecipitate showed tyrosine phosphatase activity comparable to that of control (pCB6 transfected Cos-1 cells) indicating that the mutant protein was inactive. When transfected in 1:1 stoichiometry with wild-type PTP-S4 it inhibited phosphatase activity of wild-type PTP-S4 by about 50% (Fig. 2B). Mutant and wild-type PTP-S4 proteins were expressed at

about the same level as determined by Western blotting of the immunoprecipitates (Fig. 2A). This result suggests that mutant PTP-S4 protein can inhibit enzyme activity of wild-type PTP-S4 in the cells. In similar experiments the enzyme activity of PTP-S2 was not inhibited by cotransfection with mutant PTP-S4 (Fig. 2C).

#### *Mutant PTP-S4 Inhibits Anchorage-Independent Growth*

To determine the effect of expression of mutant PTP-S4 on cell proliferation and transformation, PyF cells were transfected with wild-type and mutant PTP-S4 and pCB6 plasmids. Stable clones resistant to G418 selection were collected as pools. Equal numbers of cells from each pool were plated in soft agar and allowed to grow for 5–7 days. Mutant PTP-S4-expressing pools (PymS4) formed much smaller colonies than



**FIG. 2.** Catalytically inactive mutant PTP-S4 protein inhibits the activity of normal PTP-S4. Cos-1 cells were transiently transfected using 500 ng of mutant PTP-S4 with 500 ng of control plasmid pCB6, 500 ng of wild-type PTP-S4 with 500 ng of pCB6, and both mutant and wild-type PTP-S4 constructs in 1:1 ratio (500 ng each). One microgram of pCB6 was transfected in each experiment as control. After 48 h total protein was extracted and immunoprecipitated with G11 monoclonal antibody. Each immunoprecipitate was split such that one-third was used for Western blotting and two-thirds for activity assay using radiolabeled poly (Glu<sup>4</sup>, Tyr<sup>1</sup>) as substrate. (A) Western blot showing that equal amounts of wild-type and mutant proteins were present in immunoprecipitates, whereas, as expected, protein was twofold in mS4 + S4 lanes. (B) Enzyme activity data obtained from five independent transfections. (C) Mutant PTP-S4 does not inhibit the activity of normal PTP-S2. This experiment was carried out as described for the effect of mutant PTP-S4 on the activity of normal PTP-S4 except that normal PTP-S4 plasmid was replaced by normal PTP-S2 plasmid. The data represent mean  $\pm$  SD of four experiments.

the control (PyC) cells (Fig. 3). This inhibition was observed on comparing six independently obtained stable mutant pools with five control pools (data from only two pools are shown in Fig. 3). Quantitation of the inhibitory effect of mutant PTP-S4 on anchorage-independent growth was carried out by counting the number of colonies using GeneTools software after scanning the stained plates under identical conditions. Data from three pools each of mutants and controls are

shown in Table 1. There was a 10- to 60-fold decrease in the number of medium-size colonies formed by PymS4 cells compared with controls. These results suggest that expression of mutant PTP-S4 inhibits anchorage-independent growth of PyF cells.

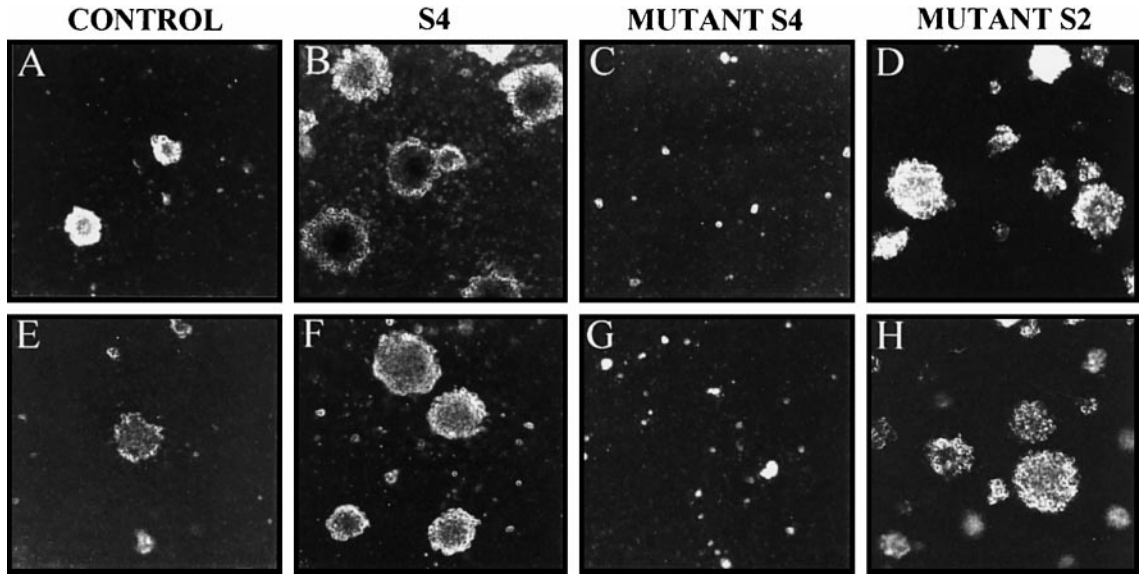
When stable pools overexpressing PTP-S4 (PyS4) were compared with control pools (PyC) for growth in soft agar after 5 days it was found that PTP-S4-overexpressing pools formed significantly larger colonies than the control cells (Fig. 3). This increase in growth was observed on comparison of four independently obtained stable PTP-S4-overexpressing pools with five control pools. Data from two pools of PTP-S4 are shown in Table 1 and Fig. 3. There was two- to fourfold increase in the number of medium-size colonies formed by PyS4 cells compared with controls.

We then examined the effect of expression of mutant PTP-S2 on the growth of PyF cells. Unlike mutant PTP-S4-expressing pools, the mutant PTP-S2-expressing pools did not show any decrease in growth in soft agar compared to controls. Instead, there was some increase in growth (Fig. 3, Table 1). These results suggest that the regulation of anchorage-independent growth by PTP-S4 is specific and independent of the activity of PTP-S2.

In order to determine the expression of exogenous PTP-S4 and mutant PTP-S4, RT-PCR method was used. For this purpose a 3' primer was designed within the transcribed pCB6 vector region and a 5' primer was designed within a region specific to PTP-S. Hence a 389-bp amplicon would be obtained only when PTP-S4 is physically contiguous with pCB6 vector sequences thereby differentiating between endogenous and exogenous transcripts. A 389-bp amplicon was seen in PyS4 and PymS4 pools and was absent in vector transfected pools and in various PCR and RT controls (Fig. 4A). The expression of normal PTP-S4 and mutant PTP-S4 was also analyzed by Western blotting using a monoclonal antibody which recognizes all isoforms of PTP-S. The level of PTP-S4 protein was increased in mutant PTP-S4-expressing pools of cells as well as in normal PTP-S4-expressing pools of cells compared to controls (Fig. 4B). This suggests that mutant PTP-S4 and exogenous normal PTP-S4 proteins are indeed expressed at significant level in these cells.

#### *Wild-Type PTP-S4 Increases whereas Mutant PTP-S4 Inhibits Tumor Formation in Nude Mice*

To determine the effect of mutant PTP-S4 on tumorigenic properties of PyF cells, we injected  $10^6$  cells each of PyS4, PyC, and PymS4 at a single site subcutaneously on the backs of 1-week-old nude mice. After 2- and 3-week intervals the size and volumes of tumors were measured. Tumors formed by PyS4 cells were larger in size and volume compared to those formed by



**FIG. 3.** Mutant PTP-S4 inhibits anchorage-independent growth of PyF cells. Stable pools overexpressing mutant PTP-S4, wild-type PTP-S4, or mutant PTP-S2 were compared with control (pCB6 transfected) pools in soft agar;  $5 \times 10^4$  cells were plated in 60-mm dishes and allowed to grow for 5 days. Colonies were photographed at  $40\times$  magnification using an inverted microscope. Representative fields are shown for two independent pools of control (A, E), wild-type PTP-S4 (B, F), mutant PTP-S4 (C, G), and mutant PTP-S2-expressing cells.

injecting PyC cells. PymS4 cells formed tumors that were smaller compared to the controls. At both the 2- and the 3-week intervals, the differences in tumor volumes between PyC and PyS4 ( $P < 0.2$ ), PyC and PymS4 ( $P < 0.5$ ), and PyS4 and PymS4 ( $P < 0.05$ ) are significant (Table 2).

#### Mutant PTP-S4 Expression in PyF Cells Does Not Affect Growth in Liquid Culture

To determine whether PTP-S4 affects anchorage-dependent cell growth, we plated  $10^5$  cells each of PyS4,

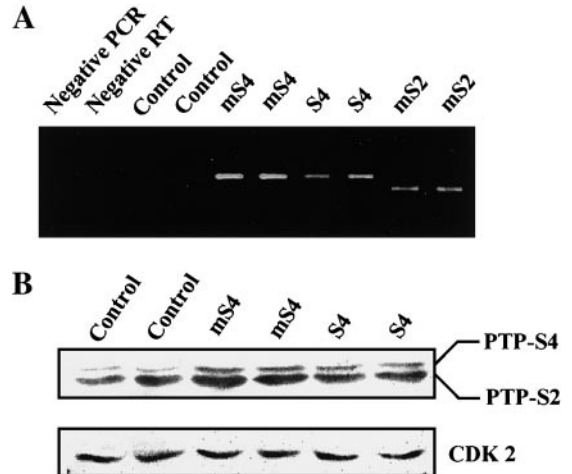
PyC, and PymS4 pools in liquid culture and enumerated by hemocytometric counting after 48 h. Mutant PTP-S4-expressing pools did not differ significantly in growth from control pools (data not shown). PTP-S4-overexpressing pools showed only a marginal increase in growth (data not shown). These results suggest that

**TABLE 1**

Anchorage-Independent Growth of Mutant PTP-S4 and Mutant PTP-S2 Overexpressing PyF Cells

	Cells	Number of colonies
Control	PyC.3	199 $\pm$ 22.0
	PyC.4	140 $\pm$ 12.7
	PyC.5	178 $\pm$ 20.4
PTP-S4	PyS4.3	579 $\pm$ 38.3
	PyS4.4	409 $\pm$ 42.6
MUTANT PTP-S4	PymS4.5	5 $\pm$ 1.3
	PymS4.7	14 $\pm$ 5.2
	PymS4.8	3 $\pm$ 0.2
MUTANT PTP-S2	PymS2.1	371 $\pm$ 14.0
	PymS2.2	481 $\pm$ 28.4

*Note.* Cells ( $5 \times 10^4$  each) of PyF stably overexpressing control (pCB6), wild-type PTP-S4, mutant PTP-S4, and mutant PTP-S2 were plated in 60-mm dishes in soft agar. After 5 days the plates were stained. The plates were then scanned using GeneTools software (SynGene) and the number of colonies larger than pixel 5 was counted. The data represent mean  $\pm$  SD of three experiments.



**FIG. 4.** Expression of exogenous wild-type and mutant PTP-S4 in pools of PyF cells. (A) RT-PCR was done using M3 and M4 primers designed such that a 389-bp amplicon would be obtained only when PTP-S4 was physically contiguous with pCB6 vector sequences. Mutant PTP-S2 gave a PCR product of 305 bp. The amplified products were run on 1.5% agarose gel. (B) Immunoblot showing expression of PTP-S4 in pools of PyF cells obtained by transfection with mutant PTP-S4 (mS4), normal PTP-S4 (S4), or control plasmid. The position of PTP-S4 band at 48 kDa and PTP-S2 band at 45 kDa is indicated. The Cdk 2 blot was used as a loading control.

**TABLE 2**

Effect of Wild-Type PTP-S4 and Mutant PTP-S4 on Tumor Growth in Nude Mice

Cells	Tumor volume after 2 weeks (mm <sup>3</sup> ) <sup>a</sup>	Tumor volume after 3 weeks (mm <sup>3</sup> ) <sup>b</sup>
PyC.3	734.20 ± 900.78 (n = 4)	7001.10 ± 4383.37 (n = 4)
PyS4.3	1740.04 ± 554.02 (n = 3)	13832.00 ± 4221.48 (n = 2) <sup>c</sup>
PymS4.5	297.26 ± 389.93 (n = 3)	3625.42 ± 1567.40 (n = 3)

Note. The data represent mean ± SD values.

<sup>a</sup> PymS4.5 < PyC.3 at  $P < 0.5$ ; PyC.3 < PyS4.3 at  $P < 0.2$ ;

PymS4.5 < PyS4.3 at  $P < 0.05$ .

<sup>b</sup> PymS4.5 < PyC.3 at  $P < 0.5$ ; PyC.3 < PyS4.3 at  $P < 0.2$ ;

PymS4.5 < PyS4.3 at  $P < 0.05$ .

<sup>c</sup> One animal died during the third week.

expression of mutant PTP-S4 causes a marked inhibition in anchorage-independent growth but does not affect growth in liquid culture significantly.

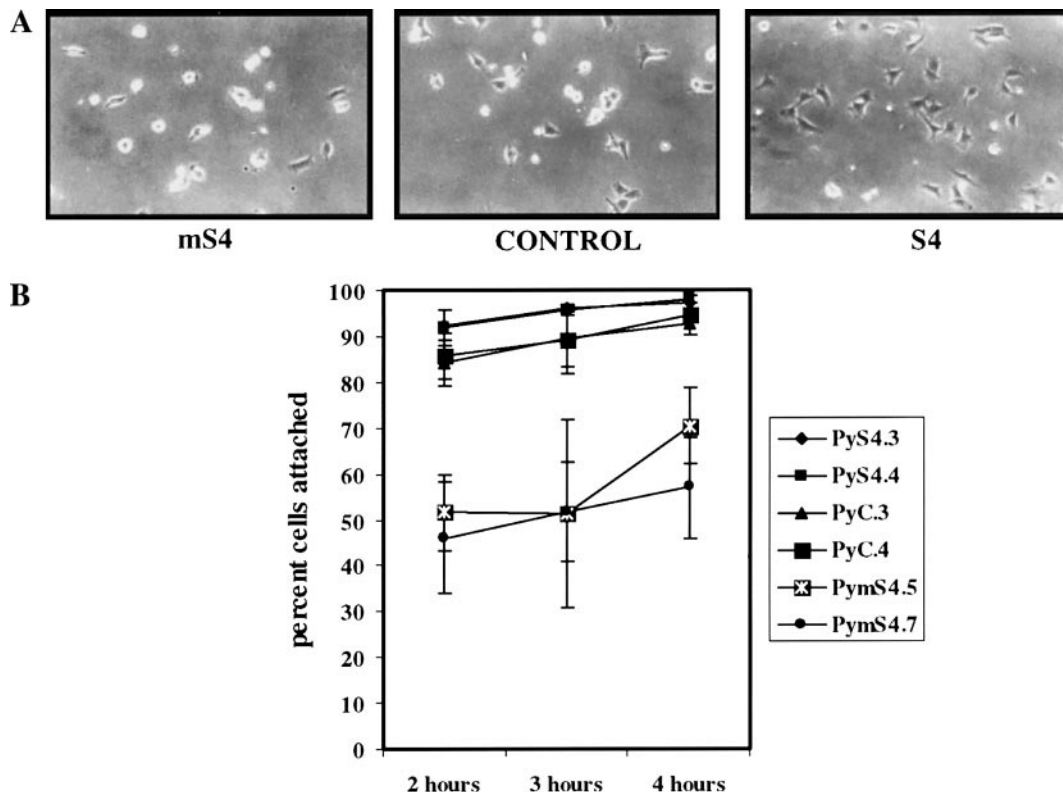
#### Mutant PTP-S4 Expression Inhibits Cell-Substratum Adhesion

To determine whether PTP-S4 affects cell-substratum adhesion, we plated  $5 \times 10^4$  cells each of PyS4, PymS4,

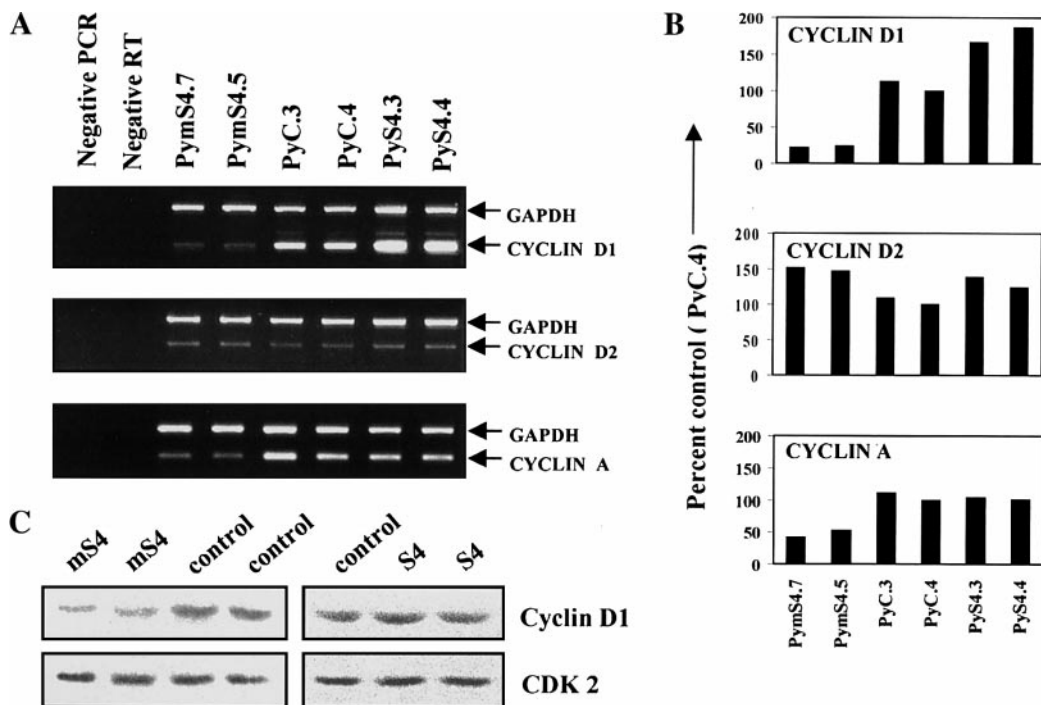
and PyC in 35-mm dishes. Random fields were photographed at 100× magnification after 2, 3, and 4 h using an inverted microscope. Attached cells could be differentiated from unattached cells ± 2%. Overexpression of wild-type PTP-S4 showed small but significant increase in the percentage of cells attached ( $P = 5.24 \times 10^{-4}$ ). However, expression of mutant PTP-S4 resulted in a large reduction in the percentage of cells attached ( $P = 1.66 \times 10^{-10}$ ) (Fig. 5). These results suggest that PTP-S4 plays a positive regulatory role in cell adhesion that is inhibited by expression of mutant PTP-S4.

#### Expression of Cyclin D1 and Cyclin A Is Affected by Mutant PTP-S4

Increased expression of some of the cyclins such as cyclin A and cyclin D1 is associated with anchorage-independent growth. We therefore analyzed the level of mRNAs of these cyclins in mutant PTP-S4 and wild-type PTP-S4-expressing PyF cells by using semiquantitative RT-PCR. The level of cyclin D1 mRNA was reduced by about fourfold in cells expressing mutant PTP-S4 compared to controls, whereas normal PTP-S4-expressing cells showed slight increase ( $P = 0.01154$ ) as shown in Figs. 6A, and 6B. The level of cyclin D2 mRNA was not



**FIG. 5.** Inhibition of cell adhesion by mutant PTP-S4;  $5 \times 10^4$  cells each of pools stably overexpressing pCB6 (control), mutant PTP-S4, and wild type PTP-S4 were plated in uncoated 35-mm dishes. (A) After 4 h random fields were photographed at 100× magnification using an inverted microscope. Attached cells could be differentiated from round and refractile unattached cells. (B) The data represent percentage of cells attached (mean ± SD of three experiments). At least 300 cells were counted for each clone expressing mutant PTP-S4, wild-type PTP-S4, or control plasmid.



**FIG. 6.** Effect of mutant PTP-S4 and wild-type PTP-S4 overexpression on mRNA levels of cyclins. Specific primers were designed for cyclins D1, D2, and A and semiquantitative RT-PCR was carried out using GAPDH as an internal control. (A) Ethidium bromide gels for RT-PCR done for cyclins D1, D2, and A. RNA obtained from two independent pools were used in each experiment and PCR was done twice. (B) Quantitation of the PCR products using GeneTools software (SynGene). Each bar represents the mean of two quantitations done for each pool, after normalization with GAPDH, relative to control PyC.4 as 100%. (C) Immunoblot for expression of cyclin D1 protein in pools of cells obtained by transfection with mutant PTP-S4 (mS4), normal PTP-S4 (S4), or control plasmid. The same blot was reprobed for Cdk 2 as loading control.

affected significantly by mutant or normal PTP-S4. Cyclin A mRNA level showed a twofold decrease in mutant PTP-S4-expressing cells ( $P = 0.00026$ ) compared with controls (Figs. 6A and 6B). Expression of normal PTP-S4 had no effect on cyclin A mRNA level. The effects of mutant and wild-type PTP-S4 on cyclin D1 and cyclin A mRNA levels were confirmed by Northern blotting, which showed about 3.5-fold decrease in cyclin D1 mRNA level and 4.5-fold decrease in cyclin A mRNA levels in mutant PTP-S4-expressing cells (not shown). Immunoblot analysis showed that there was a large decrease in cyclin D1 protein level in mutant PTP-S4-expressing pools of cells compared to controls (Fig. 6C). Expression of PTP-S4 showed a small increase in cyclin D1 protein level (Fig. 6C).

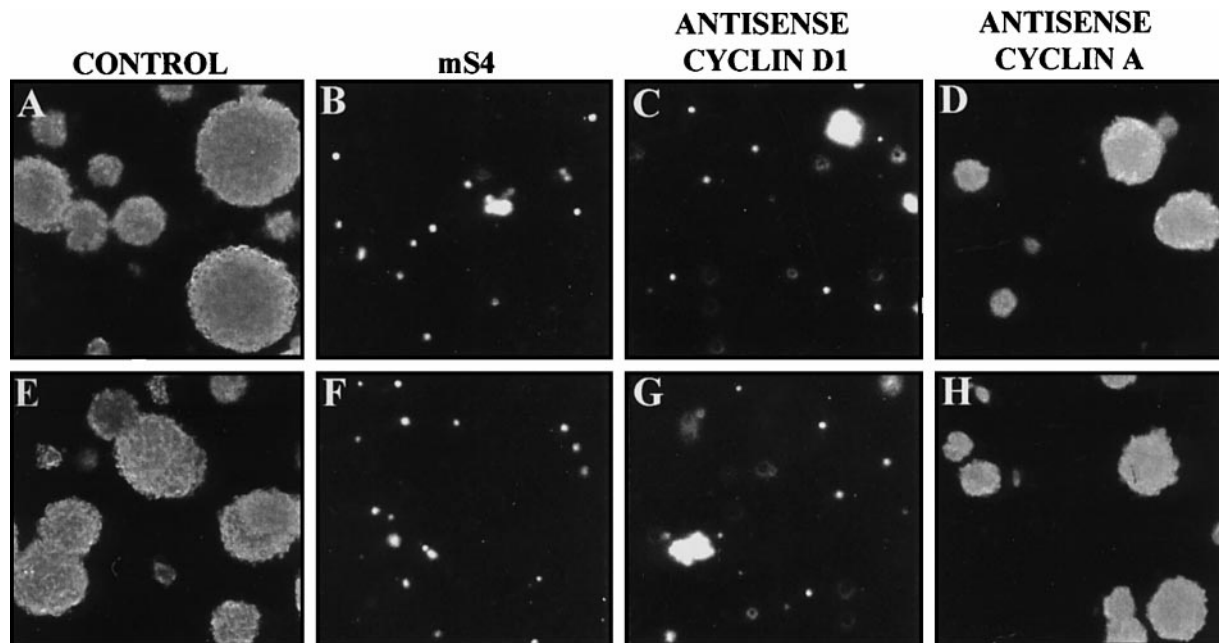
#### *Role of Cyclin D1 in Suppression of Anchorage-Independent Growth by Mutant PTP-S4*

To address the role of cyclin D1 and cyclin A in anchorage-independent growth, PyF cells were transfected with antisense cyclin D1, antisense cyclin A, mutant PTP-S4, or control plasmid. After 3 days of selection in G418 the cells were plated for growth in soft agar. After 7–10 days of growth the number of

colonies was counted. It was observed that antisense cyclin D1 strongly inhibited growth of PyF cells in soft agar, whereas antisense cyclin A showed much less inhibition (Fig. 7, Table 3). Mutant PTP-S4 expression also showed strong inhibition of growth in these experiments (Fig. 7, Table 3). These observations raised the possibility that the expression of mutant PTP-S4 results in decreased level of cyclin D1 which is largely responsible for decreased growth of PyF cells in soft agar. Therefore we next examined the effect of expression of cyclin D1 along with mutant PTP-S4. PyF cells were transfected with mutant PTP-S4 plus control plasmid, mutant PTP-S4 plus cyclin D1, or control plasmid alone. After 3 days of selection in G418 the cells were assayed for growth in soft agar. The number of colonies was counted after 8 days of growth. It was found that cyclin D1 expression largely prevented the growth inhibition by mutant PTP-S4 (Fig. 8, Table 4).

#### *Mutant PTP-S4 Expression Inhibits Anchorage-Independent Growth and Adhesion in MCF-7 Breast Carcinoma Cells*

To determine whether PTP-S4-mediated regulation of anchorage-independent growth and adhesion is a



**FIG. 7.** Inhibition of anchorage-independent growth by expression of antisense cyclinD1. PyF cells were transfected with control (pCB6), mutant PTP-S4, antisense cyclinD1, and antisense cyclinA plasmids. After 3 days of selection in G418,  $5 \times 10^4$  cells from each transfection were plated in 60-mm dishes in soft agar. After 7–10 days the plates were photographed at 40 $\times$  magnification. Representative fields are shown for two independent experiments using control (A, E), mutant PTP-S4 (B, F), antisense cyclinD1 (C, G), and antisense cyclinA (D, H) plasmids.

cell-type-specific phenotype, we prepared stable pools of MCF-7 cells expressing mutant PTP-S4 (MCMs4)- and pCB6-transfected pools (MCC). Stable pools overexpressing wild-type PTP-S4 could not be obtained in MCF-7, as its expression causes apoptosis in this and other p53-positive cell lines [23]. Growth of these pools was analyzed by plating  $10^5$  cells in soft agar. After 14 days of growth there were two- to threefold fewer colonies in mutant PTP-S4-expressing pools compared with controls (Fig. 9A, Table 5). Mutant PTP-S4 expression did not affect growth of MCF-7 cells in liquid culture (data not shown). Thus although mutant PTP-S4 inhibited anchorage-independent growth of

MCF-7 cells, the inhibition was weak compared with the inhibition of PyF cells. Expression of mutant PTP-S4 inhibited cell adhesion by 20–40% (Figs. 9B and 9C). Thus the effect of mutant PTP-S4 on cell adhesion did not appear to be cell type specific.

## DISCUSSION

We have shown that expression of an enzymatically inactive mutant of PTP-S4 in polyoma virus-transformed cells inhibited anchorage-independent growth in soft agar and cell adhesion but this mutant PTP-S4 had no effect on anchorage-dependent growth. Wild-type PTP-S4 expression increased anchorage-independent growth and cell adhesion. These results suggest that PTP-S4 affects a biochemical step that controls primarily anchorage-independent growth and cell adhesion.

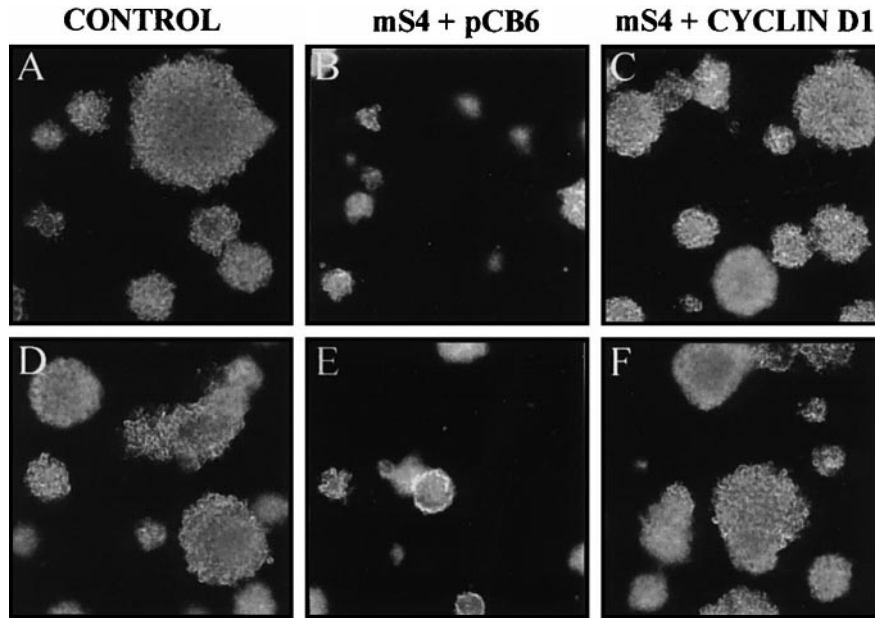
PTP-S4 is a tetrameric protein and the C-terminal hydrophobic domain is required for oligomerization of PTP-S4 [20]. Therefore a catalytic mutant of PTP-S4 may act as a dominant negative mutant. Coexpression of mutant PTP-S4 with normal PTP-S4 partially inhibited the enzymatic activity of the latter. In addition the observed effects of mutant PTP-S4 on anchorage-independent growth and cell adhesion are opposite those of normal PTP-S4. These observations taken together suggest that mutant PTP-S4 acts by a dominant neg-

**TABLE 3**

Anchorage-Independent Growth of Antisense Cyclin D1 and Antisense Cyclin A Expressing PyF Cells

Expression plasmids	Number of colonies
Control (pCB6)	$109.25 \pm 7.27$ ( $n = 4$ )
Mutant PTP-S4	$0.25 \pm 0.5$ ( $n = 4$ )
Antisense cyclin D1	$1.0 \pm 1.41$ ( $n = 4$ )
Antisense cyclin A	$73.25 \pm 6.18$ ( $n = 4$ )

*Note.* PyF cells were transfected with control (pCB6), mutant PTP-S4, antisense cyclin D1, and antisense cyclin A plasmids. After 3 days of selection in G418,  $5 \times 10^4$  cells from each transfection were plated in 60-mm dishes in soft agar. After 7–10 days the plates were stained and the number of colonies larger than 5 pixels were counted.



**FIG. 8.** Cyclin D1 expression prevents inhibition of anchorage-independent growth by mutant PTP-S4. PyF cells were transfected with pCB6 (1  $\mu$ g), mutant PTP-S4 (500 ng) + pCB6 (500 ng), and mutant PTP-S4 (500 ng) + cyclinD1 (500 ng) plasmids. After 3 days of selection in G418,  $5 \times 10^4$  cells from each transfection were plated in 60-mm dishes in soft agar. After 8 days the plates were photographed at 40 $\times$  magnification. Representative fields are shown for two independent experiments using control plasmid (A, D), mutant PTP-S4 + pCB6 (B, E), and mutant PTP-S4 + cyclinD1 (C, F) plasmids.

ative mechanism, that is, by inhibiting the normal function of PTP-S4.

Effect of expression of many PTPs on anchorage-independent growth has been analyzed in various cells transformed by oncogenes coding for tyrosine kinases. In these studies an inhibition of anchorage-independent growth was observed upon expression of PTP1B, SHP-1, PTP-MEG, low-molecular-weight PTP, and RPTP $\sigma$  [6, 7, 35–38]. Expression of a few PTPs has been shown to increase anchorage-independent growth. PRL-1 and PTP $\alpha$ , which increase anchorage-independent growth, are also able to transform normal cells [4, 5]. PTP-S4 mRNA like that of PRL-1 is

strongly induced during liver regeneration [20]. However, expression of PTP-S4/TC48 does not cause cell transformation. In PyF cells expression of PTP-S4 increased the anchorage-independent growth and mutant PTP-S4 inhibited it. Thus overall the observed effects of wild-type PTP-S4 and its mutant do not resemble that of any known PTP.

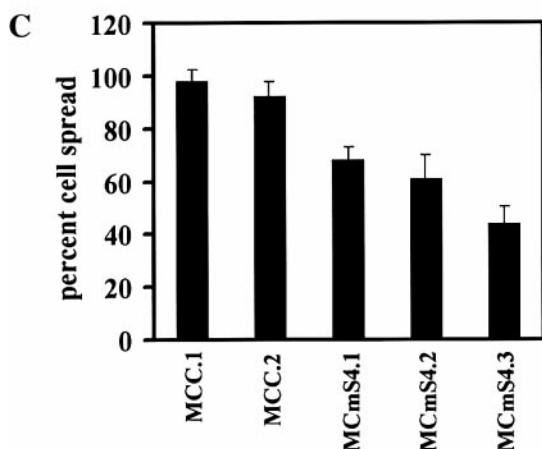
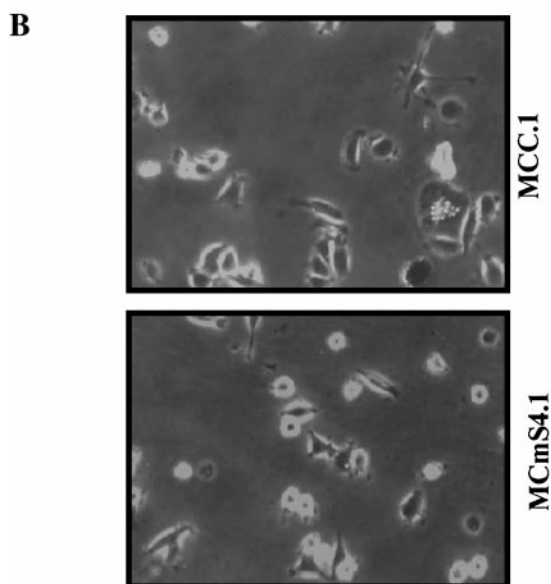
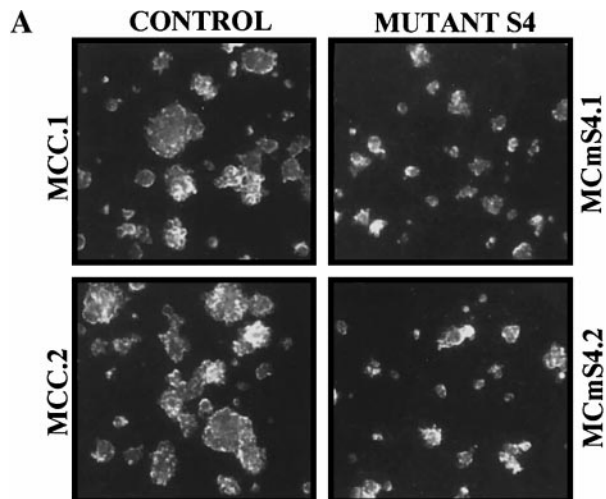
An increased level of cyclin D1 is observed in many human cancers (reviewed in Ref. 39). Transgenic mice overexpressing cyclin D1 develop mammary hyperplasia and carcinoma [40]. Expression of antisense to cyclin D1 inhibits growth and tumorigenicity of colon cancer cells [41]. Overexpression of cyclin D1 but not cyclin E induces anchorage-independent cell cycle progression [42]. Ectopic expression of cyclin D1 in human breast tumor cell line MCF-10A results in preferential stimulation of anchorage-independent growth but not anchorage-dependent growth [43]. There was good correlation between cyclin D1 mRNA levels and anchorage-independent growth of PyF cells expressing mutant or wild-type PTP-S4 (this study). Antisense cyclin D1 strongly inhibited anchorage-independent growth of PyF cells. Inhibition of anchorage-independent growth by mutant PTP-S4 was largely overcome by coexpression of cyclin D1. These results suggest that the observed effect of mutant PTP-S4 on anchorage-independent growth, at least in part, is mediated by cyclin D1. Like cyclin D1, cyclin A is also involved in

**TABLE 4**

Rescue of Anchorage-Independent Growth of Mutant PTP-S4 Expressing PyF Cells by Overexpression of Cyclin D1

Expression plasmids	Number of colonies
Control (pCB6)	121.25 $\pm$ 18.87 ( $n = 4$ )
Mutant PTP-S4 + control	7.33 $\pm$ 3.79 ( $n = 3$ )
Mutant PTP-S4 + cyclin D1	83.33 $\pm$ 6.66 ( $n = 3$ )

*Note.* PyF cells were transfected with pCB6 (1  $\mu$ g), mutant PTP-S4 (500 ng) + pCB6 (500 ng), and mutant PTP-S4 (500 ng) + cyclin D1 (500 ng) plasmids. After 3 days of selection in G418,  $5 \times 10^4$  cells of each transfection were plated in 60-mm dishes in soft agar. After 8 days the plates were stained and the number of colonies larger than 5 pixels were counted.



**FIG. 9.** Mutant PTP-S4 inhibits anchorage-independent growth and adhesion of MCF-7 cells. (A) Stable pools expressing mutant PTP-S4 were compared with control (pCB6 transfected) pools for growth in soft agar;  $10^5$  cells were plated in 60-mm dishes and

**TABLE 5**

Anchorage-Independent Growth of Mutant PTP-S4 Expressing MCF-7 Cells

	Cells	Number of colonies
Control	MCC.1	601 $\pm$ 48.8
	MCC.2	785 $\pm$ 69.3
Mutant PTP-S4	MCmS4.1	200 $\pm$ 18.2
	MCmS4.2	315 $\pm$ 28.3

*Note.* Cells ( $10^5$  each) of MCF-7 stably overexpressing control (pCB6) and mutant PTP-S4 were plated in 60-mm dishes in soft agar. After 2 weeks plates were stained with 0.1% methylene blue and scanned, and the number of colonies larger than pixel 10 was counted. The data represent mean  $\pm$  SD of three experiments.

cell adhesion-independent proliferation [44, 45]. However, cyclin A may not contribute significantly to the observed effect of mutant PTP-S4 since antisense cyclin A showed only a small effect on anchorage-independent growth.

In our experiments cyclin D1 level decreased upon expression of mutant PTP-S4 even though the cells were grown attached to substratum. Under these conditions the growth of cells was not affected significantly. One possible explanation for these observations is that the decreased cyclin D1 level in mutant PTP-S4-expressing cells is sufficient for anchorage-dependent growth but not for anchorage-independent growth of PyF cells. Alternatively or in addition, some other mechanisms such as cyclin D2 levels may contribute toward anchorage-dependent growth in liquid culture.

Cell adhesion plays an important role in many functions such as cell anchorage, proliferation, differentiation, invasion, and signal transduction. Increased cell adhesion is associated with an increase in tumorigenicity of many tumor cells [46–48]. We found that mutant PTP-S4 expression in PyF cells inhibited adhesion to substratum, which was associated with decreased anchorage-independent growth. Adhesion-dependent signaling is essential for growth factors to induce expression of cyclin D1 as well as cyclin A mRNA in many cells. It is, therefore, likely that mutant PTP-S4 affects a step which controls cell adhesion leading to changes in cyclin D1 expression and anchorage-independent growth.

In conclusion our results show that a dominant interfering mutant of PTP-S4 preferentially inhibits an-

allowed to grow for 2 weeks. Colonies were then photographed at  $40\times$  magnification. Inhibition of cell adhesion is shown in (B) and (C) Cells ( $10^5$  each) of mutant PTP-S4-expressing pools and control pools were plated in uncoated 35-mm dishes. After 4 h random fields were photographed at  $100\times$  magnification. The data represent percentage of cells attached (mean  $\pm$  SD of three experiments).

chorage-independent growth but not anchorage-dependent growth. This mutant also inhibits cell-substratum adhesion. These cellular effects of mutant PTP-S4 show good correlation with the level of cyclin D1 mRNA. Our results suggest that the effects of mutant PTP-S4 are brought about by inhibition of normal function of PTP-S4 upstream of cyclin D1 gene expression.

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