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A Nuclear Protein Tyrosine Phosphatase Induces Shortening of G1 Phase and Increase in c-Myc Protein Level

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PTP-S2 is a ubiquitously expressed nuclear protein tyrosine phosphatase which shows increased expression upon mitogenic stimulation in a variety of cells *in vitro* and *in vivo.* In order to understand the role of this enzyme in cell cycle progression, tetracycline-regulated HeLa clones expressing PTP-S2 were isolated and characterized. Tetracycline-controlled expression of PTP-S2 increased the rate of cell proliferation. An analysis of the distribution of cells in various phases of the cell cycle in an exponentially growing cell population showed that there was a large decrease in the percentage of cells in G1 phase in a PTP-S2-expressing population of cells compared to nonexpressing cells. This decrease in the percentage of cells in G1 was dependent on the level of PTP-S2 expression. There was a corresponding increase in the percentage of cells in G2/M but no significant increase in the percentage of cells in S phase. An analysis of the time course of cell cycle progression after release from double thymidine block showed that the duration of G1 phase was significantly shortened in cells induced to express exogenous PTP-S2. However, the duration of S phase was not significantly altered and the duration of G2 phase was increased to some extent. Induction of PTP-S2 expression was associated with an increase in c-Myc protein levels, although the c-Myc mRNA level was not changed. Our results suggest that overexpression of PTP-S2 promotes progression of cells through G1 to S phase and is associated with increased level of c-Myc protein through a posttranscriptional mechanism. © 2001 Academic Press

Key Words: **protein tyrosine phosphatase; cell cycle; c-Myc; PTP-S2; G1/S transition.**

INTRODUCTION

Protein tyrosine phosphorylation mediated by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) has been shown to play a pivotal role in regulating cell proliferation, differentiation, and migration [1–4]. Progression through the cell cycle is dependent on the phosphorylation of key regulatory proteins by cyclin-dependent kinases (CDKs), which in turn are regulated in a complex fashion by association with cyclins, by phosphorylation and dephosphorylation, and by CDK-inhibitory proteins [5]. The role of many PTKs, such as those which are associated with cytokine or growth factor receptors and intracellular PTKs, in cell proliferation and other cellular functions has been analyzed in detail [1, 6]. In contrast, the role of most of the PTPs in cell proliferation and other cellular activities is poorly understood although many genes coding for receptor type as well as nontransmembrane PTPs have been isolated [2]. Various intracellular or nontransmembrane PTPs have a conserved catalytic domain flanked by unique noncatalytic sequences at the carboxy- or amino-terminal ends. The noncatalytic sequences are involved in determining subcellular location and in the regulation of enzyme activity and substrate specificity [2, 3, 7].

The nontransmembrane protein tyrosine phosphatase PTP-S/TCPTP is ubiquitously expressed [7–9]. In rat cells four different splice forms of this phosphatase are generated from the primary transcript by alternative splicing [10]. Two of these are major forms, PTP-S2 and PTP-S4 (also known as TC45 and TC48, respectively), which are expressed in rat as well as human cells. PTP-S2 is located mainly in the cell nucleus, partly in association with chromatin, possibly through its interaction with DNA [7, 11–13]. The noncatalytic sequences at the carboxy-terminal end of PTP-S2 and PTP-S4 determine their subcellular location, substrate specificity, enzyme activity, and interaction with DNA and nuclear matrix [7]; PTP-S2 mRNA is transiently increased upon mitogenic stimulation of a variety of cells *in vitro* [7, 14, 15]. During liver regeneration after partial hepatectomy both the major isoforms PTP-S2 and PTP-S4 are transiently induced in G1 phase. PTP-S2 is specifically phosphorylated on its C-terminal region during mitosis by CK2 or CK2-like enzyme [16]. PTP-S-deficient mice show poor T and B cell proliferative responses and defects in bone marrow hematopoiesis [17]. These observations indicate a role for these

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PTPs in cell proliferation. Overexpression of PTP-S2 in HeLa cells at moderate levels increases cell division, providing further evidence for its involvement in this process [18]. PTP-S has been shown to associate with importin β and the hepatocyte growth factor receptor, Met [19, 20]. Using substrate trap mutants, EGF receptor and adaptor protein Shc were identified as its substrate in Cos cells but the physiological significance of these observations is yet to be elucidated [21, 22]. Overexpression of PTP-S2 at a high level by transient transfection induces apoptosis in p53-positive but not in p53-negative human tumor cell lines which is suppressed by mutant p53 [23]. In contrast to PTP-S2, overexpression of PTP-S4 results in a much lower level of apoptosis in p53-positive cells.

The proto-oncogene c-myc is a key regulator of cell proliferation and apoptosis [24, 25]. c-Myc encodes a basic helix-loop-helix leucine-zipper transcription factor (Myc) that dimerizes with Max [26] and binds to DNA in a sequence-specific manner [27]. Myc–Max heterodimers activate transcription and are required for Myc-induced cell transformation, cell cycle progression, and apoptosis [28, 29]. Homozygous inactivation of c-Myc in immortalized rat fibroblasts caused a marked prolongation of cell doubling time and accumulation of cells in the G1 and G2 phases of the cell cycle [30]. This latter observation further suggests a central role for c-Myc in regulating cell proliferation. Overexpression of c-Myc shortens the duration of G1 phase without affecting the duration of G2 phase [31]. Despite substantial effort the molecular mechanisms by which c-Myc controls proliferation and tumorigenesis are not understood. It has been suggested that c-Myc controls cell proliferation by affecting transcription of a critical set of target genes [32–35].

The nuclear tyrosine phosphatase PTP-S2 and the protooncogene c-Myc share certain properties, such as nuclear location, transient increase in expression upon mitogenic stimulation, and effect on cell proliferation and apoptosis upon overexpression. The possibility of an interplay of function between PTP-S2 and c-Myc in regulating cell proliferation led us to analyze the effect of expression of rat PTP-S2 on cell cycle progression. Our results suggest that expression of rat PTP-S2 in HeLa cells causes a significant shortening of the G1 phase and is associated with altered expression of the c-Myc protein by a posttranscriptional mechanism.

MATERIALS AND METHODS

Cell lines, antibodies, and chemicals. The premade HeLa-Tet-Off inducible cell line was a kind gift from Dr. Helen M. Blau, Stanford University. This cell line was obtained by transfection of HeLa cells with the Tet-Off plasmid followed by selection in G418. pTK-Hyg, a selection vector which confers hygromycin resistance in mammalian cells; Tet-off regulator; and response plasmids were from Clontech. Bromodeoxyuridine (BrdU) labeling reagent, anti-BrdU antibody, enhanced chemiluminescence detection reagents, and secondary antibodies were from Boehringer Mannheim. FITC-linked anti-mouse secondary antibody was from Bangalore Genei, India. Anti-Cdk2 and anti-PCNA antibodies were purchased from Santa Cruz Biotechnology. Anti-c-Myc antibody (9E 10) was from the Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa. Trizol reagent, for single-step isolation of total RNA, Geneticin, hygromycin, and Lipofectamine PLUS reagent were from GIBCO BRL. Tetracycline, thymidine, and fetal calf serum (FCS) were obtained from Sigma Chemical Co.

cDNAs and transfections. A full-length PTP-S2 cDNA fragment was cloned into the *Bam*HI site of pTRE response plasmid. HeLa-Tet-Off cells (5×10^5) were plated in 60-mm dishes and transfected with 2 μg of pTRE-PTP-S2, 1 μg of pTK-Hyg using Lipofectamine PLUS reagent. At 30 h posttransfection, 100 μg/ml hygromycin was added for selection. After 2 weeks of selection, individual clones were expanded and screened for low background and high induction of PTP-S2 by immunofluorescence using anti-PTP-S2 monoclonal antibody, which recognizes the rat PTP-S2 protein but not the endogenous human protein in HeLa cells [7]. Unless otherwise mentioned, the cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, 200 μg/ml Geneticin, 100 μg/ml hygromycin, and 2 μg/ml tetracycline in a humidified CO_2 incubator at 37°C.

Determination of growth kinetics. Growth kinetics of the HeLa Tet-inducible PTP-S2 clone was measured by plating 50,000 cells in 35-mm dishes in duplicate in DMEM with 10% FCS and cell numbers were counted after 24, 48, 72, and 96 h of growth and induction following removal of tetracycline.

Immunofluorescence staining. Cells were plated on coverslips and incubated in a 37°C incubator under a 5% CO_2, 95% air atmosphere to allow cells to adhere and spread. They were induced for PTP-S2 expression for different periods of time—0, 24, 48, 72, and 96 h—by thoroughly washing off medium containing tetracycline and replacing it with tetracycline-free medium. Cells were fixed with 3.7% formaldehyde in PBS for 10 min and processed for immunostaining with anti-PTP-S2 monoclonal antibody as described previously [11].

Western blot analysis. For Western blot analysis, proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose filters. Molecular weight markers were detected by Ponceau S stain. Membranes were blocked for 1 h in TBSTG/2% BSA (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 0.2% gelatin) and then incubated for 2 h at room temperature with primary antibody in TBSTG (wash buffer). After three washes of 5 min each in TBSTG (wash buffer) the membrane was incubated for 1 h at room temperature with horseradish peroxidase-labeled secondary antibody in TBSTG wash buffer. The membrane was washed three times for 5 min each in wash buffer and bound antibodies were detected by enhanced chemiluminescence detection system. In some experiments, color reaction generated by the action of alkaline phosphatase (conjugated to anti-mouse IgG) on the substrate BCIP/NBT was used to detect bound antibodies.

BrdU incorporation and detection. Approximately 10^4 HeLa PTP-S2 Tet-inducible cells were plated on coverslips and induced for PTP-S2 expression for 72 h. A pulse of 100 μM BrdU (in DMEM containing 10% serum) was given for 1 h, prior to fixing the cells with cold 70% ethanol for 30 min at 4°C. They were then exposed to HCl (2 N) for 30 min at room temperature to ensure DNA denaturation and neutralized by several washes with sodium tetraborate (1 mg/ml) and with PBS. For BrdU detection, cells were incubated for 2 h with anti-BrdU antibody, washed thrice with PBS, and then incubated with FITC-conjugated secondary antibody. The cell nuclei were then labeled with DAPI (1 μg/ml) for 10 min. Coverslips were mounted and observed using a fluorescence microscope.

Flow cytometric analysis. For analysis of DNA content by flow cytometry, exponentially growing cultures of cells were trypsinized uniformly, avoiding clumping, and spun down in a 15-ml centrifuge tube. They were washed once with PBS. The pellet was resuspended in 1.25 ml PBS and added dropwise to 3.75 ml cold absolute ethanol. The fixed cells were kept overnight at 4°C. Prior to analysis the fixed cells were washed twice with PBS to remove ethanol and resuspended in PBS containing 20 μg/ml propidium iodide, 50 μg/ml DNase-free RNase, and 1% Triton X-100. Cells were then incubated at 37°C for 30 min before being analyzed in a FACStar Plus flow cytometer using CELL Quest software. In the double-staining procedure for staining the cells with anti-PTP-S2 antibody, prior to propidium iodide staining, the fixed cells were washed once with PBS and permeabilized using 0.5% Triton X-100, 0.05% Tween 20 in PBS for 6 min at room temperature. The permeabilized cells were washed twice to remove detergent and blocked in PBS containing 1% BSA for 1 h at room temperature and then incubated in primary antibody overnight at 4°C. Following three washes with PBS to remove unbound primary antibody, cells were stained with FITC-conjugated secondary antibody for 1 h at room temperature. After three washes they were stained with propidium iodide as described above and analyzed by flow cytometry.

Cell synchronization. Induced and uninduced HeLa clones were synchronized at the G1/S boundary by the double thymidine block protocol. Thymidine block was initiated in cultures at a cell density that would permit active growth throughout the time course of the synchronization procedure. The first thymidine block was imposed for 14 h by removing the growth medium by aspiration and providing fresh medium containing 2 mM thymidine. Cells were released from the first block for 9 h by removing the thymidine-containing medium by aspiration and washing the monolayers thrice with an equal volume of serum-free medium (at 37°C) prior to replacement with normal growth medium. Following the 9-h release period, a second thymidine block was imposed for 14 h by adding thymidine. The cells were released from the second thymidine block, replated in normal growth medium, and fixed for flow cytometric analysis at different times after release from the block. Samples for FACS analysis were made for both induced and uninduced cells at 2-h intervals starting from the release from the second block until 22 h after release from the block.

Immunoprecipitation. Whole-cell lysates were prepared by extraction with 2× IP buffer (40 mM Tris, pH 7.4, 2% Triton X-100, 1% sodium deoxycholate, 300 mM sodium chloride, 2 mM PMSF, 2 μg/ml protease inhibitors, soyabean trypsin inhibitor, leupeptin, and aprotinin) for 15 min on ice. The cell debris was removed by spinning at 10,000 rpm for 10 min at 4°C. The supernatant was diluted 1:1 to make 1× IP buffer and incubated with the antibody for 1 h. Cdk2 antibody used was rabbit polyclonal from Santa Cruz Biotechnology (Santa Cruz, CA) and 2 μl was used for each immunoprecipitation. For PTP-S2, 30 μl of G11 antibody (hybridoma supernatant) was used. Following this 20 μl of protein A–agarose beads was added and the incubation was carried out for another 1 h. The beads with the antibody attached to them were pelleted by spinning at 5000 rpm for 3 min and washed with 1× IP buffer three to five times and processed for kinase assay or phosphatase assay.

Cdk2 activity assay. Cdk2 activity of the immunoprecipitated protein attached to the protein A–agarose beads was measured by phosphorylation of purified histone H1 (2 μg) in kinase buffer (10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2 μg/ml protease inhibitors, 0.1 mM orthovanadate, and 10 μM [γ-^{32}P]ATP in a total volume of 50 μl for 10 min at 25°C. The reaction was stopped by adding 25 μl of 3× sample buffer for SDS–PAGE and boiling for 5 min. The samples were then resolved by SDS–PAGE, transferred to Immobilon membranes, and exposed to a Fuji phosphorimager. This blot was then probed with Cdk2 antibody and Cdk2 activity (histone H1 phosphorylation) was normalized for Cdk2 protein levels.

Assay of protein tyrosine phosphatase activity. The phosphatase activity was measured essentially as described by Swarup and Subramanyam [36]. The substrate for PTP assay, ^{32}P-labeled poly(Glu^4, Tyr^1), was prepared as described by us previously [36]. PTP-S2 was immunoprecipitated from cell extracts as described above using G11 monoclonal antibody, and the PTP activity in the immunoprecipitate was determined as described [36] using labeled poly(Glu^4, Tyr^1) as substrate.

Northern blotting. Total RNA was isolated from tetracycline-controlled PTP-S2 inducible clone in HeLa cells that were induced for different times of 0, 24, 48, 72, and 96 h using Trizol reagent. RNA samples (10 μg each) were analyzed by electrophoresis through a 1% agarose/2.2 M formaldehyde gel and transferred to Hybond N$^+$ membrane for Northern hybridization as described previously [10]. Various probes were generated by PCR or RT-PCR using appropriate primers. The first-strand cDNA synthesis for amplification by RT-PCR was carried out with 2 μg total RNA, using the reagents and procedures provided with the first-strand cDNA synthesis kit from Life Technologies, Inc. The primers used for labeling rat PTP-S2 and GAPDH have been described previously [7, 10]. The other primers used for labeling the probe by PCR or RT-PCR were c-myc, human, forward primer 5′-GACCTTCATCAAAAACATCATCATC-3′, reverse primer 5′-CCTCTTTTCCACAGAAACAACATC-3′; ribonucleotide reductase, human R2 subunit, forward primer 5′CCTCTCCAAGGACATTCAGC-3′, reverse primer 5′-CCCAGTCTGCCTTCTTCTTG-3′; and cyclin E, forward primer 5′-CAGATTGCAGAGCTGTTGGA-3′, reverse primer 5′-TCCCCGTCTCCCTTATAACC-3′. After hybridization and washing, the blot was sealed in a plastic bag and exposed in a phosphorimager Fuji cassette for quantitation.

RESULTS

Establishment and Characterization of a Tetracycline-Inducible PTP-S2 Clone in HeLa Cells

To have a better understanding of the growth-promoting effects of PTP-S2 and its possible role in cell cycle progression, stable clones of PTP-S2 were made in the HeLa-Tet-Off inducible cell line, in which the expression of exogenous protein is induced by removal of tetracycline. The cell line was cotransfected with responsive plasmid encoding PTP-S2 (pTRE) and the pTK-Hyg plasmid (encoding the hygromycin resistance gene). The clones were selected with 100 μg/ml hygromycin for about 2 weeks. The stable clones so obtained were then screened for PTP-S2 expression by immunostaining after induction by removing tetracycline, using a monoclonal antibody that recognized exogenous rat PTP-S2 protein but not the human form present in HeLa cells. Two of the positive clones (C14 and C5) were analyzed for induction of PTP-S2 over a period of 24, 48, and 72 h and PTP-S2 expression was monitored by immunofluorescence (Figs. 1A and 1C). The level of expression increased significantly with time and the induced PTP-S2 protein was localized in the nucleus (Figs. 1A and 1C). Western blot analysis for PTP-S2 expression (Figs. 1B and 1D) also showed a similar time-dependent increase in PTP-S2 protein. About 50–80% of cells showed detectable PTP-S2 expression in various experiments after 72 to 96 h of induction. The PTP-S2 protein expressed upon induction was found to

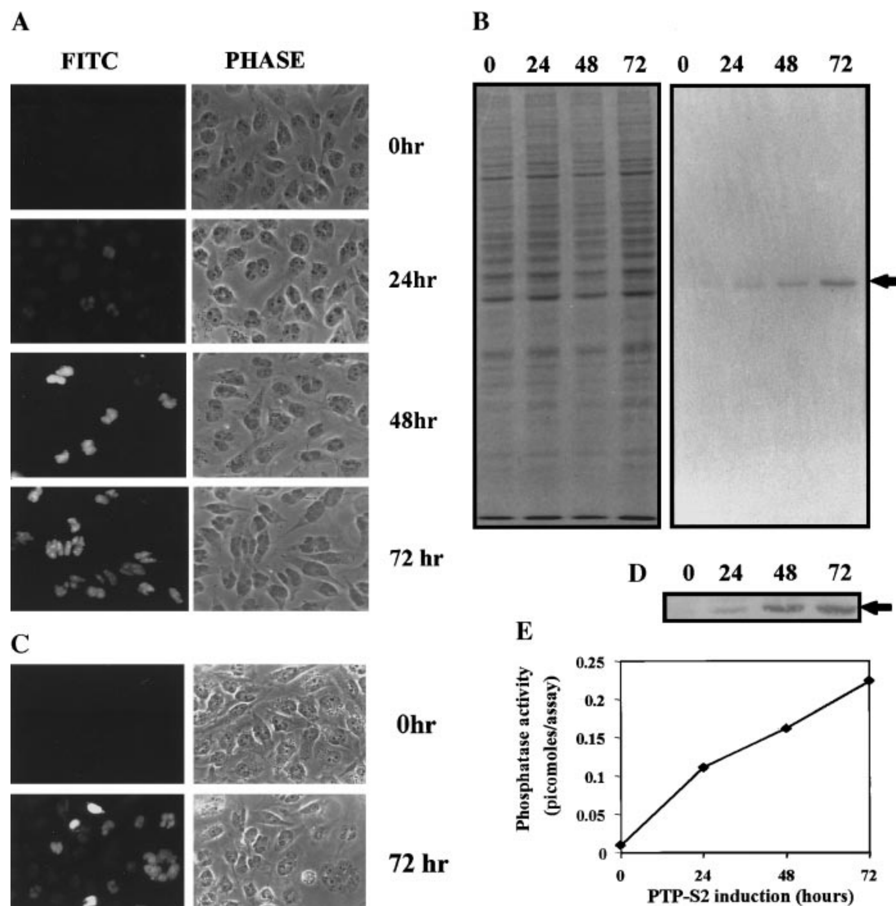


FIG. 1. Tetracycline-regulated expression of PTP-S2. (A) The Tet-off clone C14 in HeLa containing rat PTP-S2 was monitored for induction of PTP-S2 protein expression following removal of tetracycline for different periods of time. After induction the cells were fixed and stained for PTP-S2 using anti-PTP-S2 monoclonal antibody (G11). (B) Immunoblot showing expression of rat PTP-S2 in tetracycline-regulated HeLa-tTA inducible clone C14. The left shows a Coomassie blue-stained gel showing the protein profile of lysates used for the blot on the right. The arrow indicates the position of rat PTP-S2 protein overexpressed upon induction for the indicated time periods of 0, 24, 48, and 72 h. (C) Immunostaining of clone C5 after 72 h of induction. (D) Immunoblot showing PTP-S2 expression in clone C5. (E) PTP activity of PTP-S2 in clone C14 after 24–72 h of induction. PTP-S2 was immunoprecipitated from cell extracts and PTP activity of the immunoprecipitate was determined.

be enzymatically active as determined by dephosphorylation of phosphorylated poly(Glu^4, Tyr^1) by the G11 antibody immunoprecipitates (Fig. 1E). Clone C14 was used for all subsequent experiments.

Effect of PTP-S2 Expression on Cell Growth

Growth kinetics of the Tet-inducible PTP-S2 clone (C14) was measured by plating 50,000 cells in duplicate and cell numbers were counted after 24, 48, 72, and 96 h of growth and induction (Fig. 2). Following PTP-S2 induction, there was a significant increase in cell number, compared to the uninduced clone. This was in agreement with earlier data in stable clones of HeLa cells overexpressing PTP-S2 [18]. The results indicated that overexpression of PTP-S2 in HeLa increased cell proliferation.

BrdU pulse labeling of induced and uninduced cells during exponential growth also indicated that PTP-S2 overexpression increased the growth rate of cells. A comparison of the percentage of BrdU-positive cells in induced (48.7% ± 2.8) and uninduced cells (35.3% ± 2.5) showed a significantly higher value in the induced cells, suggesting that more induced cells entered S phase compared to uninduced cells within a given period of time (Fig. 3). In addition cells induced to express PTP-S2 showed brighter BrdU staining than the uninduced cells.

Effect of PTP-S2 Expression on Cell Cycle Progression

To investigate how overexpression of PTP-S2 affects cell cycle progression, we performed flow cytometric analysis on exponentially growing cell cultures. The cells were induced for PTP-S2 expression for 48 or 72 h and processed for double staining as described under

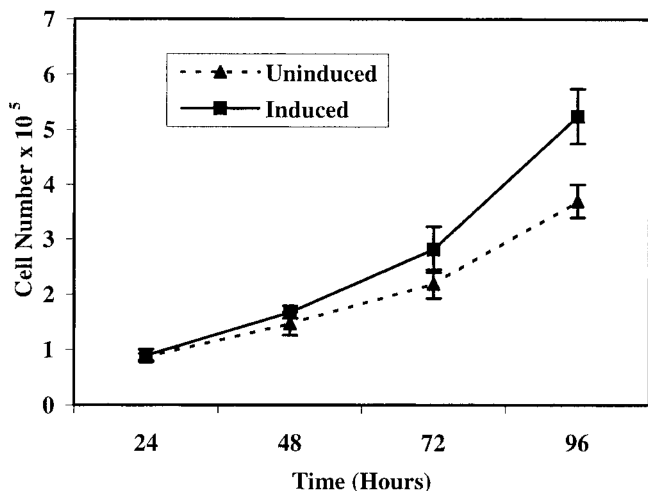
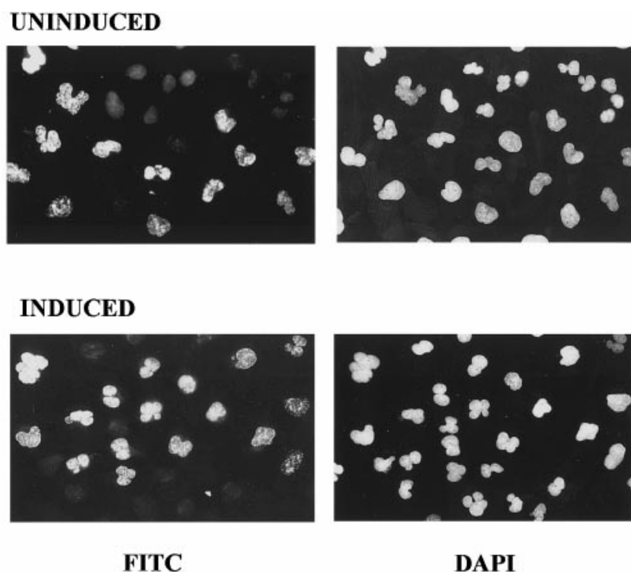


FIG. 2. Growth kinetics of tetracycline-regulated induced and uninduced PTP-S2 clone in HeLa. Details of the experiment are described under Materials and Methods. The data represent the means ± SE of three experiments done in duplicate dishes.

Materials and Methods. PTP-S2 was stained with a monoclonal antibody that recognized the exogenous rat PTP-S2 and the DNA was stained with propidium iodide. The distribution of cells in various phases of the cell cycle was analyzed in the PTP-S2-expressing and nonexpressing populations of cells (Fig. 4, Table 1). Following induction of PTP-S2 expression, there was a drastic fall in the percentage of cells in G1 phase in the expressing population compared to the nonexpressing population. This decrease in the percentage of cells in G1 is accompanied by an increase in the percentage of cells in G2/M. This suggested a faster progression of cells from G1 to S phase following induction of PTP-S2 expression. Despite a drastic fall in the percentage of cells in G1 phase in expressing cells, only a marginal increase in the percentage of S phase cells was observed. The increase in the percentage of PTP-S2-expressing cells in G2/M phase indicates that there is lengthening of the G2 phase. Similar changes in G1 and G2/M phases were observed in the cell cycle profile of expressing cells when PTP-S2 was transiently overexpressed in Cos-1 and HeLa cells (data not shown), suggesting that these effects are not cell type specific.

In order to determine the effect of increasing levels of PTP-S2 expression on cell cycle progression, the tetracycline-regulated PTP-S2 clone was induced for 72 h and cell cycle distribution was analyzed by flow cytometry after staining the cells for PTP-S2 (Figs. 5A, 5B, and 5C). The PTP-S2-expressing population was analyzed by subdividing on the basis of increasing FITC fluorescence, which represents PTP-S2 level. With increasing fluorescence intensity, the percentage of cells in G1 phase fell drastically with a corresponding increase in percentage of cells in G2/M phase. The distribution of cells in S phase did not change much except in a very small percentage of highly expressing cells in which the percentage of cells in S phase was drastically reduced (Fig. 5C).

To confirm our finding that overexpression of PTP-S2 causes shortening of the G1 phase and show conclusively the effect on the S and G2/M phases, the uninduced and induced cells expressing PTP-S2 were synchronized at G1/S phase by double thymidine block. Distribution of cells at various time points after release from the block was determined by flow cytometry as



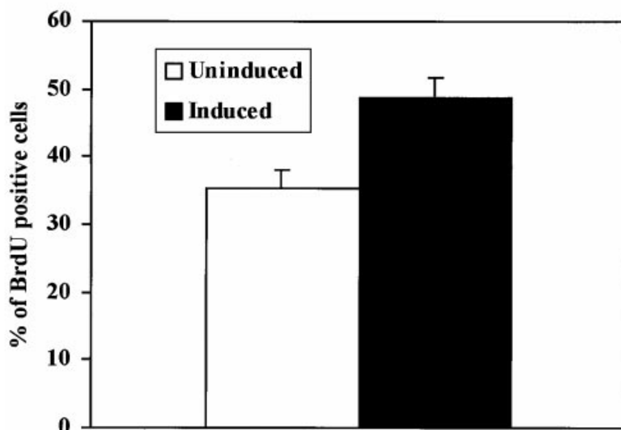


FIG. 3. Effect of PTP-S2 expression on DNA synthesis determined by BrdU staining of induced and uninduced cells. The cells were grown on coverslips and one set was induced to express PTP-S2 by removing tetracycline for 72 h. The cells were then labeled with BrdU for 1 h, followed by staining with anti-BrdU antibody (FITC), and nuclei were stained with DAPI. BrdU-labeled cells were counted. The data represent the means ± SE of three experiments done in duplicate.

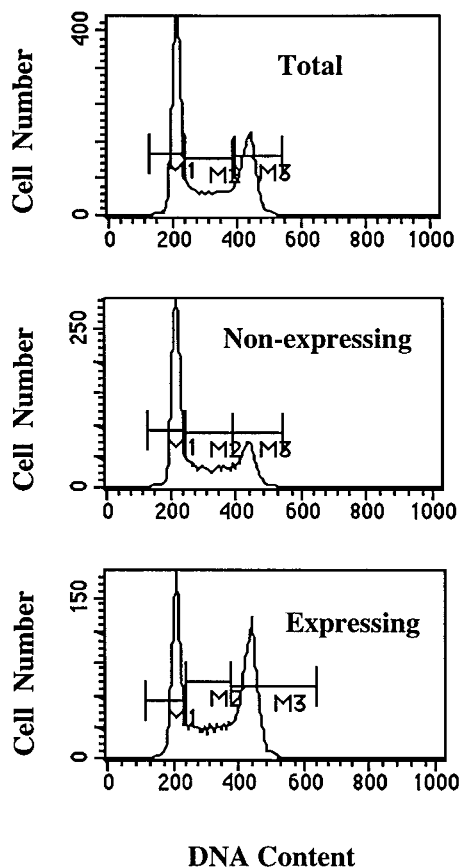


FIG. 4. Effect of PTP-S2 expression on cell cycle distribution of exponentially growing cells. Exponentially growing cells were induced for PTP-S2 expression for 48 h. Within the induced sample, the expressing population was distinguished from the nonexpressing population by staining with anti-PTP-S2 monoclonal antibody prior to staining of DNA for flow cytometric analysis. Details of the experiment are described under Materials and Methods.

described under Materials and Methods. The majority of the cells were arrested in G1 phase during double thymidine block. By 16 h after release from the block, induced cells had already started exiting out from G1 phase of the next cycle (Fig. 6). After 18 h of release from the block, 30–40% of the induced cells were in S phase of the next cycle, whereas only 10–15% of uninduced cells reached S phase. These results suggest that overexpression of PTP-S2 causes faster progression of cells from G1 to S phase, resulting in significant shortening of G1 phase by about 2–3 h in the induced population. Duration of S phase did not show any significant change. However there was a small increase in the duration of G2/M phase (Fig. 6). In these experiments the PTP-S2-expressing population also had nonexpressing cells and the two populations in the induced samples were not analyzed separately. Therefore the data in Fig. 6 represent an underestimate of the effect of PTP-S2 on cell cycle progression, since generally only about 60% of the cell population showed measurable PTP-S2 expression by fluorescence.

Mechanism of Action of PTP-S2 on Cell Cycle Progression

To understand the molecular mechanism of the effect of PTP-S2 expression on cell cycle progression, the level of some cell-cycle-regulatory proteins was analyzed by Western blotting. This analysis was performed using total proteins isolated from an exponentially growing population of cells that had been induced for PTP-S2 expression for 24, 48, and 72 h. There was an increase in the level of c-Myc protein in the induced cells in comparison to the uninduced cells (Fig. 7). No significant change was detected in the expression of another cell cycle protein, proliferating cell nuclear antigen (data not shown). There was a small increase in Cdk2 protein level (Fig. 7). The increase in c-Myc protein expression correlated with the increase in PTP-S2 protein level in different experiments. To further investigate whether this change in c-Myc protein levels was associated with changes at the mRNA levels, we carried out Northern blot analysis with RNA from exponentially growing population of cells that had been induced for 0, 24, 48, 72, and 96 h. The results as shown in Fig. 8 indicated that there was no significant change in the mRNA levels of c-Myc upon induction of PTP-S2 expression. This suggested that overexpression of PTP-S2 affects the expression of c-Myc post-transcriptionally and not transcriptionally. We did not detect any significant change in the mRNA levels of another cell-cycle-regulated gene, ribonucleotide reductase (R2 subunit) (Fig. 8).

Since the c-Myc protein level was found to increase upon induced expression of PTP-S2, it was possible that transcriptional regulation of target genes by c-Myc may contribute to the observed effect of PTP-S2 on cell cycle progression. We analyzed the level of expression of cdc 25A, cyclin D1, cyclin E, and cyclin A upon induction of PTP-S2 expression. There was no significant change in the levels of cdc 25A, cyclin D1, or cyclin A mRNA as determined by RT-PCR (data not shown). However, some increase in the cyclin E mRNA level

TABLE 1

Effect of PTP-S2 Expression on Cell Cycle Distribution in Exponentially Growing Cells

	Nonexpressing cells	PTP-S2-expressing cells
G1	55.85 ± 2.83	44.17 ± 4.46
S	26.05 ± 2.97	28.23 ± 3.44
G2/M	18.57 ± 1.70	28.00 ± 6.75

Note. The data represent the means ± SE from 10 independent experiments carried out as for Fig. 4.

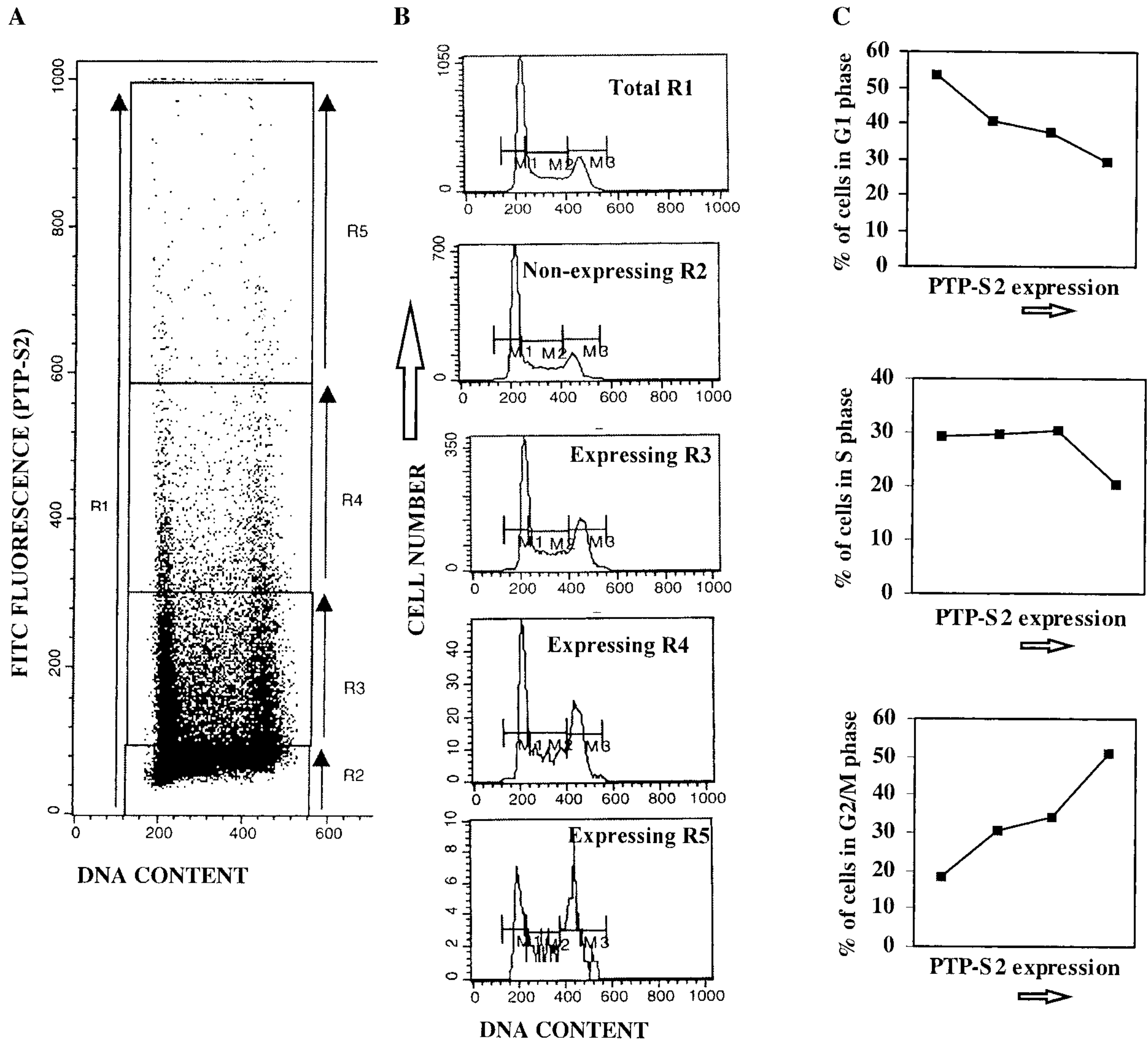


FIG. 5. Effect of increasing levels of PTP-S2 expression on cell cycle distribution. (A) The profile of distribution of cells with increasing fluorescence intensity is plotted as DNA content versus intensity of FITC fluorescence (representing level of PTP-S2 expression). (B) Cell cycle distribution of various populations of induced cells. R1 represents the entire population of cells, both expressing and nonexpressing, within the same induced sample. R2 represents the nonexpressing population, while R3, R4, and R5 represent populations of cells with increasing fluorescence intensity. (C) Percentage of cells in G1, S, and G2/M phases plotted against level of PTP-S2 expression.

was observed (Fig. 8). The activity of cyclin-dependent kinase Cdk2 is one of the major regulators of entry of cells into S phase. Therefore we analyzed the effect of PTP-S2 expression upon Cdk2 activity. We found that there was a 58% increase in Cdk2 activity, upon induction of PTP-S2 expression for 72 h in asynchronous cells (Fig. 9), as measured by phosphorylation of histone H1 with Cdk2 immunoprecipitate. The activity of Cdk2 was normalized for the amount of Cdk2 protein in the immunoprecipitate by Western blotting.

DISCUSSION

In this study we have shown that overexpression of the nuclear tyrosine phosphatase PTP-S2 in HeLa cells, using a regulated expression system, results in increased rate of cell proliferation. The rate of DNA synthesis also increased as measured by BrdU incorporation. This increase in cell proliferation upon PTP-S2 expression was due to shortening of the duration of G1 phase. The results indicate that the duration of S phase did not change significantly, whereas the duration of G2 phase increased to some extent. The decrease in the percentage of cells in the G1-phase population was dependent on the level of PTP-S2 expression (Fig. 4). This shows that the observed effects on cell cycle distribution are not due to removal of tetracycline itself but are due to PTP-S2 expression. Overall our results suggest that PTP-S2 acts in the G1

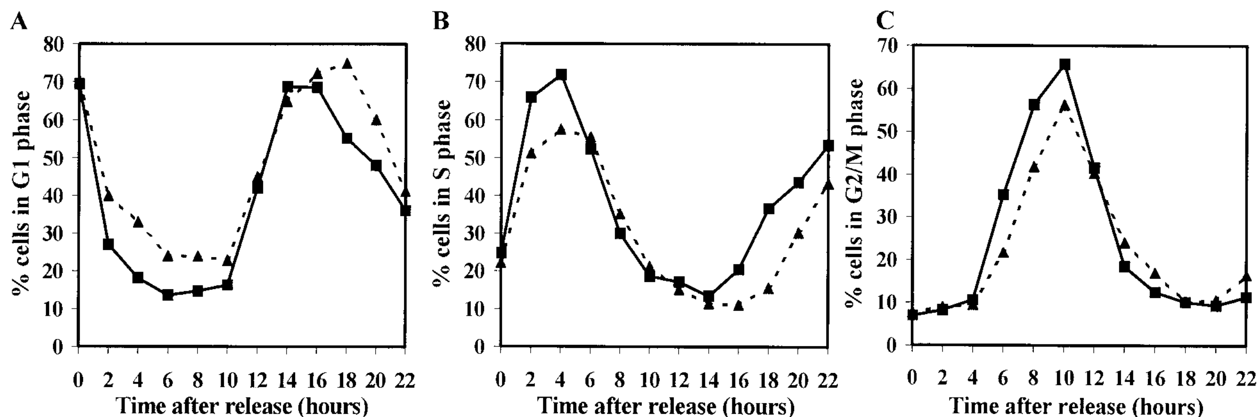


FIG. 6. Effect of PTP-S2 expression on cell cycle progression after release from a double thymidine block. (A, B, and C) The percentages of cells in G1, S, and G2/M phases, respectively, at various time points in induced and uninduced cells after release from a double thymidine block. Cell synchronization and flow cytometric analyses were performed as described under Materials and Methods. Broken and solid lines show data from uninduced and induced samples, respectively. These data show an average of two experiments.

phase of cell division cycle. This is in agreement with the observations showing that mitogenic stimulation of a variety of cells results in transient increase in expression of PTP-S2 mRNA in G1 phase [7, 12, 14, 15]. Thus PTP-S2 positively regulates a step in the G1 phase of cell cycle which allows the cells to progress faster through G1 phase.

Several proteins are known to regulate progression of cells through G1 phase and increase the rate of DNA replication. In our attempt to identify proteins (regulated directly or indirectly by PTP-S2) which could account for the observed effects of PTP-S2 on cell cycle progression, we found that the level of c-Myc protein was increased by expression of this phosphatase. The regulation of c-Myc is quite complex and occurs at multiple levels including control of transcription, stability of both mRNA and protein, and control of translation [32, 37]. Although PTP-S2 expression increased the level of c-Myc protein, the level of its mRNA did not show any change. This suggests that a posttranscriptional mechanism is involved in the regulation of c-Myc protein level by PTP-S2.

Overexpression of c-Myc is known to decrease the duration of G1 phase without affecting G2 phase, whereas S phase is slightly shortened or unaffected [31]. Since the effect of PTP-S2 on cell cycle progression (on G1 and S phase) is very similar to that of c-Myc, it is likely that c-Myc mediates at least in part the observed effects of PTP-S2 on cell cycle progression. Another property shared by PTP-S2 and c-Myc is that both these proteins induce p53-dependent apoptosis upon overexpression at high level [23, 38]. However, c-Myc-induced apoptosis requires serum starvation, whereas PTP-S2 induces apoptosis even in the presence of serum. Thus it appears that although increased level of c-Myc may contribute to PTP-S2-induced apoptosis in p53-positive cells, it is not sufficient to explain serum starvation-independent apop-

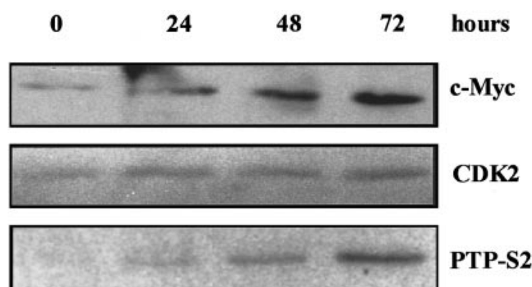


FIG. 7. The increase in the level of c-Myc protein upon overexpression of PTP-S2. Total proteins were isolated from uninduced (0 h) and induced (24, 48, and 72 h) cells. The control sample was from cells grown for 72 h without induction (0-h induction). Western blotting was performed as described under Materials and Methods using antibodies against c-Myc, Cdk2, and PTP-S2.

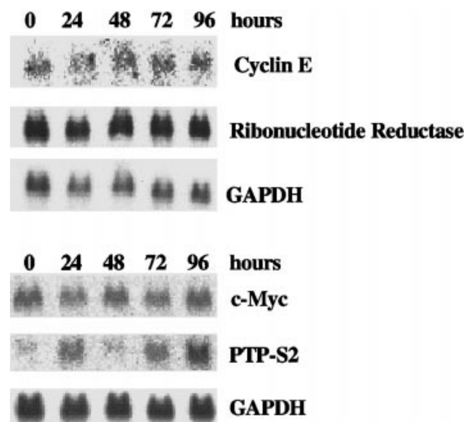


FIG. 8. c-Myc mRNA level is not affected by PTP-S2 expression. Total RNA was isolated from uninduced (0 h) and induced (24, 48, 72, and 96 h) cells. Northern blotting was performed using probes for cyclin E, c-Myc, PTP-S2, ribonucleotide reductase, and GAPDH.

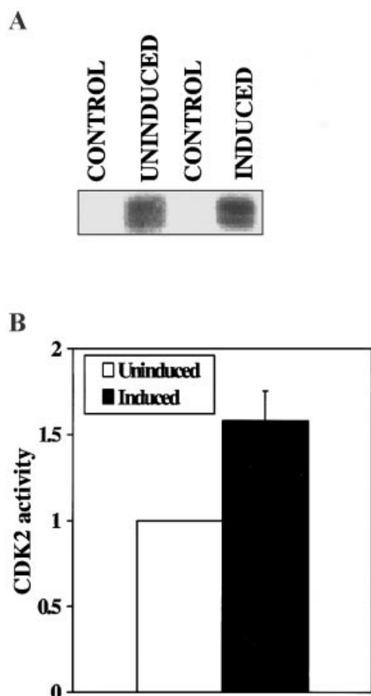


FIG. 9. Cdk2 activity is increased by PTP-S2 expression. (A) Cells were induced for PTP-S2 expression for 72 h and Cdk2 activity was determined in the anti-Cdk2 and control immunoprecipitates by histone H1 phosphorylation. Autoradiograph shows histone bands. (B) Quantitation of Cdk2 activity from three experiments is shown relative to uninduced samples, after normalization for Cdk2 protein levels determined by immunoblotting.

tosis induced by PTP-S2. Therefore other proteins, whose activity is affected by PTP-S2, are likely to contribute to PTP-S2-induced apoptosis.

The biochemical steps involved in the progression of cells through G1 phase are not completely known. The progression of cells from G1 to S phase is regulated by retinoblastoma protein (pRb), which in its active, less phosphorylated state inhibits E2F transcription factors [39]. Phosphorylation of pRb by cyclin E/Cdk2 results in inactivation of pRb, leading to activation of E2F transcription factors, which allows transcription of certain genes whose function is required for DNA synthesis [40]. In HeLa cells retinoblastoma protein is functionally inactive and therefore increased progression of HeLa cells from G1 to S by PTP-S2 is not likely to be mediated by the pRb/E2F pathway. In Rb-negative cells, ectopic expression of G1 CDKs has been shown to accelerate S-phase entry [41]. It has been suggested that c-Myc controls a G1/S-promoting pathway which is parallel to the classical pRb/E2F pathway [42]. This Myc-regulated pathway functions through cyclin E/Cdk2 but does not require pRb or E2F. Two members of the cdc 25 family tyrosine phosphatases—cdc 25A and 25B—are direct transcriptional targets of c-Myc in murine fibroblast cell lines [43]. The Rb/E2F-independent alternate pathway mediated by c-Myc leading to S-phase entry of cells has been shown to require cyclin E and cdc 25 activation, and ecotopic expression of cdc 25A accelerates G1/S transition by activation of Cdk2 [44]. Though we have not found any changes in cdc 25A mRNA levels upon PTP-S2 expression, Cdk2 activity was enhanced, suggesting that PTP-S2 expression promotes G1 to S progression through a Myc-controlled Cdk2-dependent pathway.

c-Myc is known to be activated in response to stimulation of growth factor receptors, and induction of c-Myc appears to be dependent on the Src family tyrosine kinases [45]. The low-molecular-weight PTP, which decreases Src kinase activity in PDGF-stimulated cells, was shown to downregulate c-Myc mRNA expression [46]. Therefore PTPs appear to act positively (this report) as well as negatively [46] in regulating c-Myc protein or mRNA level. For a complete understanding of the role of c-Myc in cell proliferation (and other cellular functions) the molecular mechanism underlying regulation of c-Myc protein level by PTP-S2 needs to be elucidated.

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