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Enhanced expression of a chromatin associated protein tyrosine phosphatase during G_0 to S transition

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Abstract. The non-transmembrane protein tyrosine phosphatase, PTP-S, is located predominantly in the cell nucleus in association with chromatin. Here we have analysed the expression of PTP-S upon mitogenic stimulation and during cell division cycle. During liver regeneration after partial hepatectomy, PTP–S mRNA levels increased 16-fold after 6 h (G_1 phase) and declined thereafter. Upon stimulation of serum starved cells in culture with serum, PTP-S mRNA levels increased reaching a maximum during late G_1 phase and declined thereafter. No significant change in PTP-S RNA levels was observed in growing cells during cell cycle. PTP$^-$S protein levels were also found to increase upon mitogenic stimulation. Upon serum starvation for 72 h, PTP–S protein disappears from the nucleus and is seen in the cytoplasm; after 96 h of serum starvation the PTP-S protein disappears from the nucleus as well as cytoplasm. Refeeding of starved cells for 6 h results in reappearance of this protein in the nucleus. Our results suggest a role of this phosphatase during cell proliferation.

Keywords. Protein tyrosine phosphatase; nuclear phosphatase; liver regeneration; cell cycle; cell proliferation.

1. Introduction

Protein tyrosine kinases as well as protein tyrosine phosphatases (PTPases) play an important role in the signal transduction processes by regulating the phosphorylation state of substrate proteins. The importance of PTP ases in regulating various cellular functions has been brought out by various studies relatively recently (Fisher *et al* 1991; Saito and Streuli 1991; Swarup and Radha 1992; Brautigan 1992). Two types of PTP ases have been identified in various cells. The receptor type PTPases such as CD45 are transmembrane proteins. The non-transmembrane PTPases differ from one another in sequences found at the N-terminal and/or C-terminal ends of the catalytic domain. Specific roles of individual intracellular PTPases are, in general, yet to be identified.

A non-transmembrane PTPase cDNA (known as T-cell PTPase) was isolated by Cool *et al* (1989) from a human T-cell cDNA library, which codes for a polypeptide of 415 amino acids showing 65% homology with PTP-1B. Subsequently two different forms of this PTPase, which probably arise due to differential splicing, were isolated from rat, human and mouse tissues (Swarup *et al* 1991; Champion-Arnaud *et al*

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1991; Mosinger *et al* 1992). One of these forms (PTP-S) was isolated in our laboratory from rat spleen cDNA library (Swamp *et al* 1991). The important feature of PTP-S is the presence of a region rich in basic amino acids in the non-catalytic C-terminal domain which is highly conserved in various forms isolated from human and mouse. When expressed in *Escherichia coli* the PTP-S protein was enzymatically active and bound to DNA through its C-terminal non-catalytic domain (Radha *et al* 1993). The cellular PTP-S gene products were identified by using specific monoclonal and polyclonal antibodies as 42–44 kDa polypeptides; these are located predominantly in the nucleus in fibroblasts in association with chromatin and to a lesser extent with nuclear matrix (Radha *et al* 1994). The cellular PTP-S protein is capable of binding to DNA *in vitro* (Radha *et al* 1993). Mitogenic activation of T-lymphocytes by Concanavalin A results in an increase in PTP-S mRNA levels after 48–72 h due to increase in half life of mRNA (Rajendrakumar *et al* 1993). The relationship of this increase in PTP-S levels in T-cells with cell cycle progression has not been analysed.

Here we have analysed the expression of PTP-S upon mitogenic stimulation and during cell division cycle using rat fibroblast cell line F 111, human cell line HeLa and regenerating rat liver as experimental model systems.

2. Materials and methods

2.1 *Cell culture and synchronization of cells*

The various cell lines used in this study are Rat2, F111 (rat fibroblast cell lines), NIH 3T3 (mouse fibroblast cell line), J774 (mouse macrophage cell line), PA1 (human ovarian teratocarcinoma cell line), MDCK (dog kidney epithelial cell line), HeLa (human cervical epithelial cell line) and KBP (human epidermoid carcinoma cell line). All the cells were grown as monolayers in DMEM supplemented with 10% fetal calf serum and antibiotics at 37°C in a humidified 5% CO_2 atmosphere. Cells were washed with phosphate buffered saline (PBS) and harvested for total RNA preparation or lysed in SDS sample buffer for Western blotting.

Cells were arrested in G_0 phase by maintaining in 0·05% serum containing medium for 48–96 h. Cells were released from serum starvation by addition of 10% serum containing DMEM and samples were taken at various times as indicated. Cells were treated with 3 mM hydroxyurea (Sigma) for 16 h or 1 μg/ml nocodazole (Aldrich) for 16 h to arrest them at G_1-S phase or mitosis, respectively. The hydroxyurea block was released by washing the monolayer with PBS thrice and with medium once and then growing with fresh medium containing 10% serum. The nocodazole treated cells which were arrested in mitosis were removed by shaking the flask, washed twice with PBS and with medium and plated freshly in 10% FCS containing DMEM.

2.2 *Liver regeneration*

Adult male Wistar rats weighing approximately 200 g were subjected to partial (70%) hepatectomy (PHT) as described (Grisham 1962; Alison 1986). Experimental as well as control animals were provided with normal diet continuously. At different

times after PHT ranging from 3–72 h animals were sacrificed, their livers were removed, frozen in liquid nitrogen after mincing quickly and stored at -70°C until used for RNA isolation. Total RNA was isolated from these samples essentially as described (Chomczynski and Sacchi 1987).

2.3 *Anti-PTP-S monoclonal antibody and immunoblotting*

A monoclonal antibody which specifically recognizes polypeptides coded by PTP-S has been described by us previously (Radha *et al* 1994). Immunoblotting using this or PCNA antibody was carried out as described (Radha *et al* 1994).

2.4 *Northern blotting*

RNA isolation was carried out as described (Chomczynski and Sacchi 1987). For Northern blotting l0 μg of total RNA was fractionated on formaldehyde agarose gel, transferred onto positively charged nylon membrane (Hybond N^+) with 50 mM sodium hydroxide under vacuum. Ethidium bromide (0·5 μg) was added to the samples to help in visualization of RNA on the gel. Hybridization was carried out at 65°C for 16–20h in a solution containing sodium phosphate (0·5 M with respect to sodium) pH 7·2 and sodium dodecyl sulphate (7%). Filters were washed at 65°C as described previously (Rajendrakumar *et al* 1993) and kept for autoradiography.

2.5 *Indirect immunofluorescence*

F111 cells were grown on coverslips, fixed with formaldehyde and indirect immunofluorescence was carried out using anti-PTP-S monoclonal antibodies as described previously (Radha *et al* 1994). Serum starvation and refeeding of cells grown on coverslips was carried out as for cells in the flasks.

3. Results

For initial experiments we had used 3 different cell lines. In all cases growing cells showed higher PTP-S expression than growth arrested cells. For PTP-S protein work only F111 cells were used since the antibody recognizes rat PTP-S and not human protein. Use of different cell lines gives an idea about how generalized is the increase in PTP-S expression in growing cells.

3.1 *Expression of PTP-S in various cell lines*

PTP-S was cloned from a rat spleen cDNA library and is known to express in rat spleen, brain, lymphocytes and fibroblasts. We analysed the expression of PTP-S in various cell lines like HeLa, NIH 3T3, PA1, J774 and MDCK by Northern blotting with PTP-S cDNA probe. All the cell lines showed a 1·7 kb transcript (figure 1A) corresponding to PTP-S mRNA as reported previously (Swarup *et al* 1991). The levels of PTP-S mRNA was lower in serum starved or confluent Rat2 cells as compared to growing cells (figure 1B, C).

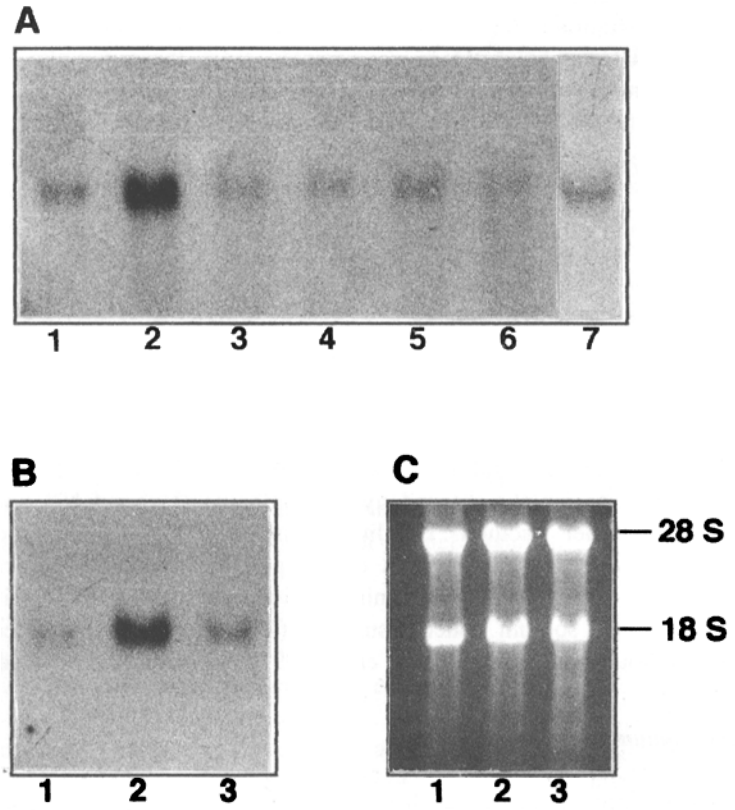

Figure 1. Expression of PTP-S in various cell lines. Total RNA was fractionated on formaldehyde agarose gel transferred onto nylon membrane and hybridized with PTP-S cDNA probe. **(A)** Various cell lines analysed were: lane 1–KBP, lane 2–MDCK, lane 3 – PA1, lane 4 – F 111, lane 5 – Rat 2, lane 6 – NIH 3T3 and lane 7– J774. We have no explanation as to why PTP-S expression is high in MDCK cells. **(B)** Lane 1 – serum starved Rat2 cells, lane 2 – growing Rat2 cells and lane 3 – confluent Rat2 cells. **(C)** Ethidium bromide stained gel of RNA samples used in **(B)** for Northern blotting.

Since expression of PTP-S mRNA appeared to be related to growth of fibroblasts, we examined in detail its expression during cell division cycle and upon mitogenic stimulation of resting cells using *in vitro* and *in vivo* experimental systems.

3.2 *Expression of PTP-S during liver regeneration after partial hepatectomy*

Regeneration of rat liver after partial (70%) hepatectomy provides an experimental model system for studying transition of cells from resting state (G_0 phase) to replicative state *in vivo.* After partial hepatectomy different polypeptides generate sequential signals which lead the cells to initiate DNA synthesis and subsequently to mitosis (Alison 1986; Michalopoulos 1990). In order to analyse the level of PTP-S expression, total RNA was isolated from control, Sham operated and regenerating rat livers after 70% PHT at various times. Northern blot analysis of

these RNA samples showed that PTP-S mRNA levels increased after partial hepatectomy and maximum levels were observed after 6 h (figure 2A). After reaching maximal levels at 6 h PTP-S mRNA level declined gradually and reached almost control levels by 72 h. PTP-1 mRNA levels showed a small increase over the control levels. Sham operated control (6 h) showed no increase in PTP-S mRNA which shows that elevated PTP-S mRNA level after PHT was not because of surgical stress. Quantiation of autoradiograms by densitometric scanning showed that the level of PTP-S mRNA increased 16 ± 3·6 (n = 4)– fold after 6 h of PHT. The level of histone H4 mRNA showed an increase after 18 h and reached a maximum after 24 h (figure 2). Histone H4 synthesis occurs mainly in S phase and therefore we have used it as a marker for S phase. DNA synthesis during rat liver regeneration starts after 13–14 h and reaches a peak within 22–24 h after partial hepatectomy (Grisham 1962; Michalopoulos 1990). This suggests that PTP-S mRNA levels reach maximum levels during G_1 phase of cell cycle after partial hepatectomy.

3.3 *Changes in PTP-S mRNA levels upon stimulation of serum starved, G_0 arrested cells in culture by serum*

We compared the level of PTP-S mRNA in growing and serum starved rat fibroblasts Fl11 and Rat2, and also in HeLa cells by Northern blotting. Growing Rat2 cells

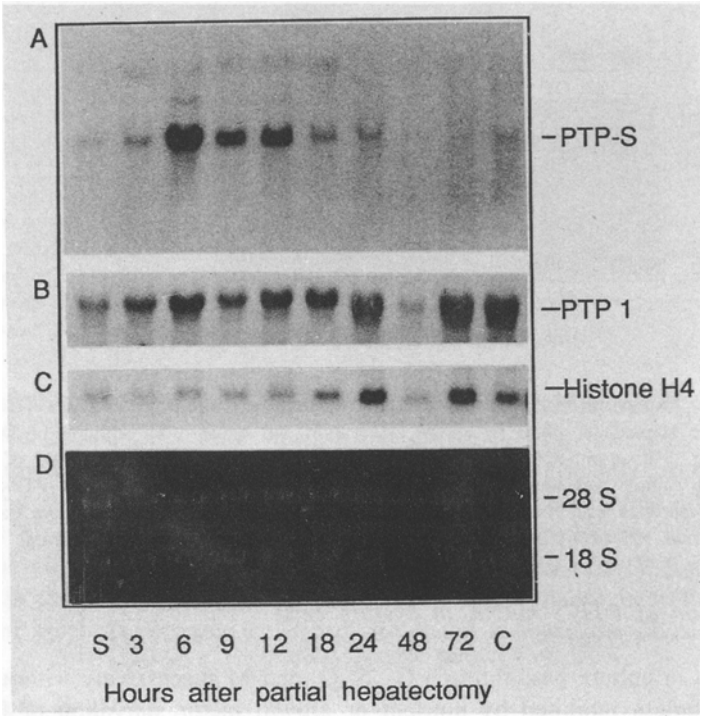

Figure 2. Expression of PTP-S, PTPI and histone H4 mRNA during liver regeneration in adult rats. Total RNA prepared from livers at various time points after PHT was separated by gel electrophoresis, transferred to a nylon membrane and hybridized with probes for PTP-S **(A),** PTP1 **(B)** and histone H4 **(C). (D)** Ethidium bromide stained gel before transfer to nylon membrane. The position of ribosomal RNA bands 28S and 18S is indicated. S, Sham operation; C, normal liver control.

showed 3–4-fold higher level of PTP-S mRNA as compared with the cells serum starved for 48 h (figure 1B,C). Similar result was obtained with F111 and HeLa cells (data not shown). We next examined the time course of expression of PTP-S after addition of serum to serum starved HeLa cells. RNA was isolated after 3, 6, 8, 10, 12, 15 and 18 h after serum addition. Northern blot analysis of these RNA samples showed that the level of PTP-S transcripts increased after 3–6 h of addition of serum reaching a maximum (4-fold higher than control) after 8 h (figure 3). The same blot was also hybridized with histone H4 cDNA probe which showed that histone H4 mRNA levels reach maximum levels after 10 h of stimulation of these cells by serum. These observations suggest that PTP-S mRNA levels reach the peak level in late G_1 phase and decrease thereafter as the cells enter the S phase.

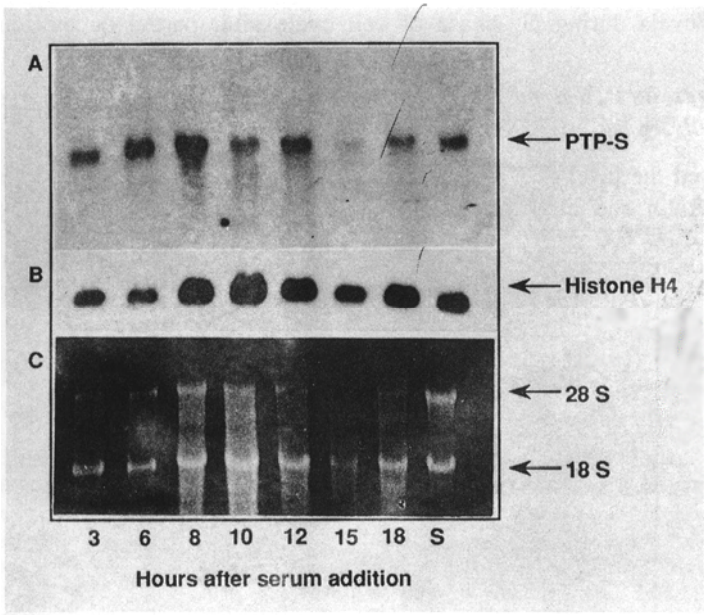

Figure 3. Expression of PTP-S after addition of serum to serum starved HeLa cells. HeLa cells were starved for 48 h by serum deprivation and released by addition of DMEM containing 10% serum. RNA was isolated at the indicated time points and 10 μg of RNA from each sample was used for Northern blotting. The blot was probed-with PTP-S **(A)** and later reprobed with Histone H4 (**B**). (**C**) Ethidium bromide stained gel before transfer. The 28S and 18S rRNA positions are indicated by arrowheads. S, Serum starved.

3.4 *Expression of PTP-S mRNA in cycling cells*

Growing cells in culture pass through G_1 S, G_2 and M successively without entering the G_0 state unless inhibited by nutrient or growth factor starvation or by density inhibition (Pardee 1989; Muller *et al* 1993). It has been recently proposed that several genes which are induced by mitogenic stimulation of quiescent cells in culture, do not show the same alterations during normal cell cycle progression (Wick *et al* 1994; Burger *et al* 1994). Therefore we examined the levels of PTP-S mRNA in cycling HeLa cells by Northern blotting using two different methods of

cell synchronization. Treatment of cells with nocodazole arrests the cells in mitosis. The mitotic cells were washed to remove nocodazole and replated in medium containing 10% serum. RNA was isolated after various intervals for Northern blotting. PTP-S mRNA levels did not show any significant change after release from mitosis as shown in figure 4. The cells were entering S phase after 8–10 h of release from mitosis as shown by histone H4 expression (figure 4).

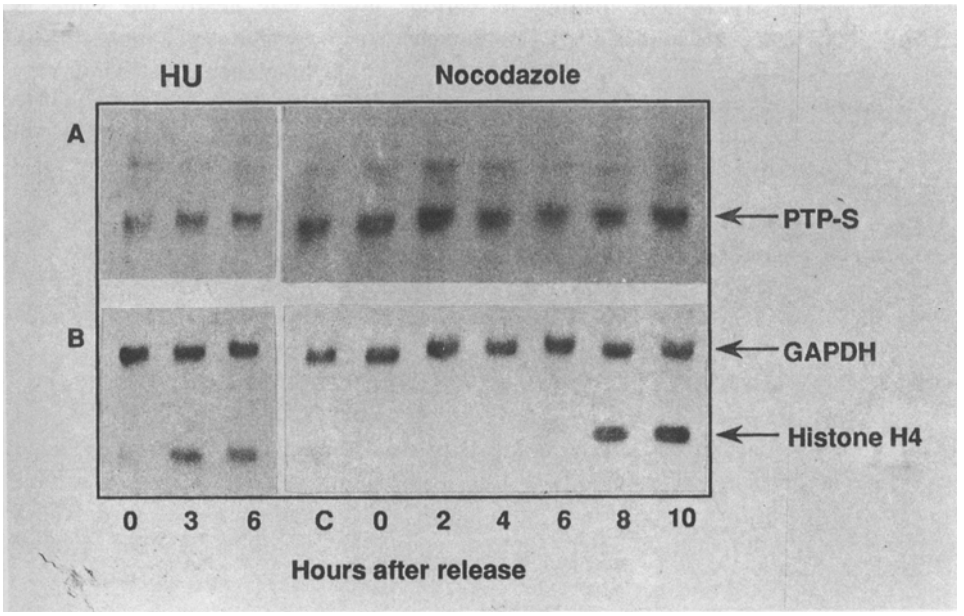

Figure 4. Expression of PTP-S in growing HeLa cells. HeLa cells were arrested at G_1-S using hydroxyurea (HU) or with nocodazole in metaphase. They were released from arrest by washing with PBS and medium and then grown in fresh medium. RNA was isolated at various time points and analysed by Northern blotting using PTP-S cDNA probe (**A**). The same blot was reprobed with GAPDH cDNA and histone H4 (**B**).

Treatment with hydroxyurea blocks the growing cells at G_1-S boundary. Hydroxyurea treated cells were washed and grown in fresh medium. As shown in figure 4, the PTP-S mRNA levels do not change significantly at G_1-S or after release from this block. Histone H4 mRNA levels increased after release of cells from G_1-S block, as expected. Although from these experiments it is difficult to rule out the possibility of some small changes in the level of PTP-S mRNA during cell division cycle in growing cells, there was no large change in PTP-S expression as observed when G_0 arrested cells are stimulated to enter the division cycle.

3.5 *Changes in PTP-S protein levels*

We analysed the levels of PTP-S protein during cell cycle in growing cells. For this F1ll cells were arrested at G_1-S boundary using hydroxyurea or at mitosis using nocodazole. The cells were washed and released for 3 and 6 h in culture and Western blotting was done using the monoclonal antibody against PTP-S (Radha

et al 1994). There was no significant change in the levels of PTP-S gene product in the arrested cells nor in the cells released after the arrest. This monoclonal antibody recognizes rat PTP-S protein but not the human protein (data not shown).

When F1ll cells were serum starved for 96 h, the level of PTP-S protein diminished as determined by Western blotting. These cells were stimulated with 10% serum containing medium and samples were made at various time points from 6 h to 24 h. The level of PTP-S went up by 6 h and remained more or less constant thereafter (figure 5). Protein loading in various levels was nearly the same as determined by staining samples with Coomassie blue in a parallel gel (not shown). PTP-S polypeptides of 42–44 kDa, identified by using a monoclonal antibody, may represent two splice variants, or a post-translational modification may give rise to these forms. The same blots were probed with antibody against proliferating cell nuclear antigen (PCNA). PCNA is known not to be expressed in resting cells and its expression starts in G_1 and peaks at S phase. As expected PCNA was hardly detectable in the starved cells while by 9 h it was very high. In our experiments the level PTP-S protein was found to decrease significantly upon serum starvation only after 72–96 h, while its mRNA level went down after 24–48 h.

3.6 *Localization of PTP-S in serum starved cells*

The level of PTP-S protein does not go down up to 72 h in G_0 arrested F111 fibroblast cells. However we wanted to see if there are any changes in its subcellular localization upon serum starvation. For this indirect immunofluorescence was done using F111 cells grown on coverslips with anti PTP-S monoclonal antibodies. PTP-S was localized in the nucleus up to 48 h of starvation; however by 72 h most of

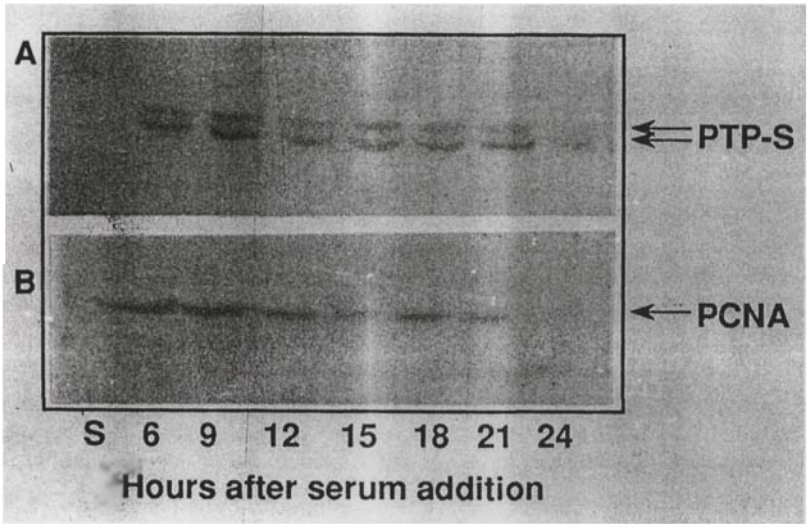

Figure 5. Levels of PTP-S protein in scrum starved and growing F 1ll rat fibroblasts. F111 cells were growth arrested by scrum deprivation for 96 h and then released by addition of DMEM containing 10% fetal calf serum. Cells were collected at the indicated time points and cell lysates were analysed by Western blotting using anti-PTP-S monoclonal antibody. **(A)** Shows the PTP-S levels at indicated time points. The same blot was reprobed with PCNA antibody (**B).** S, Serum starved cells.

the protein was present in the cytoplasm and by 96 h the protein had more or less disappeared (figure 6). Upon refeeding the starved cells with serum containing medium, within 6 h, the protein was present in the nucleus and the protein level was like in growing cells.

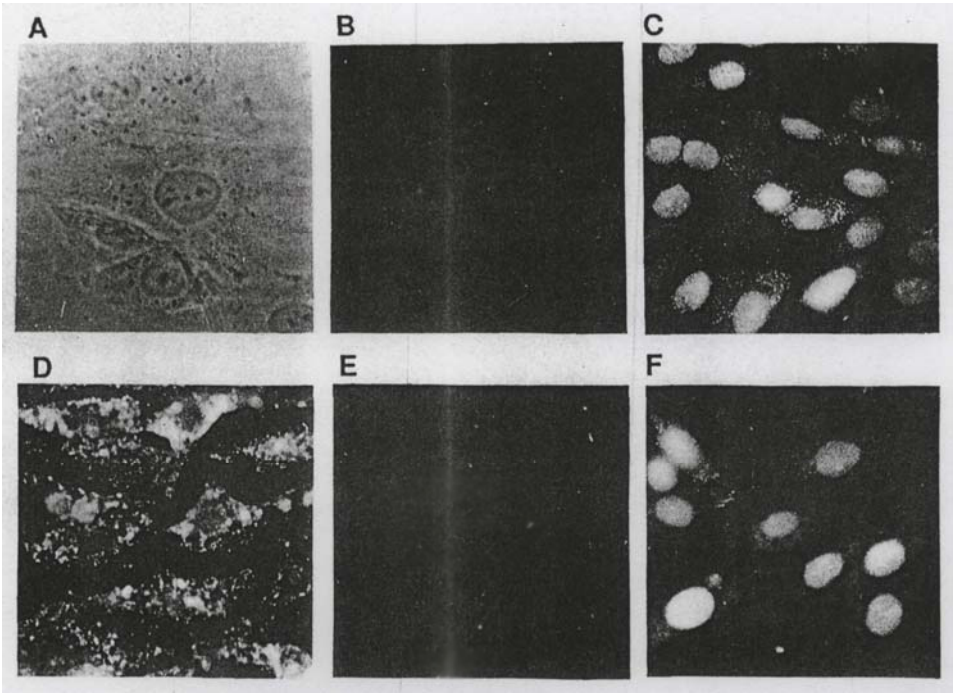

Figure 6. Localization of P'TP-S in serum starved and re fed F111 cells. F111 cells were grown on coverslips and serum starved up to 96 h. Indirect immunofluorescence was carried out using anti PTP-S monoclonal antibody. (**A**) Phase contrast micrograph of cells in (**B**); (**B**), cells treated in the absence of primary antibody; (**C**), growing F111 cells; (**D**), cells starved for 72 h; (**E**), cells starved for 96 h and (**F**), 6 h after addition of serum to starved cells. Cells were photographed at × 400. (**C**) to (**F**) are samples stained with anti-PTP-S antibody.

4. Discussion

The results presented here show that PTP-S expression is ubiquitous and the level of PTP-S mRNA is low in growth arrested (G_0 state) cells. Upon mitogenic stimulation as the cells enter G_1 phase, the level of PTP-S mRNA increases several fold reaching a maximum in G_1 phase or just before the entry of cells in S phase. As the cells enter S-phase, the level of PTP-S mRNA declines. In normally growing cells in culture there was little change in the level of PTP-S mRNA at various stages of cell cycle. Therefore PTP-S is one of those genes which are induced by mitogens but do not show significant changes once the cells have entered the division cycle (Muller *et al* 1993; Burger *et al* 1994; Wick *et al* 1994). Previously we have shown that upon mitogenic stimulation of T-cells PTP-S mRNA and

protein levels increase 3–4-fold (Rajendrakumar *et al* 1993). This increase occurs just before the entry of cells in S-phase (G V Rajendrakumar and G Swarup, unpublished observations). All these results show that PTP-S mRNA levels increase during G_0 to S transition *in vitro* as well as *in vivo*.

Recently, it has been reported that the level of MPTP (mouse homologue of PTP-S) RNA increases during G_1 phase of cell cycle, after addition of serum to serum starved NIH 3T3 cells (Tillmann *et al* 1994). When cells were induced after hydroxyurea or nocodazole arrest, then also an increase in MPTP RNA was observed in G_1 phase. Our results reported in this paper differ from those of Tillmann *et al* (1994) with respect to the expression of this PTPase mRNA in growing cells. One possible explanation for this could be that different cells were used in these studies.

Earlier studies on NIH 3T3 cells have shown that many of the nuclear proteins, localize differently in a proliferation dependent manner. Analysis of PCNA, c-Fos, c-Myc and DNA polymerase alpha shows that in growth arrested cells they leave the nucleus and enter the cytoplasm (Vriz *et al* 1992). DNA polymerase alpha is the first to disappear from the nucleus by about 8 h while PCNA, c-Myc and c-Fos levels in the cytoplasm increase by about 24 h and by 48 h these proteins have completely disappeared from the whole cell. However upon stimulation c-Fos and c-Myc are the earliest to localize to the nucleus by 6 h (Vriz *et al* 1992). All these proteins are known to have role in mitogenic signalling or cell proliferation. PTP-S too shows similar behaviour in that it can be seen outside the nucleus by 72 h after starvation and by 96 h it disappears completely. Again upon serum stimulation it is seen in the nucleus within 6 h.

The possible role of human T-cell phosphatase in cell division has been analysed previously (Cool *et al* 1990, 1992; Gould *et al* 1990). Over expression of full length T-cell PTPase in BHK cells had no effect on cell morphology or cell division (Cool *et al* 1990), whereas over expression of truncated form of this enzyme resulted in cytokinetic failure and asynchronous nuclear division (Cool *et al* 1992). The T-cell PTPase can dephosphorylate yeast cdc2 kinase *in vitro* and can complement an yeast cdc25 mutant *in vivo* (Gould *et al* 1990). All these studies are indicative of a role of T-cell PTPase in cell cycle. The results presented in this paper provided some clues about the stages of cell cycle where PTP-S might act.

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