autoregulation of protein phosphatase type 2A expression*

Zora Baharians and Axel H. Schönthal‡

From the Department of Molecular Microbiology and Immunology, K. Norris Jr. Comprehensive Cancer Center, University of Southern California, Los Angeles, California 90033

Protein phosphatases are involved in many cellular processes. One of the most abundant of these enzymes, the serine/threonine-specific protein phosphatase type 2A (PP2A), is present in most eukaryotic cells and serves a variety of functions. However, the detailed study of its regulation and function has been hampered by the difficulty of manipulating its expression level in cell culture. By using a new mammalian expression vector to forcibly overexpress PP2A in the mouse fibroblast cell line NIH3T3, we now show that the catalytic subunit of PP2A is subject to a potent autoregulatory mechanism that adjusts PP2A protein to constant levels. This control is exerted at the translational level and does not involve regulation of transcription or RNA processing. Thus, our results demonstrate tight control of PP2A expression, and provide an explanation for the difficulty of increasing PP2A expression experimentally.

Reversible protein phosphorylation events play a key role in many cellular processes, such as metabolic pathways, ion channel regulation, signal transduction pathways, and the regulation of gene expression. While one component of this regulation, the protein kinases, has been studied intensively, the importance of the other component, the protein phosphatases, has only lately received more acknowledgment. The finding that some phosphatases are crucial components of pathways that regulate cellular growth and, consequently, may play a role in the process of tumorigenic transformation, has brought this fact.

One of the best studied members of this class is protein phosphatase type 2A (PP2A),‡ an abundantly expressed enzyme which targets mainly phosphoryl and phosphothreonyl residues in its substrates (6). Its role in cell growth regulation has been suggested by several findings. For example, in frog oocytes, PP2A has been shown to be a negative regulator of maturation promoting factor, a cyclin-dependent kinase complex that is essential for cell cycle progression (1). Furthermore, the small and medium T antigens of the DNA tumor viruses SV40 and polyoma virus form stable complexes with PP2A (4). This interaction inhibits phosphatase activity and leads to the activation of mitogen-activated protein kinase pathways in the absence of growth factor-initiated signaling (7). Moreover, a negative role of PP2A in the regulation of immediate early gene expression has been demonstrated by microinjection studies (8) and by the use of okadaic acid (9), a tumor promoter that inhibits PP2A (and certain other phosphatases) (10). In human breast cancer cells, PP2A was found to inhibit the activity of telomerase, a ribonucleoprotein complex that catalyzes the elongation of telomeres, the length of which regulates cell proliferation (11).

In mammalian cells, the native forms of the PP2A holoenzyme consist of oligomeric complexes of three subunits, termed A, B, and C (2, 4). The core of these structures are the catalytic C subunit complexed with the regulatory A subunit. This dimer exists alone, or in association with one of the B subunits, which is a diverse group of regulatory proteins that determine substrate specificity and subcellular localization (12–14). Several isoforms of the various subunits exist; for example, the C subunit is encoded by two isoforms, α and β, which are 97% conserved at the amino acid level and likely serve redundant functions (15, 16). Besides subunit composition, the activity of the C subunit is determined by post-translational modifications, such as phosphorylation and methylation, and by interactions with heat-stable protein inhibitors (17–22).

Despite PP2A’s negative function in cell growth control, it is ubiquitously expressed at high levels and appears to be essential for cell viability. Genetic inactivation of PP2A in yeast (23), or inhibition of PP2A by okadaic acid in mammalian cells (9), severely impairs cell growth and survival. However, the opposite experimental approach, the stable overexpression of PP2A, has proven difficult to achieve, despite the availability of an expression vector that produces functional PP2A in mammalian cells (24). It is therefore conceivable that the expression of PP2A is tightly regulated in order to ensure constant amounts of PP2A protein, which may constitute an essential component of cellular function. To test this hypothesis, we have analyzed the regulation of expression of the catalytic C subunit of PP2A. We found a potent autoregulatory control that adjusts the amount of PP2A C subunit to constant levels. This control is exerted at the translational level and does not involve transcriptional mechanisms.

**Experimental Procedures**

Materials—Okadaic acid was obtained from Alexis/LC Laboratories (San Diego, CA) and dissolved in dimethyl sulfoxide to a final concentration of 100 μM. Rabbit anti-PP2A antibodies were obtained from Upstate Biotechnology Inc. (UBI, Lake Placid, NY), mouse anti-HA-tag antibodies were from Boehringer Mannheim (Indianapolis, IN).

Cell Culture and Transfection—Mouse NIH3T3 and human HeLa cells were grown in 10% calf serum/Dulbecco’s modified Eagle’s medium as described (25). Transfections were performed in 6-cm tissue culture dishes using the calcium-phosphate-DNA precipitation technique. The precipitate was added into the medium for 8 h. Then the cells were rinsed twice with phosphate-buffered saline and incubated with fresh medium. For transient transfections, the cells were harvested 16 to 36 h after transfection. For stable transfections, the cells were split 1:5 and selected in 500 μg/ml G418. Individual colonies were isolated and propagated in the continued presence of 200 μg/ml G418. For mass cultures, >500 colonies were pooled.

Transfection Analysis—Transiently transfected cells were harvested

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and analyzed for luciferase activity exactly as described (25). After lysis and clearing by centrifugation, the protein concentration of each lystate was determined, and the same amount of total cellular protein was used for each assay. Each plasmid was tested by transfection and luciferase assay at least three times. For the determination of transfection efficiency, a CMV-β-galactosidase plasmid was co-transfected in some of the experiments, and β-galactosidase activity was determined in parallel to luciferase activity.

**Plasmid Constructs**—A full-length cDNA encoding the catalytic subunit of PP-2A (α isoform) was obtained from Brian E. Wadzinski (24). This cDNA contained a 9-amino acid HA-tag at its N terminus. The coding sequence of this cDNA clone (including its HA-tag but not its 3′-UTR), was subcloned into vector pRD114, which contains the murine leukemia virus (MLV) long terminal repeat (26). pRD114 was kindly supplied by Ralph Dornburg. In our experiments, pRD114 was renamed MLV-control, and the same vector containing the HA-tagged PP2A insert was named MLV-PP2A. In addition, we generated a construct similar to MLV-PP2A that contained parts of the PP2A 3′-UTR with its polyadenylation signal (called MLV-PP2A-3′).

**Northern Blot Analysis**—The isolation of poly(A)+ RNA and further details of Northern blot analysis are described in Ref. 27. For hybridization, the following radioactively labeled probes were used. For the detection of the total amount of PP2A, we used a 2-kilobase fragment from plasmid MLV-PP2A that represented the complete PP2A coding sequence plus the vector-derived 3′-UTR. The probe recognized exogenous PP2A mRNA, as well as both isoforms, α and β, of the endogenous PP2A mRNA. For the specific detection of the α and β isoforms of PP2A mRNA, we used the same probes as described earlier in Ref. 15: the α-specific probe was a 400 bp fragment from the 3′-UTR of the PP2A α-isoform, the β-specific probe was a 470-base pair fragment from the 3′-UTR of the PP2A β isoform. As control probes for equal mRNA loading in each lane, we used either β-actin or choA. ChoA is an abundant, ubiquitous RNA which was originally isolated from Chinese hamster ovary cells as clone A (28).

**Western Blot Analysis**—Cells were lysed in RIPPA buffer as described (25). 20 μg of each sample was separated by polyacrylamide gel electrophoresis and blotted onto nitrocellulose. After blocking with blotto (5% milk, 0.1% Tween 20, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 h, the membrane was exposed to the primary antibody diluted in blotto at 4 °C overnight. All antibodies were diluted according to manufacturers’ instructions. The secondary antibodies were coupled to horseradish peroxidase, and were detected by chemiluminescence using the SuperSignal substrate (Pierce Chemical Co.).

**Polyacrylamide Extraction and Sucrose Gradient Analysis**—The procedure for isolation of cytoplasmic ribosomes from monolayer cultures was modified from that of Thomas et al. (29). Ten 10-cm dishes of logarithmically growing cells were used per gradient. The monolayers were rinsed three times with ice-cold Dulbecco’s modified Eagle’s medium containing 10 μg/ml cyclohexamide. Cells were then scraped into 5 ml of ice-cold Dulbecco’s modified Eagle’s medium/cyclohexamide and harvested with a brief centrifugation. The cell pellet was suspended in 1 ml of polysome extraction buffer (125 mM KC1, 12.5 mM MgCl2, 10 mM Hepes, pH 6.8, 0.1 mM dithiothreitol, 10 μg/ml cyclohexamide, 0.5% Triton X-100, and 0.5% deoxycholate), followed by seven passes of a Dounce homogenizer to lyse the cells. The resulting cell extracts were then layered onto 11 ml of a 10–40% sucrose gradient (125 mM KCl, 12.5 mM MgCl2, 10 mM Hepes, pH 6.8, 0.1 mM dithiothreitol, and 10 μg/ml cyclohexamide) and centrifuged at 4 °C in a SW41 rotor at 36,000 rpm for 1.75 h. The gradients were analyzed with a UV monitor and flow cell fractions were collected with a Gilson Micro-Fractionator. The gradient fractions were adjusted to 1% SDS and extracted twice with phenol/chloroform (CHCl3/isoamyl alcohol (25:24:1, v/v)), followed by a single extraction with CHCl3/isoamyl alcohol (24:1, v/v). The samples were adjusted to 0.3 M sodium acetate and precipitated in 2 volumes of ethanol at −20 °C. The RNA was collected by centrifugation, washed twice with 70% ethanol, and suspended in 10 μl of H2O.

**Determination of PP2A Protein Half-life**—Cells were grown in 6-cm culture dishes. The growth medium was removed and the cell monolayers were rinsed three times with medium deficient in methionine and cysteine. Then medium lacking methionine and cysteine was added together with 10% dialyzed fetal bovine serum and 10 μCi/ml Trn35S-label (ICN, Costa Mesa, CA), which contained [35S]methionine and [35S]cysteine at 1200 Ci/mmol. After 8 h incubation, the radioactive medium was replaced by fresh medium containing excess non-radioactive methionine and cysteine. At different times thereafter, the cells were harvested in RIPPA buffer (30). Each lysate was subjected to immunoprecipitation with anti-PP2A antibodies. The antigen-antibody complexes were harvested with protein A-Sepharose and separated by polyacrylamide gel electrophoresis. The gel was dried and exposed to Kodak X-AR film. The amount of radioactivity in each lane was determined with the AMBIS radioanalytic imaging system.

**RESULTS**

To investigate a possible autoregulatory control of PP2A expression, NIH3T3 mouse fibroblasts were treated with okadaic acid, a phosphatase inhibitor. We reasoned that, should there be an autoregulatory control, the inhibition of phosphatase activity by this drug might stimulate a compensatory increase in PP2A expression in the cells. We used 50 nM okadaic acid, which we have shown before inhibits PP2A by 80% in these cells (25). Another major serine/threonine phosphatase, type 1 (PP-1), is not inhibited at this concentration (25). The cells were incubated in the presence of okadaic acid for 24 and 48 h, then the amount of PP2A protein was determined by Western blot analysis. As shown in Fig. 1A, there was a substantial increase in PP2A protein levels when PP2A activity was blocked by okadaic acid. This effect was completely reversible, as after the removal of okadaic acid the amount of PP2A protein returned to pretreatment levels. This effect did not appear to be cell type-specific, because a similar effect could be observed in the human cervix carcinoma cell line HeLa (Fig. 1B).

Should this autoregulation exist indeed, we would expect that elevated PP2A activity should generate the opposite effect, namely the down-regulation of PP2A synthesis. We tested this by transfecting expression vectors for PP2A, and analyzing the

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**FIG. 1. PP2A protein levels in response to okadaic acid treatment.** A, NIH3T3 fibroblasts were treated with 50 nM okadaic acid for 24 and 48 h (+OA). In addition, after 48 h of culture in the presence of okadaic acid, the cells were washed and further incubated in fresh growth medium for another 48 h (−OA). B, human HeLa cervix carcinoma cells were treated with okadaic acid as indicated. In all cases, cell lysates were prepared and subjected to Western blot analysis with anti-PP2A specific antibodies. The lysates of NIH3T3 cells were also subjected to Western blot analysis with antibodies against p34cdc2 (bottom panel). This served as a control, as it has been shown before that the expression of p34cdc2 is down-regulated by okadaic acid (48). Numbers on left indicate the molecular weight marker (kDa).
consequences for endogenous PP2A expression. NIH3T3 cells were stably transfected with a cDNA encoding the PP2A catalytic subunit under the control of the MLV-long terminal repeat. The two plasmids we used were MLV-PP2A and MLV-PP2A-3’ (see “Experimental Procedures” for details). In each case, the PP2A coding sequence contained an N-terminal HA-tag, which allowed the specific identification of transfected PP2A. As a control, the vector without PP2A cDNA (MLV-control) was transfected in parallel. Mass cultures were established and analyzed for the expression of PP2A. As shown in Fig. 2, the transfected HA-tagged PP2A protein was expressed efficiently in cells that had received construct MLV-PP2A or MLV-PP2A-3’. In control cells (either untransfected or transfected with MLV-control), there was no expression of HA-tagged PP2A. Intriguingly, however, when the amount of total PP2A protein (endogenous plus endogenous) was analyzed, only a minor increase (<20%) in the overall amount of PP2A could be detected in the cells transfected with PP2A cDNA (Fig. 2). This suggested that in the presence of transfected PP2A, the synthesis of endogenous PP2A was reduced.

We confirmed this by comparing the amount of endogenous PP2A protein between cells that were transfected with either MLV-control or MLV-PP2A. Because antibodies against PP2A cannot distinguish between endogenous and transfected HA-tagged PP2A, we performed an immunoblot to solve this issue (Fig. 3). We first immunoprecipitated the transfected PP2A with an antibody against the HA-tag, which left only the endogenous PP2A back in the lysate. Then the amount of endogenous PP2A in this precleared lysate could be determined by Western blot analysis. As shown in Fig. 3, the amount of endogenous PP2A was reduced by 75% in cells that had been transfected with PP2A cDNA. Thus, it appeared that in response to increased PP2A levels (by transfection), the cells reduced the synthesis of endogenous PP2A to adjust to pretransfection levels of the protein. Similarly, when the enzymatic activity of PP2A was determined in crude cell lysates, no statistically significant difference could be found between non-transfected cells and cells transfected with MLV-control, MLV-PP2A, or MLV-PP2A-3’ (not shown).

In order to investigate the mechanism underlying this auto-regulation of PP2A expression, we first analyzed the mRNA levels of transfected and endogenous PP2A. For this purpose, poly(A)+ RNA was harvested from untransfected cells, or from cells stably transfected with MLV-control, MLV-PP2A, or MLV-PP2A-3’ and subjected to Northern blot analysis. As shown in Fig. 4, the cells containing the PP2A expression vectors produced 9–10-fold more PP2A mRNA than the control cells. However, when the two isoforms of the endogenous PP2A mRNA were visualized, there was no obvious difference in the amount of either the α or β subunit mRNA (Fig. 4). Thus, despite the presence of high levels of exogenous PP2A mRNA, no down-regulation of the endogenous PP2A mRNA became apparent.

The previous experiment indicated that there was no negative effect of transfected PP2A on the transcription of the endogenous PP2A gene. This was confirmed by transient co-transfection of the PP2A expression vector together with a luciferase reporter construct under the control of the PP2A promoter. For these experiments we used the α and β isoforms of the PP2A promoter fused to the luciferase gene, and NIH3T3 cells as recipient cells. As shown in Fig. 5, co-transfected PP2A did not result in altered PP2A promoter activity, consistent with our observation above that endogenous PP2A mRNA levels were not altered in response to transfected PP2A.

Because the experiments in Figs. 2–4 were performed with mass cultures of transfected cells, with the chance of obscuring strong effects in individual PP2A-transfected clones, we also analyzed a few isolated clones of transfected cells. Three clones of MLV-PP2A transfected cells were compared with three clones of MLV-control transfected cells. High levels of HA-tagged PP2A could be detected in the MLV-PP2A transfected clones, but not in the clones transfected with vector alone (Fig. 6A). However, in keeping with our results obtained with mass cultures, the overall amount of PP2A protein (endogenous plus exogenous) was only weakly increased (Fig. 6A).

When the levels of PP2A mRNA were analyzed in these clones, the lack of transcriptional or post-transcriptional auto-regulation became even more apparent. Despite 8–10-fold elevated overall levels of PP2A mRNA, the amount of the endogenous α and β isoform mRNAs remained the same (Fig. 6B).
Nevertheless, in agreement with our results shown in Fig. 3 above, the amount of endogenous, non-HA-tagged PP2A protein was greatly reduced (not shown), suggesting that autoregulation took place at translational or post-translational levels.

In order to further characterize this autoregulatory loop, we compared the rate of PP2A mRNA translation in MLV-control cells to that in MLV-PP2A cells. For this purpose, the association of PP2A mRNA with cytoplasmic polysomes was determined. In control cells without transfected PP2A (MLV-control), the great majority (81%) of the PP2A mRNA was associated with high molecular weight polysomes (Fig. 7), indicating an efficient rate of PP2A translation. In contrast, in PP2A-transfected cells (MLV-PP2A), only half (48%) of the PP2A mRNA was located in these structures, and the percentage of low molecular weight polysomes and monosomes each increased 2.5-fold from 14 to 36% and from 5 to 16%, respectively (Fig. 7B), demonstrating a lower rate of translation. Therefore, this experiment indicates that in PP2A-transfected cells the overall rate of PP2A mRNA translation is significantly reduced, and provides an explanation for the observed discrep-

Fig. 4. Expression level of PP2A mRNA. Poly(A)+ RNA was prepared from mass cultures of NIH3T3 cells stably transfected with MLV-control, MLV-PP2A, or MLV-PP2A-3, and subjected to Northern blot analysis. Different types of probes were used to visualize the amount of PP2A mRNA: probe 1 detects endogenous as well as transfected PP2A (PP2A (total), top panel); probe 2 detects only the endogenous PP2A α mRNA (PP2A-α (endo), second panel from top); probe 3 detects only the endogenous PP2A β mRNA (PP2A-β (endo), second panel from bottom). Note that the two probes for the α and β isoforms do not detect transfected PP2A (see “Experimental Procedures” for details). To confirm that equal amounts of RNA were loaded in each lane, the filter was also hybridized with a probe for choA (bottom panel).

Fig. 5. PP2A promoter activity. NIH3T3 cells were transiently co-transfected with luciferase reporter constructs containing the α or β isoforms of the PP2A promoter together with expression vectors without (MLV-control) or with PP2A cDNA (MLV-PP2A). 24 h after transfection the cells were harvested and luciferase activity was determined. Shown is the average of five independent experiments.

Fig. 6. PP2A expression in individual, transfected clones. NIH3T3 cells were stably transfected with either MLV-control or MLV-PP2A together with pSV2neo. The cells were selected in G418, and several individual clones were analyzed (A) by Western blot analysis and (B) by Northern blot analysis. In A, the top panel shows the amount of transfected, HA-tagged PP2A protein as visualized by the use of an anti-HA-tag antibody. The middle panel was reacted with anti-PP2A antibody and shows the total amount of PP2A (transfected plus endogenous). The bottom panel shows the amount of p34cdc2 protein which was used as a loading control. In B, poly(A)+ RNA was analyzed with specific probes as described in the legend to Fig. 4. These probes detected total (transfected plus endogenous) PP2A, only the endogenous α isoform (PP2A-α (endo)), or only the endogenous β isoform (PP2A-β (endo)), respectively. As a control for equal loading in each lane, a probe for β-actin was used.
As the above experiment does not exclude potential additional autoregulatory mechanisms at the post-translational level, we next investigated the turnover of PP2A protein in MLV-control and MLV-PP2A cells. The cells were pulse-chased with \[^{35}S\]methionine, then PP2A protein was immunoprecipitated 6, 12, 18, and 24 h thereafter. We detected only a minor difference in the rate of decay of PP2A protein between the two cell lines (not shown). In MLV-control cells, the half-life of PP2A protein was determined to be 16.5 h, whereas in MLV-PP2A cells the half-life was shortened to 15 h. This difference, however, was not statistically significant, and especially during the first 12 h there was no difference in the decay rate. We therefore conclude that turnover of the protein is not a major factor in the autoregulatory process.

DISCUSSION

In this paper we have investigated the control of PP2A catalytic subunit expression. We found a strong autoregulatory mechanism that appears to ensure relatively constant levels of PP2A synthesis. We show that in cells with highly elevated amounts of PP2A mRNA (due to transfection), there is no corresponding increase in PP2A protein levels because of a control mechanism at the translational level that reduces the efficiency of PP2A translation.

Our data may provide an explanation for earlier seemingly contradictory findings by others who investigated PP2A expression. For example, in various mammalian cells and in fission yeast, it has been shown that the level of PP2A protein remains constant throughout the cell cycle (31–33). In contrast, analyzing the amount of mRNA, others have demonstrated increased PP2A mRNA levels during the early stages of G1 in mammalian cells (34, 35). Moreover, Kakinoki et al. (36) by performing partial hepatectomy, presented evidence of almost constant levels of PP2A protein in regenerating liver, despite a 30-fold increase in PP2A mRNA. These observed discrepancies between elevated mRNA levels and rather constant protein levels can be resolved by our finding of a potent autoregulatory mechanism of PP2A protein synthesis.

However, even though PP2A protein levels remain constant, there are examples of altered enzymatic activity of PP2A (1, 4, 7, 37). These alterations of PP2A activity can be brought about by post-translational mechanisms, e.g. by interactions with regulatory subunits, such as the various B subunits (13, 14) or heat-stable protein inhibitors (21). Moreover, PP2A has been shown to interact with various other cellular proteins which affect its phosphatase activity (38, 39). In addition, post-translational modifications of the catalytic subunit have been implicated in the regulation of PP2A activity (19, 20, 40). Together with our data, it therefore appears that the main regulation of PP2A occurs at the post-translational level. Even though there are occasions where PP2A mRNA levels are dramatically increased, the above described autoregulatory mechanism seems to prevent these changes from translating into elevated levels of PP2A protein.

Our findings have important implications for the study of PP2A function in cells. As noted previously (24), the study of serine/threonine protein phosphatases in mammalian cells using genetic strategies have been frustrating due to difficulties in expressing functional phosphatases with standard gene transfer techniques. Wadzinski et al. (24) modified the N terminus of the PP2A protein by the addition of a 9-amino acid peptide sequence derived from the influenza hemagglutinin protein which allowed, for the first time, functional expression of PP2A in mammalian cells. However, as our results with this same modified PP2A cDNA show, an efficient overall increase in PP2A protein cannot be achieved due to an autoregulatory block of translation. Therefore, for the purpose of functional studies, other avenues need to be pursued to manipulate phosphatase activity.

One possibility, which was successful in the past, is the microinjection of purified PP2A (8, 41). This assay, however, is limited by the low number of cells that can be used per experiment. Another approach is the transient transfection of HA-tagged PP2A, which has been shown to generate certain cellular effects (25). The drawback of that assay is its transient nature, which most likely is rapidly terminated by the autoregulatory feedback loop of PP2A expression. A further strategy to increase PP2A activity in cells was recently presented by Ruediger et al. (42). These authors generated an N-terminal mutant of the regulatory A subunit that was able to bind to the catalytic C subunit, but not to the regulatory B subunit. This resulted in an increase in the amount of core protein (A-C) and a decrease in the amount of holoenzyme (B-A-C) with concomitant alterations in phosphatase activity. Combined with the observed difficulty of increasing the overall amount of the catalytic C subunit (our manuscript), it is therefore likely that approaches which manipulate the expression levels of the regulatory subunits will be more successful in altering phosphatase activity to allow the study of PP2A function.

In the same vein, it will be important to establish the precise mechanism by which PP2A autoregulates expression of its...
catalytic subunit at the translational level. Several translation factors have been found to be reversibly phosphorylated and thus are potential targets for PP2A. For example, okadaic acid causes increased phosphorylation of translation elongation factor 2 (EF-2) (43, 44), and phosphorylated EF-2 is a substrate for PP2A in vitro (44, 45). In addition, in reticulocyte lysates, PP2A appears to be the principal enzyme responsible for dephosphorylating the β-subunit of translation initiation factor 2 (46), and, to a lesser extent, the α-subunit of eukaryotic initiation factor-2 (45). Moreover, the catalytic subunit of PP2A has been found associated with translation termination factor eRF1 (eukaryotic release factor 1) in vitro (47), and the authors postulate that this interaction may serve to recruit PP2A into polysomes and into contact with putative targets among the components of the translational apparatus.

However, neither of the above findings would suffice as a basis for the specific autoregulatory control of PP2A translation, because these translation factors are part of the general machinery involved in the synthesis of a multitude of different proteins. Furthermore, it is unlikely that autoregulation is controlled via specific sequences in the 5'- or 3'-UTR of the PP2A mRNA, as the transfected construct MLV-PP2A, which is controlled via specific sequences in the 5'-UTR of the translational apparatus.

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