Regulation of the human spermidine synthase mRNA translation by its 5′-untranslated region

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Received 30 March 1995

Abstract An increased mRNA content of spermidine synthase was found in phytohemagglutinin stimulated human peripheral lymphocytes and in cultured human myeloma (Sultan) cells stimulated to grow by change of the culture medium. The many-fold increase in the amount of the message was accompanied by stimulation of the enzyme activity in activated lymphocytes, but not in stimulated myeloma cells. In the present study the effect of the 5′-untranslated region of spermidine synthase mRNA on the post-transcriptional control of its expression was studied both in vitro in rabbit reticulocyte system and in cultured mammalian cells. The results show that the GC-rich 5′-untranslated region of spermidine synthase mRNA has an inhibitory effect on its translation.

Key words: Polyamine; Spermidine synthase; Translational regulation

1. Introduction

Spermidine synthase (EC 2.5.1.16) is one of the four enzymes in the polyamine-biosynthetic pathway and catalyzes the conversion of putrescine into spermidine. The polyamines, putrescine, spermidine and spermine, are known to be essential for cell growth and differentiation [1–3]. Studies with lectin-stimulated bovine lymphocytes have shown that polyamines, especially spermidine, are required for optimal DNA synthesis [4]. In addition, it has been shown that spermidine is crucial for continued protein synthesis by virtue of its role as a precursor of hypusine, a post-translational modification of initiation factor eIF-5A essential for its activity [2,5–7].

In mammalian tissues spermidine synthase enzyme appears to be a stable enzyme and present in excess [8]. The activity of spermidine synthase is supposed to be regulated mainly by the amount of enzyme protein and availability of its substrate, decarboxylated *S*-adenosyl-L-methionine [8,9]. Its expression has been reported to respond to mitogenic stimuli, such as lymphocyte activation [10], and hormone-induced tissue proliferation [11,12].

In order to examine the regulation of the enzyme, the cDNA and the gene for human spermidine synthase have been cloned and sequenced [13,14]. In the present study the regulation of spermidine synthase expression was studied in lectin-stimulated human lymphocytes and growth-stimulated myeloma cells. The results show that elevated amounts of mRNA are not necessarily reflected as an enhanced enzyme activity and suggest that the expression of the enzyme is translationally regulated under these conditions. Recently the 5′UTR of several eukaryotic mRNAs have been shown to influence the rate of their translation [15,16]. Since the 82 nucleotides long 5′UTR of spermidine synthase cDNA is very GC-rich and has been predicted to form a stable stem–loop structure [13], its effect on the translation of spermidine synthase was studied in a rabbit reticulocyte cell-free system and in cultured cells. These results indicate that the 5′UTR in mRNA plays a role in regulation of human spermidine synthase expression.

2. Experimental

2.1. Materials

5′-[α-^{32}P]dCTP (specific radioactivity 400 Ci/mol) and L-[^{35}S]methionine (1200Ci/mmol) were purchased from Amersham International (Amersham, Bucks., UK). Radioactive *S*-adenosylmethionine was prepared by the method of Pegg et al. [17] and the decarboxylated adenosylmethionine as reported by Pösö et al. [18]. Cell culture media and fetal calf serum were obtained from Gibco (Glasgow, UK), Percoll from Pharmacia LKB (Uppsala, Sweden) and phytohemagglutinin M from Difco Laboratories (Detroit, Michigan, USA)

2.2. Cell culture and transfection

Human peripheral lymphocytes were isolated from the buffy-coat fraction supplied by the Finnish Red Cross Blood Transfusion Service (Kuopio, Finland). Lymphocytes were purified with the aid of Percoll density gradient [19] and were cultured in RPMI-1640 medium at a density of 10^6 cells/ml supplemented with 5% fetal calf serum and gentamycin (50 μg/ml). The lymphocytes were stimulated with phytohemagglutinin-M (0.2 mg/ml) and samples were collected at 24, 48 and 72 h after the stimulation. Human Sultan myeloma cells were cultured in RPMI-1640 medium with the above-mentioned supplementations. For analysis the cells were seeded at a density of 3×10^5 cells/ml. Green monkey kidney cells (CV-1) were cultured in Minimum Essential Medium supplemented with 5% fetal calf serum and gentamycin in humidified incubator with 5% CO_2. Transfections were performed by the calcium phosphate coprecipitation method described by Chen and Okayama [20]. CV-1 cells were transfected using 5 μg circular spermidine synthase constructs cloned in pSG5 expression vector. Plasmid pCH110 (5 μg, Pharmacia), coding *E. coli* β-galactosidase gene was used as an internal reference. Cells were harvested by scraping at 48 h after transfection.

2.3. Analytical methods

Total RNA was isolated from the cell cultures by using the guanidinium isothiocyanate method of Chomczynski and Sacchi [21]. Total RNA was electrophoresed in 1.4% agarose gels in the presence of formaldehyde, blotted onto Hybond-N membranes (Amersham) and hybridized to nick-translated phSD1 [13]. The activity of spermidine synthase was assayed as described by Raina et al. [22]. For statistical analyses two-tailed *t*-test was used.

2.4. cDNA constructs

A blunted *Eco*RI fragment of human spermidine synthase cDNA containing the full-length 5′UTR and 3′UTR (1238 bp) was subcloned into the *Sma*I site of vector pSP73 (Promega) to form pSPD-82. To obtain plasmid pSPD-6, spermidine synthase cDNA was amplified by PCR using 5′ primer 5′-CCCGAATTCCCGCCATGGAG-3′ and 3′ primer corresponding to nucleotides 1045 to 1065 of the cDNA. The

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Abbreviations: 5′UTR, 5′-untranslated region.

SSDI 0014-5793(95)00437-8

PCR product was digested with *Eco*RI and *Apa*I and ligated with the *Apa*I–*Eco*RI fragment from pSPD-82 containing the vector and the 3′ end of the spermidine synthase cDNA. Since there is an extra ATG codon in the polylinker of the pSP73 vector which might also start translation it was deleted from both plasmid constructs by *Eco*RI and *Eco*RV digestion. For transfection experiments the intact spermidine synthase cDNA and the cDNA having a deletion in the 5′UTR were inserted into the *Eco*RI–*Bam*HI site of the pSG5 expression vector (Stratagene) governed by the SV-40 early promoter. The sequences were confirmed by using the Autoread fluorescent sequencing kit and an A.L.F automated DNA sequencer (Pharmacia).

2.5. Transcription and translation in vitro

m^7GpppG-capped RNAs were synthesized in vitro from *Bam*HI linearized DNA templates using SP6 RNA polymerase and the supplied reagents (Promega) according to the Promega protocol. The quality of the RNA synthesized was determined using gel electrophoresis. For in vitro translation 0.25 μg of RNA transcribed in vitro were added to 50 μl of a reaction mixture containing rabbit reticulocyte lysate and L-[^{35}S]methionine to label the synthesized protein according to the suppliers specifications (Promega). Reaction mixtures were incubated at 30°C for 5–60 min, and the products were analyzed by 12.5% SDS-PAGE and protein bands were visualized by fluorography.

3. Results

As depicted in Fig. 1, the stimulation of human peripheral lymphocytes with phytohemagglutinin resulted in a distinct stimulation of spermidine synthase activity. The maximum activity was attained at 72 h after the addition of the mitogen. The enhanced enzyme activity was accompanied by corresponding or greater increases in the level of spermidine synthase mRNA (Fig. 2). By the time of the maximum enzyme activity the amount of the message had already returned to the level found in unstimulated cells (Fig. 2). The faint bands seen in the Northern blot probably resulted from the homology of the GC-rich region of the cDNA probe with ribosomal RNA, since they disappeared when only a fragment of cDNA was used as a probe.

The stimulation of human myeloma cells to proliferate by dilution and fresh medium change was accompanied by a marked and early enhancement of the accumulation of spermidine synthase mRNA as shown in Fig. 3. In fact, the level of the message was substantially elevated already at 4 h and peaked at 24 h after the medium change. In spite of the manyfold increase in the amount of the message, the activity of spermidine synthase remained entirely unaltered during the whole period of observation (Table 1). Neither were there changes in the amounts of enzyme protein (results not shown). The same kind of results were seen while using mouse L1210 leukemia cells (results not shown). The measurement of the decline of spermidine synthase activity after protein synthesis was stopped with cycloheximide did not reveal changes in enzyme turnover. The half-live of spermidine synthase was over 12 h both in confluent and stimulated Sultan cells (results not shown). This phenomenon apparently means that for some

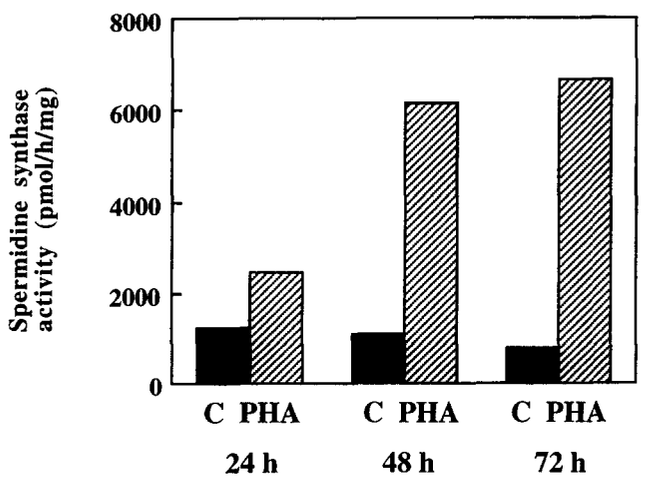

Fig. 1. Spermidine synthase activity in human peripheral lymphocytes stimulated with phytohemagglutinin (PHA). Human peripheral lymphocytes separated with Percoll were seeded at a density of 10^6 cells/ml (5 ml) and stimulated with PHA-M (0.2 mg/ml). Cells were collected at 24, 48 and 72 h after the stimulation and spermidine synthase activity measured. The results are means of two parallel samples.

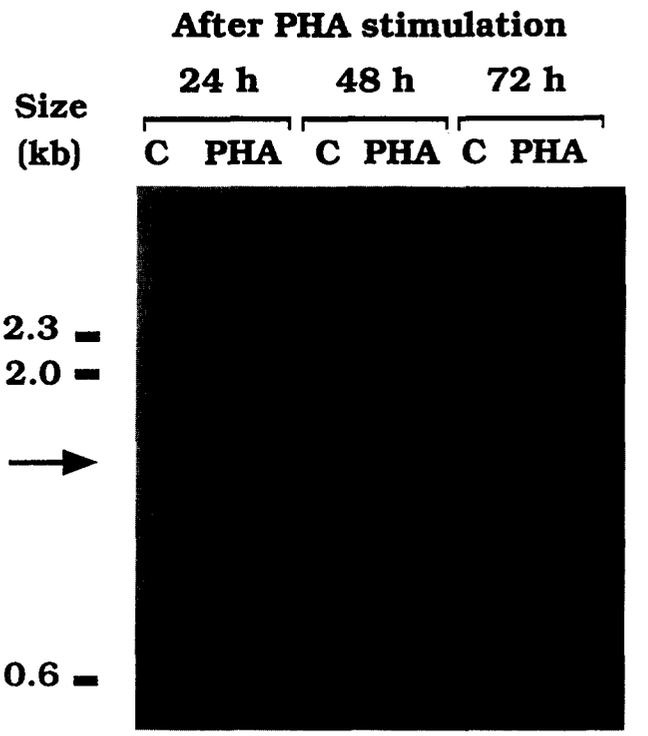

Fig. 2. Northern blot analysis of total RNA from human peripheral lymphocytes stimulated with phytohemagglutinin (PHA). Total RNA (10 μg) extracted from untreated lymphocytes (C lanes) and from PHA-stimulated lymphocytes (PHA lanes) were electrophoresed, blotted and probed with spermidine synthase cDNA (phSD1). The arrow indicates the spermidine synthase mRNA.

Table 1
Spermidine synthase activity in human myeloma cells (Sultan) stimulated to proliferate by dilution in fresh medium

Time of incubation after medium change (h)	Spermidine synthase activity (nmol/h/mg)
0	12.3 ± 0.3
4	10.4 ± 0.9
8	10.9 ± 0.3
12	10.5 ± 0.7
24	12.4 ± 0.7
30	12.6 ± 0.4
36	12.9 ± 0.5
48	12.8 ± 0.2

Values are means ± S.D.; $n = 3$.

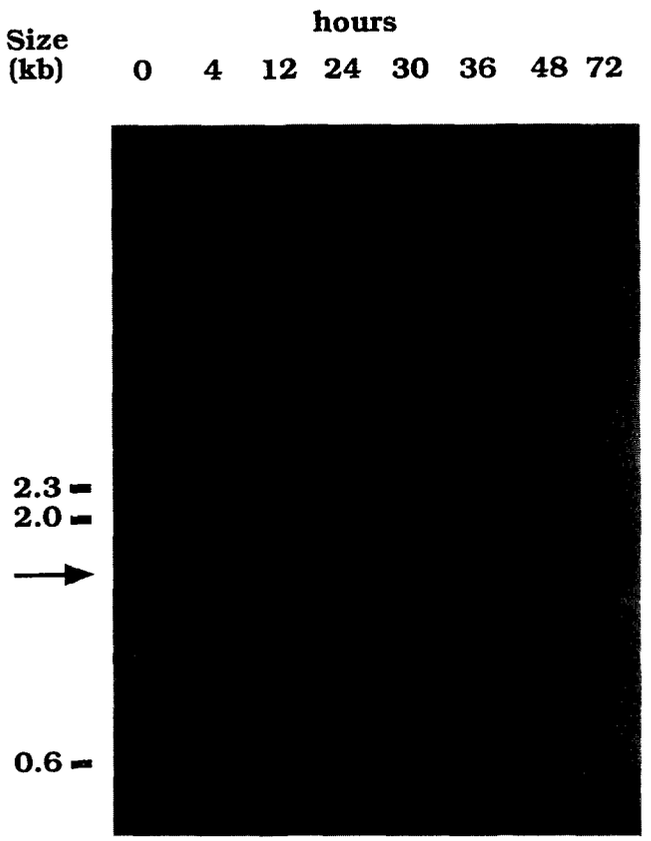

Fig. 3. Spermidine synthase mRNA levels in human myeloma cells (Sultan) stimulated to grow by dilution and fresh medium. Confluent Sultan cell cultures were diluted to a density of 0.3×10^6 cells/ml with fresh medium and the cells were harvested for RNA extraction after 4, 12, 24, 30, 36, 48 and 72 h of stimulation. Total RNA (10 μg) was electrophoresed, blotted and probed with phSD1. The arrow indicates the spermidine synthase mRNA.

unknown reasons the increased mRNA content was not translated under these conditions.

To examine the effect of the 5′-untranslated region of human spermidine synthase mRNA on translation efficiency, two recombinant plasmids were constructed composed of human spermidine synthase cDNA containing the entire 82 bp (pSPD-82) or 6 bp (pSPD-6) of 5′UTR in the pSP73 expression vector. To achieve 5′UTR deletion in spermidine synthase cDNA a PCR reaction was performed with a 5′ primer containing a *Eco*RI restriction site and the complete Kozak's consensus sequence corresponding to the intact cDNA. SP6 RNA polymerase was used to generate m7G-capped transcripts in vitro. According to Northern blot analysis the in vitro transcripts were of the same size as the messages in human lymphocytes and in Sultan cells. In vitro transcripts were translated in a rabbit reticulocyte lysate system. When aliquots of the lysates were analyzed on SDS-PAGE the predominant translation products were of the size of M_r 34,000 corresponding to human spermidine synthase (Fig. 4A). However, a time course experiment showed that deletion of the first 76 nucleotides from the 5′UTR of the mRNA resulted in a 4- to 5-fold increase in the translation of spermidine synthase (Fig. 4B).

To examine whether the 5′UTR of spermidine synthase mRNA confers the inhibition of translation in intact cells, the entire cDNA and the 5′-deleted cDNA were cloned into pSG5 eukaryotic expression vector and transfected transiently into CV-1 cells. The spermidine synthase activities in CV-1 cells transfected with the entire spermidine synthase cDNA and with the deleted cDNA were 928 ± 229 and 2510 ± 467 pmol/mg/h, respectively. Thus there was a 2- to 3-fold increase in spermidine synthase expression in cells transfected with the construct without 5′UTR, in comparison with the entire cDNA. These same kind of results obtained both in in vitro translation experiments and in intact cells demonstrated that the 5′UTR of mRNA negatively influences the translation of the human spermidine synthase enzyme.

4. Discussion

The increased spermidine synthase expression in mitogen-induced lymphocytes, as indicated by elevated mRNA level and enzyme activity (Figs. 1 and 2), is in all likelihood related to the critical role of polyamines in the transition of lymphocytes from

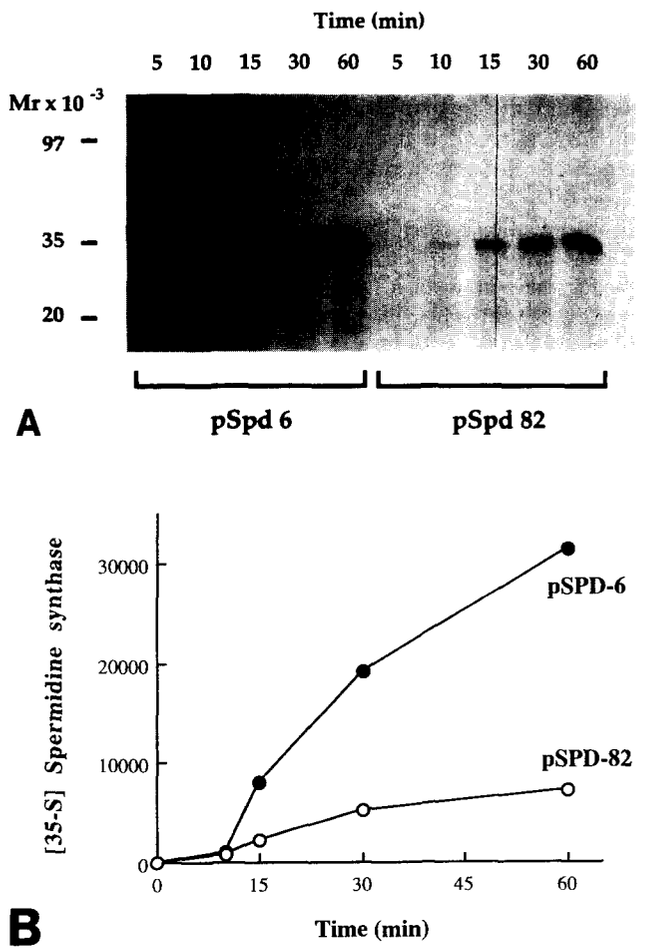

Fig. 4. Time course of in vitro translation of spermidine synthase mRNAs in reticulocyte lysates. SP6-generated mRNAs (250 ng) were translated for 60 min at 30°C in rabbit reticulolysates in the presence of [^{35}S]methionine. Aliquots were removed at indicated times and subjected to SDS-PAGE. To determine the amount of spermidine synthase protein synthesized, translation products were visualized by autoradiography (A), and the M_r 34,000 band corresponding to spermidine synthase was excised from the gel and assayed for radioactivity by scintillation counting (B).

the resting to the growing state. Fillingame and coworkers [4] have shown that there are large increases in cellular levels of the polyamines in lymphocytes induced to transform by mitogen concanavalin A and that increased cellular levels of spermidine or spermine are required for optimal DNA synthesis and progression of a cell through S-phase [4]. Moreover, it has been shown that inhibition of polyamine synthesis in activated lymphocytes resulted in a suppression of protein synthesis [23,24]. Elevated protein synthesis in activated lymphocytes seems to be regulated by mRNA level and there is no change in the translational efficiency of proteins upon activation of lymphocytes [25]. However, the increased expression of *S*-adenosyl-methionine decarboxylase in mitogen activated lymphocytes has been shown to arise from elevated translational initiation which results in a change in the number of ribosomes associated with this mRNA [26].

After refeeding confluent human Sultan myeloma cells with fresh culture medium spermidine synthase mRNA accumulated rapidly indicating increased transcription after stimulation to growth. However, the activity and amount of enzyme remained unchanged. The results indicate that the translation of spermidine synthase is repressed in these cells. Earlier studies with transgenic mice over-expressing human spermidine synthase gene have revealed that the tissue spermidine synthase mRNA amounts correlate poorly with the enzyme activities [27]. These findings suggest that the expression of spermidine synthase is regulated post-transcriptionally. Down-regulation of the enzyme synthesis may be important in preventing polyamine overproduction, which would result in cytotoxicity in constantly dividing myeloma cells where spermidine content is higher than in resting lymphocytes. Spermidine is already known to negatively regulate the translation of other polyamine biosynthetic enzymes, ornithine decarboxylase, *S*-adenosylmethionine decarboxylase [28–30] and to stimulate the translation of the interconversion enzyme, spermidine/spermine N^1-acetyltransferase [31,32]. Whether intracellular polyamine content is likewise regulating spermidine synthase translation remains to be studied.

Factors which have been shown to influence the translability of an mRNA are the extensive stability of secondary structure in the 5′-untranslated leader sequence, the presence of short open reading frames upstream of the actual translation start site and alterations in phosphorylation status and activity of initiation factors [16,33]. 5′-Noncoding sequences with a GC content of 70–90% have been reported to be typical of mRNAs that encode oncoproteins, growth factors, signal transduction components, and transcription factors, as well as many housekeeping proteins [34]. In primer extension experiments it has been shown that the 5′-untranslated region of human spermidine synthase is 83 nucleotides in length [13,35] but no upstream open reading frames were found. Computer analysis of the mRNA has identified a highly stable stem–loop structure in the 5′UTR the GC content of which is 87%. The calculated free energy associated with this structure is −73 kcal/mol. The in vitro translation experiments reported here showed that the 5′UTR of spermidine synthase mRNA is involved in the regulation of its translation and that the removal of the 5′UTR increased the translation fivefold. To confirm the differences in the in vitro translation results the same constructs were cloned into an expression vector and transfected into green monkey kidney cells (CV-1). Again, the spermidine synthase cDNA lacking the 5′UTR was expressed more efficiently. However, the difference in cultured cell was only about twofold.

Kozak has shown that a hairpin structure in mRNA will inhibit translation only if it is close enough to the 5′ end to prevent the initial binding of the 40S ribosome [36]. Moreover, it has been shown that some secondary structures located 3′ to the initiation codon can stimulate translation initiation, depending upon their stability and position with respect to initiation codon [37]. In spermidine synthase mRNA the GC-rich area extends from the 5′ leader sequence beyond the start codon in to the coding region [13]. One possible explanation is that the removal of the non-coding 5′ end of the spermidine synthase mRNA can support the unwinding of mRNA and therefore stimulate the initiation of translation.

Present data are insufficient to suggest how the 5′ end of the mRNA inhibits translation and how this suppression is circumvented in activated lymphocytes. The utilization of functional mRNAs in the cytoplasm of eukaryotic cells can be regulated by controlling the stability of individual mRNAs or by altering their ability to bind ribosomes and be translated. It has been shown that 5′ secondary structures can influence on ribosome binding to mRNA and mRNA distribution on polysomes. Also the secondary structures present in the 5′UTR of mRNAs may serve as binding site for mRNA-binding proteins or specific translational inhibitors which may be cell type-specific. Recently results on eIF-4E initiation factor (Gap binding protein) have shown that it is a limiting factor in the translation of mRNAs with extensive secondary structures in their 5′UTR and demonstrated how ribosomes in vivo circumvent the unwinding of stable 5′UTR [38,39]. Preliminary results with NIH 3T3 cells overexpressing eIF-4E have shown that spermidine synthase activity is likewise elevated in these cells. Further studies are needed to understand the details of the mechanisms by which spermidine synthase expression is regulated.

Acknowledgements: I thank Ms. Anne Karppinen for the synthesis of oligonucleotides and MSc Sanna Myöhänen for performing the automated DNA sequencing. I also thank Dr. Terho Eloranta for supplying me with decarboxylated *S*-adenosylmethionine. I am grateful to Prof. Juhani Jänne and Dr. Leena Alhonen for their advice and helpful comments on the manuscript. This work was supported by grants from Academy of Finland, University of Kuopio and Finnish Foundation for Cancer Research.

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