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Posttranscriptional Regulation of Thymidylate Synthase Gene Expression

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Abstract Thymidylate synthase (TS) is an essential enzyme that catalyzes the formation of thymidylic acid in the de novo biosynthetic pathway and is the target enzyme for a variety of chemotherapeutic agents. The TS gene is expressed at a much higher level in proliferating cells than in quiescent cells. Control is primarily exerted at the posttranscriptional level. Studies with chimeric TS minigenes have shown that regulation of TS mRNA content in growth-stimulated mouse fibroblasts requires the presence of sequences located upstream of the essential promoter elements. In addition, an efficiently spliced intron must be present within the transcript. Neither sequence by itself is sufficient for proper regulation, suggesting that the upstream and downstream sequences may communicate to effect regulation. A possible mechanism by which the upstream sequences influence the efficiency of splicing of TS transcripts in a cell cycle specific manner is described.

Expression of the human TS gene is also controlled at the translational level. The TS enzyme is able to block the translation of its own mRNA by binding to the message in the vicinity of the AUG start codon. The translational block is relieved in the presence of substrates or inhibitors of the enzyme. The autogenous translational regulation of TS mRNA is likely to be responsible for the rapid increase in TS enzyme level that occurs when cells are exposed to certain TS inhibitors. Elucidation of the mechanism by which the translational control is exerted may lead to the design of more effective TS inhibitors. © 1994 Wiley-Liss, Inc.

Key words: growth regulation, cell cycle, RNA processing, intron, splicing, translational control, autogenous control

Thymidylate synthase (TS) is the enzyme that synthesizes thymidylic acid (TMP) in the de novo biosynthetic pathway. The enzyme catalyzes the reductive transfer of the methylene group from 5,10-methylene-tetrahydrofolate to the 5 position of the substrate, deoxyuridylic acid, to form TMP and dihydrofolate. TS is an essential enzyme in proliferating cells and an important target for a variety of chemotherapeutic drugs that mimic either the substrate or the cofactor.

The TS gene is a member of the family of S-phase genes whose expression is greatly increased at the G1/S phase boundary, after the cell is committed to initiate DNA replication. It appears reasonable that expression of this family of genes might be coordinated by a common factor or mechanism. In support of this possibility, recent studies have shown that the transcription of several S-phase genes, including the DHFR and TK genes, may be controlled in part at the transcriptional level by the E2F family of transcription factors [Farnham et al., 1993]. The amount of E2F, in turn, is regulated during the cell cycle by changes in the amount of the protein and by modulating its activity via association with proteins related to the RB tumor suppressor protein as well as various cyclins and cyclin-dependent kinases [Nevins, 1993; Lees et al., 1992]. Although several potential E2F binding sites can be identified in the 5′ flanking region of the mouse TS gene [Deng et al., 1989], it is not clear if this trans-acting factor plays an important role in regulating TS gene transcription.

Transcriptional control represents only one aspect of the regulatory mechanisms that modulate the expression of many S-phase genes. Control has also been observed at the levels of RNA processing, mRNA translation, mRNA stability, protein stability, and so forth. In fact, each gene appears to be regulated by a unique combination of mechanisms. This review will discuss recent

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observations on the posttranscriptional mechanisms that are responsible for controlling the TS gene in mammalian cells.

REGULATION OF TS mRNA CONTENT IN GROWTH-STIMULATED CELLS

TS enzyme level is much greater in rapidly proliferating cells than in resting cells. When resting cells are stimulated to proliferate, TS activity remains at the level found in resting cells until the cells begin DNA replication, then increases at least 10-fold as the cells traverse S phase [Navalgund et al., 1980]. TS mRNA content also increases about 10-fold as cells progress from G0 through S phase. However, nuclear run-on transcription assays showed that the rate of TS gene transcription increases very little during the G1-S transition [Jenh et al., 1985a; Ayusawa et al., 1986]. Therefore it appears that TS mRNA content is controlled primarily at the posttranscriptional level in growth-stimulated human or mouse cells. The half-life of poly(A)+ TS mRNA is about 8 h in both resting and growing mouse cells [Jenh et al., 1985a], indicating that regulation of mRNA stability is not likely to be critical.

Regulation of TS Minigenes

To identify the sequences that are responsible for regulating TS gene expression in response to growth stimulation, chimeric TS minigenes were constructed. These contain different promoters linked either to the TS coding region (with or without various introns at their normal locations) or to the CAT indicator gene. The TS coding regions were "tagged" with a small deletion to allow for simultaneous detection of mRNA derived from the endogenous TS gene as well as the TS minigene by S1 nuclease protection assays. The minigenes were stably transfected into wild-type 3T6 cells and the expression of the minigenes and the endogenous TS gene was determined in growth-stimulated cells.

A TS minigene consisting of the TS promoter, the TS coding region (including introns 1 and 2 at their normal positions), and the TS polyadenylation signal was regulated normally in response to growth stimulation. The amount of mRNA derived from the TS minigene increased at the same time and to the same extent as that of the endogenous TS gene. Similar results were obtained when the minigene contained introns 5 and 6 instead of introns 1 and 2, although the extent of the increase was not as great as that observed with the endogenous gene [Li et al., 1991]. These observations demonstrate that sequences sufficient for normal regulation are included in the minigenes, and that they are not uniquely located within a single intron of the TS gene.

When the TS promoter was replaced with the SV40 early promoter in minigenes that contained introns 5 and 6, there was almost no change in the level of expression of the minigene as the cells progressed from G1 through S phase [Li et al., 1991]. This shows that the promoter/5′ flanking region of the TS gene contains sequences that are necessary for normal regulation. In addition, this shows that the sequences downstream of the AUG codon are not sufficient for normal regulation. Preliminary analyses have suggested that important regulatory sequences are within a 40 nucleotide region that is immediately upstream of the essential promoter elements (J. Ash and L.F. Johnson, unpublished observations).

Although sequences in the 5′ flanking region are necessary, they are not sufficient for normal growth-regulated expression. A minigene that consisted of the TS 5′ flanking region linked to the CAT coding region was expressed at a constant level in growth-stimulated cells [Li et al., 1991]. Similar observations have also been made with the luciferase indicator gene (M. Graham and L.F. Johnson, unpublished observations). Therefore, sequences that are downstream of the AUG codon are also necessary for normal regulation.

Introns Are Required for Regulation

To identify the downstream regulatory sequences, additional chimeric minigenes were analyzed. The polyadenylation signal and 3′ flanking sequences do not appear to be important for normal regulation. Ash et al. [1993] found that replacement of the TS 3′ flanking region with the polyadenylation signal of the human beta globin gene or the bovine growth hormone gene did not affect normal regulation. The fact that regulation was observed when introns 1 and 2 or 5 and 6 were present suggested that regulatory sequences were not uniquely located within a single TS intron.

It was possible that simply the presence of an intron (but perhaps not a specific intron) was essential for proper regulation. To test this idea, intronless derivatives of various TS minigenes were constructed and stably transfected into

3T6 cells. When all of the introns were removed, regulation was abolished [Ash et al., 1993]. These observations suggest that an intron must be present in the minigene for proper regulation to occur.

Similar analyses have been performed with the human TS gene. Takayanagi et al. [1992] showed that sequences within intron 1 as well as in the 5′ flanking region of the human TS gene are both necessary for proper S-phase regulation. However, they did not examine the effects of other introns on regulation of the human TS gene. Therefore it is not known if there are specific regulatory sequences within intron 1 or if other introns would have the same effect.

It was not clear from our studies if regulation of the mouse TS gene depended on the presence of TS introns or if introns from other genes would suffice. Since the TS-cat minigene is not regulated, it appears that the small t intron of the SV40 gene (which is included in the CAT indicator gene downstream of the open reading frame) will not suffice. However, this could be due to the fact that this intron is inefficiently or aberrantly spliced [Huang and Gorman, 1990] or because it is too distant from the 5′ regulatory sequences.

To further explore this question, we have recently constructed a minigene that consists of the TS 5′ flanking region linked to the coding region of the human beta globin gene. When the globin gene included both introns, the gene was regulated in a growth-dependent manner that was similar to that observed for the TS gene. However, when the introns were removed (by substituting globin cDNA for the genomic sequences), the minigene was not regulated (Y. Ke, J. Ash, and L.F. Johnson, in preparation). These observations strongly support the idea that regulation requires the presence of an efficiently spliced intron but that the sequences within the intron are not important for regulation.

Recent studies have shown that introns play an important role in controlling the expression of other S-phase genes. For example, intron 4 of the human proliferating cell nuclear antigen (PCNA) gene appears to be important for proper down-regulation of the gene in quiescent cells [Ottavio et al., 1990]. Intron 1 of this gene also contains a negative element [Alder et al., 1992]. The mechanism(s) by which these intron sequences down-regulate expression of the PCNA gene have not been established. In addition, Gudas and co-workers [Gudas et al., 1988] examined the accumulation of nuclear splicing intermediates of the mouse TK gene in growth-stimulated cells. They found evidence for an increase in the efficiency of processing of TK hnRNA as cells progress from G1 to S phase.

Obviously, introns alone are not sufficient to direct S phase-specific expression since most cellular genes contain introns but are not induced during S phase. Proper regulation is observed only when the TS minigenes contain both the upstream regulatory sequences and introns. These observations are consistent with the idea that proper regulation requires some form of communication between nontranscribed upstream sequences and introns.

Model for TS Regulation

How might this communication occur? One possibility is that the upstream elements modulate the efficiency of RNA splicing (rather than transcription) in a cell cycle specific manner. For example, the factor(s) that bind to the upstream control elements might interact with a component of the splicing machinery which could be transferred to the nascent transcript, either directly or via the RNA polymerase. A transcript tagged in this manner might be processed and exported efficiently, whereas transcripts that are not tagged might be inefficiently processed and rapidly degraded within the nucleus. If the concentrations or activities of the upstream regulatory factors are much greater in S phase cells than in G0/G1 cells, this would lead to an increase in the efficiency of conversion of TS hnRNA into TS mRNA as cells enter S phase.

Communication between upstream elements and processing factors is not unprecedented. For example, correct 3′ end formation of U1 and U2 snRNAs requires a special transcription complex which is specified by sequences in the 5′ flanking region of the snRNA gene. Substitution of other RNA pol II promoters results in incorrect 3′ end formation [Hernandez and Lucito, 1988; Hernandez and Weiner, 1986; Neuman de Vegvar et al., 1988]. Second, splicing and polyadenylation occur very inefficiently if RNA polymerase I or III promoters are used to drive transcription of a protein-coding gene [Sisodia et al., 1987; Smale and Tjian, 1985]. Third, the necessity of introns for efficient expression of the immunoglobulin μ mRNA can be eliminated if a cytomegalovirus or heat shock promoter is used to drive transcription [Neuberger and Williams,

1988]. Finally, promoter sequences have been shown to have an effect (in *trans*) on transport of mRNA from the nucleus to the cytoplasm in microinjected Xenopus oocytes [de la Pena and Zasloff, 1987].

Experimental analysis of this model will be difficult due to its biochemical complexity. One possible approach might be to develop a coupled transcription-processing system using mammalian nuclear extracts. Earlier studies have shown that splicing does occur in in vitro mammalian transcription reactions, although at a low efficiency [Kole and Weissman, 1982; Padgett et al., 1983]. It might be possible to improve the efficiency and use such a system to determine if the TS upstream regulatory sequences are able to affect the efficiency of splicing in a cell cycle specific manner. For example, transcripts from templates that contained the upstream regulatory sequences might be efficiently spliced in extracts prepared from S-phase cells but not in extracts from G0/G1 phase cells. In contrast, transcripts derived from templates that lacked the upstream regulatory sequences might be spliced with the same efficiency in extracts from cells in G0/G1 or S phase.

Splicing is believed to occur on the nuclear matrix in specialized regions of the nucleus [Sharp, 1987; Wang et al., 1991; Xing et al., 1993]. These regions may contain complex aggregates of proteins and enzymes that are required for splicing and polyadenylation. For this reason, soluble in vitro splicing systems might not provide an accurate reflection of the processes as they occur within the nucleus. If the studies with nuclear extracts are not successful, it might be possible to add templates to permeablized nuclei instead of the nuclear extracts.

REGULATION OF TS mRNA TRANSLATION

A variety of in vitro, in vivo, and clinical studies have shown that TS enzyme levels increase following exposure of cells to TS inhibitors. Because such increases will reduce the therapeutic effectiveness of the inhibitors, there is considerable interest in determining the mechanisms that are responsible for the increase in enzyme levels. Cultured human and mouse cell lines that are resistant to high concentrations of TS inhibitors have been isolated. A high level of resistance is observed only after many months of continuous selection in increasing concentrations of the inhibitor. Resistance is due to overproduction of TS which is the result of a corresponding increase in TS mRNA and the number of copies of the TS gene [Jenh et al., 1985b; Berger et al., 1985]. Resistance via gene amplification has been observed with a variety of chemotherapeutic drugs [Kellems, 1993].

Rapid Increase in TS Following Exposure to Inhibitors

Acute exposure of cells to TS inhibitors such as fluorinated pyrimidines or folate analogs also leads to a rapid increase in TS enzyme level. The biochemical basis for this rapid increase is unlikely to be caused by gene amplification since amplification requires months of continuous selection. Keyomarsi et al. [1993] recently found that when normal or tumor-derived human mammary epithelial cells were exposed to the folate analogue ICI D1694, the TS enzyme level was elevated up to 40-fold within a few hours following drug exposure. Remarkably, the amount of TS mRNA remained constant during this interval. TS enzyme level increased to a lesser extent when the drug was administered in the presence of cycloheximide (a protein synthesis inhibitor). However, DRB (a transcriptional inhibitor) had no effect on the increase. These observations suggest that the rapid increase in TS enzyme level is due to an increase in the efficiency of translation of TS mRNA that is somehow caused by the drug treatment.

This conclusion makes testable predictions regarding the translation of human TS mRNA. It is difficult to conceive of a mechanism by which the rate of peptide chain elongation could increase by a factor of 40. Therefore the mechanism that is responsible for this increase in translation almost certainly involves a large increase in the efficiency of initiation of protein synthesis. This would predict that in the absence of inhibitors, the rate of initiation of translation of human TS mRNA is very low relative to that of other mRNAs. Assuming that the elongation rate is normal, this would lead to a small number of ribosomes per TS mRNA, and a relatively large fraction of TS mRNA that is not associated with ribosomes. The inefficient initiation of protein synthesis may be due, in part, to inverted repeats that are present in the 5′ untranslated region of human (but not mouse) TS mRNA [Kaneda et al., 1987]. Exposure of cells to the TS inhibitor might lead to an increase in the rate of initiation, leading to a significant increase in the number of ribosomes associated with the message and a decrease in the amount

of TS mRNA that was not associated with ribosomes.

TS Enzyme Controls Translation of Its Own mRNA

Chu et al. [1991] have uncovered evidence for a novel mechanism by which this translational regulation may occur. They found that the translation of human TS mRNA in vitro in reticulocyte extracts can be inhibited by the addition of pure human TS enzyme. The enzyme had no effect on the translation of other mRNAs. The inhibitory effect was prevented if TS substrates (dUMP or 5,10-methylene-tetrahydrofolate) or inhibitors (FdUMP) are added to the extracts. These observations raised the possibility that human TS enzyme regulates the translation of its own mRNA. The fact that translational inhibition was relieved by substrates or inhibitors of the enzyme are consistent with an autoregulatory loop in which the cell rapidly fine-tunes the rate of synthesis of the enzyme in response to changes in need for the enzyme.

TS appears to exert its translational control by directly binding to its own mRNA [Chu et al., 1991]. Mobility shift experiments have shown that human TS enzyme forms a specific complex with TS mRNA but not with other RNA species. Preliminary mapping of the binding sites revealed that two different regions of the mRNA form specific complexes with the enzyme. One binding site is within a 36 nucleotide fragment that encompasses the site of translational initiation while the other is within the coding region. Site directed mutagenesis has shown that specific nucleotides in the vicinity of the AUG start codon are important for the interaction between the fragment and the enzyme [Chu et al., 1993a]. It is not clear if secondary structure within this region is also required for the interaction.

More detailed analyses into the mechanism of interaction between TS and its mRNA will be required to define more precisely the sequence that is required for mRNA-protein interaction and to determine if additional proteins are required in vivo for translational regulation. It will also be important to define the RNA recognition domain on the enzyme and to determine if it bears any resemblance to RNA recognition domains on other proteins. Finally, it will be interesting to determine if the translational regulatory element of the mRNA, or the RNA binding domain of the enzyme, can be transferred to other mRNAs or proteins, respectively. If so, it may be possible to engineer novel translational regulatory controls into foreign genes simply by transferring the appropriate RNA or protein domains.

It will also be of interest to determine if the autogenous translational control mechanism has been conserved across evolution. It is logical to expect that an important regulatory mechanism will be retained in other mammalian organisms that are separated from humans by relatively brief evolutionary intervals. For example, mouse TS enzyme and mRNA are highly conserved across most of the open reading frame and should certainly be examined for this level of control. Interestingly, the sequences in the vicinity of the AUG codons of mouse and human TS mRNA are poorly conserved [Perryman et al., 1986], so it is possible that this type of control may not be observed with mouse TS mRNA. The phenomenon is not unique to TS mRNA since autogenous translational regulation has recently been reported by Chu and co-workers for human DHFR mRNA [Chu et al., 1993b].

Elucidation of the mechanism for the translational control of these mRNAs is important from a practical as well as a theoretical point of view. Such knowledge may suggest novel strategies for overcoming the rapid increase in enzyme level and the concomitant resistance to the inhibitor. For example, it might be possible to design novel inhibitors that block enzyme activity without disrupting the interaction between the enzyme and its mRNA.

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