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Coordinate Control of S Phase Onset and Thymidine Kinase Expression

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INTRODUCTION

Cell proliferation is a stringently regulated process in normal mammalian cells. Regulation of proliferation becomes deranged in cancer cells. Exogenous growth factors are generally believed to activate the proliferation of normal cells. In successive cell cycle stages, preparation for the final division involves duplication of the cell's initial components through a multitude of reactions. But only a few events are readily observed, such as the onset of DNA synthesis at the beginning of S phase and the terminal morphological events of nuclear and cellular divisions.[1] Homeostatic control mechanisms must balance the myriad of biochemical events in each of the different phases of the cell cycle. However, these are secondary controls, in contrast to a few regulatory processes which are critical for proliferation, such as those placed upon the onset of DNA synthesis.[2] Altered biochemical events are observed in transformed and tumorigenic cells at these major control points. The relaxed growth control of tumor cells may result from mutational deregulation of genes acting at critical points in the sequence initiated by growth factor-receptor interactions.[3–5]

In this review, we shall discuss some of the events leading to the onset of DNA synthesis. The events also correlate with thymidine kinase gene induction, thus providing an approach for molecular biological investigation of S phase onset.

Early G_1 Events

A reasonable hypothesis is that cell proliferation is controlled by two major signal transduction systems (STS). These are functionally distinct in their times of action, growth factor dependency, second messengers involved, target genes activated, and resulting biochemical events. The first STS brings quiescent cells, within which many proteins and mRNAs have been depleted, back into the cell cycle. This primary STS operates in early G_1, within the first 6 h after growth factors are provided to quiescent fibroblasts.[6] For 3T3 cells, the principal growth factors known to be involved include

platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and probably insulin.[7, 8] The principal biochemical consequences are the restoration of mRNA and protein biosynthesis to their three-fold more rapid rates in cycling cells.[1] Many new mRNAs appear,[9–12] including those for the protooncogenes c-*fos* and c-*myc*.[13–15] The induction of these early genes is necessary to make cells competent, but not sufficient for DNA synthesis and cell proliferation.

The second STS, which becomes dominant in late G_1, about 6 h prior to DNA synthesis, is required to make the stimulated, competent cells progress to the G_1/S boundary. The principal growth factor required by fibroblasts at this time is insulin-like growth factor (IGF-1).[8, 16, 17] Second messengers for these later events are unknown, but may require new proteins, since rapid *de novo* RNA and protein synthesis are obligatory events for passage of cells beyond the restriction (R) point.[1] The R point is defined as that time during late G_1 after which cells become independent of exogenous growth factors and *de novo* protein synthesis; they have become committed to replicate their DNA.[2, 18]

Inhibition of total protein synthesis by 50 to 70% dramatically lengthens the pre-R point part of G_1 in the cell cycle.[19] If protein synthesis is totally blocked with cycloheximide (Chx), after the drug is removed the interval that is required for G_1 cells to enter S phase considerably exceeds the inhibition time.[8] To explain these results, we have proposed that a labile protein must accumulate to a threshold level in late G_1 for cells to continue into S phase. In order for any labile protein to accumulate, rapid synthesis would be required to surpass its degradation. From the excess delay data, a half life of about 2.5 h was calculated for this putative labile protein. Strikingly, transformed cells do not delay their entry into S phase after being treated identically with Chx.[8] These cells presumably can make sufficient quantities of the necessary protein, owing either to increased stabilization or enhanced synthesis. A protein that fulfills all of these criteria has been described.[20]

Late G_1 Events

One possible role for the proposed labile R point protein(s) is in the induction of DNA synthesis. Evidence that inducers of DNA synthesis are produced in late G_1 has been provided by fusing cells in early G_1 to

either mid- or late G_1 cells. These fusions resulted in a significant reduction in the length of the DNA pre-replicative period in the early G_1 nuclei.[21,22] Also, cytoplasmic extracts taken from proliferating cells stimulated the initiation of DNA synthesis in nuclei isolated from resting tissues such as frog liver and spleen.[23] Factors that control DNA initiation were also demonstrated by injection of cytoplasm from pro-liferating cells into eggs of the toad *Xenopus laevis*.[24] All of these results indicate that cytoplasmic factors made during G_1 can promote the synthesis of DNA. The nature of these factors is unknown. One candidate enzyme that may be involved in timing the onset of S phase is DNA helicase, since a DNA replication origin unwinding activity that appears to be critical for priming the onset of S phase was reported to be induced in late G_1.[25]

Coordinate Control of S Phase

The events leading from quiescence to the onset of DNA synthesis are extremely complex, requiring many hours and involving a variety of signals transduced from the cell's exterior to its nucleus. This, together with the complexity inherent in the DNA replication machinery,[26] makes the elucidation of controls that regulate DNA synthesis onset a formidable problem.

Concomitant with the onset of DNA synthesis, how-ever, the activities of a number of enzymes involved in DNA synthesis rise sharply, as does histone synthesis.[1] Induction of thymidine kinase (TK) activity parallels the onset of DNA replication,[16,27] and therefore it provides a suitable molecular marker for entry into S phase. An understanding of the mechanisms responsible for TK induction at the onset of S phase could provide clues for elucidating the sequence of requisite molecular events, and for defining factors that control gene induction and, perhaps, S phase onset.

Rapidly proliferating cells in tissues and in culture express high levels of cytosolic TK mRNA and enzyme[28,29]; slowly growing, resting, or terminally differentiated cells have very low levels.[30,31] Recently, the cytosolic TK enzyme has been purified and characterized from HeLa cells.[32] Pulse-labeling of cells that have been synchronized by elutriation indicates that the synthetic rate of TK protein is about 10-fold greater in S phase cells. As the steady-state level of TK mRNA is relatively constant during the cell cycle, the periodic accumulation of TK enzyme activity and protein has been attributed to an increased efficiency of TK mRNA translation during S phase.[33]

TK activity is almost undetectable in quiescent Balb/c 3T3 cells, and increases dramatically (approximately 40-fold) 12 to 14 h after restimulation with fresh serum.[27] These kinetics correlate well with the entry of these cells into S phase, as determined by labeling nuclei or flow cytometry. The induction of TK activity in Balb/c 3T3 cells, as well as the onset of DNA synthesis, appears to be regulated by a labile protein(s). A pulse of Chx given to cells in mid-G_1 resulted in an excess delay of 2 h in the increases of TK and *in vivo* thymidylate synthase (TS) activities, as well as in the onset of DNA synthesis.[16,27] In contrast, benzo[*a*]pyrene-transformed cells, which have no excess delay for the onset of DNA synthesis after a pulse of Chx, also had no excess delay for the induction of TK activity.

IGF-1 has been shown to be the only growth factor needed for the induction of TK and *in vivo* TS activities during the interval from 6 h to 2.5 h prior to S phase. Moreover, both RNA transcription and unimpeded pro-tein translation are required for the induction of these enzymatic activities during this interval. The observation that the growth factor requirement and metabolic condi-tions necessary for the induction of TK and TS activities and DNA synthesis are very similar suggests that all three processes may be controlled by the same cellular signal.[16]

The gene for cytoplasmic TK has been cloned from chicken,[34,35] hamster,[36] mouse,[37,38] rat[39] and human[40] cells. In addition, the genomic organization and pro-moter regions of these genes have been sequenced and characterized.[36,41,42] This information has provided the requisite tools for undertaking a detailed study of the molecular mechanisms that underlie TK mRNA induc-tion at S phase onset. Using these probes, one can hope to gain a better understanding of regulatory events involved in the onset of DNA synthesis.

Transcriptional Component of the Control of TK Expression

The ultimate production of TK enzyme begins with transcription to produce hnRNA, which is then pro-cessed to mRNA, and exported to the cytoplasm for translation. These steps may all be important in the regulation of gene expression (reviewed by Darnell[43]). Inducible and tissue-specific gene transcription depends upon proteins that bind to promoter and enhancer DNA sequences.[44,45] During selective processing of hnRNA and its subsequent export to the cytoplasm, there can be a substantial enrichment for a particular species of mRNA. Stability of both nuclear hnRNA and mature mRNA is a factor in determining the steady-state levels of mRNA. The rate of message translation and the stability of the enzyme are also important determinants of the level of enzyme activity. For instance, the TK protein is quite labile, with a half life of 4 h.[46]

Following stimulation of quiescent Balb/c 3T3 cells with fresh serum, TK mRNA is first detected in the cytoplasm at the G_1/S boundary, slightly preceding the burst of TK enzyme activity.[47–49] The increase of TK

mRNA at the G_1/S boundary might in part result from transcriptional regulation involving temporal changes in the binding of specific transcription factors to the TK promoter, since transcriptional regulation of mammalian genes is mediated by specific DNA-protein interactions.[44, 50] To test this hypothesis, many DNA fragments spanning about 1 kb upstream of the human TK gene were examined for their ability to bind protein in a cell-cycle dependent manner using electrophoretic mobility shift assays.[51, 52] Complex formation was detected between nuclear factors and three DNA regions. Two were located in the promoter region,[53] within 100 bp of the transcriptional start site of the TK gene.[41, 54] Nuclear extracts derived from mouse cells at various times during the G_0 to S phase transition formed nucleoprotein complexes with this promoter region, which contained an inverted CCAAT motif and a TATA box. The mobility pattern changed dramatically at the G_1/S boundary[53]; this change correlated well with increased TK gene transcription at this time.[48, 49] Several nucleoprotein complexes that exhibited different mobilities were observed during the G_1 to S phase transition, suggesting that more than one protein was involved.

The precise location of nuclear factor binding sites was determined by methylation interference[55-57] experiments using a DNA fragment between base pairs -63 and $+4$ from the human TK promoter. Nuclear extracts prepared from both G_0 and G_1/S cells demonstrated specific binding to two guanosines comprising part of an inverted CCAAT motif at -36 on the coding strand. Two CCAAT motifs are present in the TK promoter. Using oligonucleotides, specific competition with both inverted CCAAT sequences for the binding protein was demonstrated. Dimethylsulfate protection of the β-globin CCAAT box also occurred at the corresponding guanosines.[58]

A variety of CCAAT-binding proteins have been described.[59-63] Partial purification and Southwestern blot analysis of nuclear extracts from 3T3 fibroblasts revealed one protein having a molecular weight of 33 kD, which binds the CCAAT sequence within the human TK promoter (G. B. Knight and Y-D. Guo, unpublished results). Arcot et al.[66] have presented evidence which suggests the protein is NF-Y.

Functional assays of TK transcription were performed by fusing the TK promoter to the chloramphenicol acetyltransferase (CAT) gene, and transfecting these constructs into mammalian cells. TK transcriptional elements within the promoter region appear to be required for CAT transcription in parallel to the onset of S phase[64-66] (and G. B. Knight, unpublished results).

Posttranscriptional Control of TK Expression

Posttranscriptional mechanisms must also be impor-

tant for TK mRNA accumulation at the onset of DNA synthesis. By measuring rates of TK gene transcription with nuclear run-on assays only a 3- to 4-fold increase was observed at the G_1/S boundary with mouse cells.[49, 67] With CV-1 cells, Stewart et al.[48] found a 6- to 7-fold burst in TK gene transcription, which then declined to approximately 2- to 3-fold during S phase. In contrast, no differences in chicken TK gene transcription were detectable in isolated nuclei derived from either dividing or non-dividing cells.[68] As the steady-state level of TK mRNA varies to a much greater degree (at least 20-fold) in all of these cell types, other controls, in addition to increased transcription are involved in TK mRNA accumulation. Experiments were performed in which heterologous promoters linked to cellular TK cDNA sequences were transfected into recipient cells and examined for cell cycle-regulated expression. Genetic elements capable of conferring some growth phase-dependent regulation on TK mRNA levels[48] and TK enzyme activity[67, 69] were reported to be contained entirely within sequences of the mature cytoplasmic mRNA, again suggesting several levels of TK mRNA control.[31, 37, 38, 48, 70]

Evidence has recently accumulated to indicate that mRNA regulation also occurs at the levels of processing and stabilization of hnRNA.[71, 72] When serum-starved cells were released from quiescence, we observed a dramatic change in the processing of TK hnRNA at the onset of DNA synthesis, characterized by the appearance of a series of high-molecular-weight precursor bands. Evidence that these bands represent *bona fide* processing intermediates derives from hybridization analyses. A TK cDNA probe hybridized to the entire series of precursors, but specific intron probes hybridized only to discrete subsets of the larger bands. These results suggest that quiescent cells may be inefficient in processing certain mRNAs, such as TK, that are required during S phase, and this activity might be gained just prior to the onset of DNA synthesis.[73] The failure to process TK hnRNA may somehow signal its degradation in the nucleus, thus accounting for the low levels of TK mRNA in G_0 cells.

The presence of relatively few high-molecular-weight TK hnRNA precursors indicates an ordered removal of intron sequences from this gene. Moreover, the ability to chase high-molecular-weight precursors to lower-molecular-weight species in the presence of actinomycin D suggest a precursor-product relationship.[73] We have recently obtained DNA sequence data for all of the exons comprising the murine TK gene. In addition, we have defined the intron-exon splice junctions and determined the size for each of the six TK introns. The genomic organization of the TK genes from human,[41] Chinese hamster,[36] and mouse[67] has been highly conserved in all

three species. Using specific probes derived from each of the TK introns, we have recently demonstrated that there is, indeed, a precursor-product relationship among the higher-molecular-weight species and that TK intron processing occurs by two predominant routes. Our results concur with data from studies of phospho*enol*pyruvate carboxy kinase[74] and ovomucoid and ovalbumin gene expression,[75] showing that these intervening sequences are removed by more than one pathway.

CONCLUSIONS

The information that we and others have derived from molecular studies of TK gene induction at the onset of S phase clearly indicates that this gene is controlled at

multiple levels in the cell. The challenge for the future will be to identify and characterize the regulatory factors that are responsible for the increased rate of TK gene transcription and its nuclear posttranscriptional processing at this critical point in the cell cycle. In addition, much can be learned about how a cell integrates the myriad of external and internal signals, and ultimately coordinates the multiple regulatory events involved in TK mRNA transcription and processing, as well as the initiation of DNA synthesis at nearly the same time. A better understanding of these processes in normal cells will help shed light on how these critical control circuits become deranged in tumor cells.

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