

Quantification of thymidine kinase (TK1) mRNA in normal and leukemic cells and investigation of structure-function relationship of recombinant TK1enzyme

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Quantification of thymidine kinase (TK1) mRNA in
normal and leukemic cells and investigation of
structure-function relationship of recombinant TK1
enzyme

Ph.D Thesis
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Paper 2: Kristensen, T., Jensen, H.K and Munch-Petersen, B.: Quantitation of TK1 mRNA in patients with chronic lymphatic leukemia. Purine and pyrimidine metabolism in man VIII. *Advances in Experimental Medicine and Biology.* (1995), 370, 23-26.

Preface

My experimental work during the last three years is collected in this thesis to complete my Ph.D-study. The work was performed in the laboratory of associate professor Birgitte Munch-Petersen at Roskilde University. The purpose of my work was first to establish a sensitive system for quantification of thymidine kinase (TK1) mRNA and to compare TK1 mRNA and TK activity in healthy and malignant cells. This project was supported by the Danish Cancer Society. Secondly, I have investigated the structure-function relationship in recombinant TK1 mutants. The TK1 mutants were purified and characterized. This work was supported by the Danish Research Council.

Both projects are collected in this thesis "***Quantification of thymidine kinase (TK1) mRNA in normal and leukemic cells and investigation of structure-function relationship of recombinant TK1 enzyme***".

I am very grateful to my supervisor Birgitte Munch-Petersen for her valuable support and inspiration throughout the period. Likewise, I am grateful to associate professor Ole Skovgaard (Roskilde University) for helpful discussion in the cloning experiments.

I am thankful to Sven Erik Nielsen (Roskilde Hospital) for material from leukemic patients, to Reinhold Hofbauer (University of Vienna) and Gerd Folkers (ETH, Zürich) for the cloning vector, to Poul-Erik Jensen (University for Agriculture) for giving me the opportunity to work in his laboratory, to perform the Northern Blot technique.

I will like to thank the people from the laboratory and the office; Ph.D Helle Kock-Jensen, Ph.D students' Dvora Berenstein, Solveig Nygaard Ipsen and the technician Marianne Lauridsen.

Included in the thesis are 2 papers:

- 1: Kristensen, T., Jensen, H.K and Munch-Petersen, B.: Overexpression of human thymidine kinase mRNA without corresponding enzymatic activity in patients with chronic lymphatic leukemia. *Leukemia Res.* (1994), 18, 861-866.

- 2: Kristensen, T., Jensen, H.K and Munch-Petersen, B.: Quantitation of TK1 mRNA in patients with chronic lymphatic leukemia. *Purine and Pyrimidine Metabolism in man VIII. Advances in Experimental Medicine and Biology* (1995), 370, 23-26.

Roskilde University, 25. marts 1996

Ping Kristensen

Introduction

Thymidine kinase (TK) is a key enzyme in the salvage pathway of nucleotide metabolism catalysing the transfer of the terminal phosphate group of ATP to the 5'-hydroxyl group of thymidine. The product dTMP is subsequently phosphorylated by nucleoside kinases to dTTP, which is used for the synthesis of DNA.

In mammalian cells the total TK activity is expressed by two genetically distinguishable isoenzymes: TK1 and TK2. TK1 is only present in dividing cells and TK2 is present both in non-dividing and dividing cells but at a much lower level. Of the two isoenzymes TK1 is the most studied and the one generally referred to when talking about TK activity.

For several reasons, TK1 has become increasingly important in molecular biology and medicine. TK1 is cell cycle regulated, with the amount of TK1 protein and activity increasing markedly with the onset of DNA synthesis (S phase) and then declining to a very low level in G₂ phase. As TK1 is expressed concomitantly with other proteins and enzymes involved in DNA synthesis, it is assumed that TK1 plays a role in the replication of DNA. The combination of being cell cycle regulated and involved in DNA synthesis make TK1 useful as a model system for gene expression and particular for the cell-cycle specific events taking place at the G₁/S transition. An investigation of TK1 gene expression will not only provide insight into regulation mechanisms in normal cells but also into cancer cells.

In a variety of cancer and virus-infected cells the expression of TK1 differs from the expression in normal cells. Generally, the level of TK activity is increased. Cancer cells with a changed ratio of TK1 and TK2, e.g. expression of TK1 in quiescent malignant cells and/or expression of TK enzymes with biochemical properties different from the normal counterparts, TK1 and TK2 have been observed. Biochemical differences of TK's in cancer cells together with many DNA viruses encoding for their own TK, which have a broader substrate specificity than the human host TK, are extremely useful in the treatment of cancer and virus-infected cells with nucleoside analogs. Nucleoside analogs are modified nucleosides

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that, when phosphorylated by nucleoside kinases inhibit further DNA synthesis either by incorporation into DNA or by inhibition of DNA polymerases. A very efficient treatment with nucleoside analogs is seen in the treatment of Herpes Simplex Virus type 1 infections. Herpes Simplex virus encodes for its own TK, which can phosphorylate the nucleoside analog acyclic guanidine, while the human host TK cannot. Only cells infected with Herpes simplex virus will be affected - and killed - from the treatment with acyclic guanidine. However, not all nucleoside analogs are so selective in their effect. In the treatment of Human immunodeficiency virus (HIV) the nucleoside analog azidothymidine (AZT) is used. The triphosphate of AZT inhibits the virus transcriptase and thus blocks for further DNA synthesis. As AZT also is phosphorylated by the host TK1, HIV infected and non-infected cells are influenced by the AZT treatment.

To ensure the development of nucleoside analogs that will only interact with the altered or the virus-encoded TK's, it is important that the phosphorylating enzyme is characterized with respect to structure, activity and specificity. However, even though TK1 has been investigated throughout at the genetic level, TK1 is not very well characterized at the protein level. This is due to a low amount of TK1 in the cells and a high instability of TK1 during the purification. Achieving enough materials from mammalian tissue for structural analysis, which demands protein in the mg-scale, is therefore an overwhelming task. However, expression of a cloned TK1 gene in *E.coli*, it will be possible to achieve adequate amounts of protein for structure analyses by Nuclear Magnetic Resonance or X-ray diffraction. The structure-function relationship can be investigated by performing site-directed mutagenesis. This will give a knowledge about the structure of the active site in TK1 and facilitate the development of new nucleoside analogs.

Aims

The complex regulation of TK1 gene expression differs from one cell type to another. To create selective nucleoside analogs, it is of high value with a detailed knowledge about the phosphorylating enzyme regarding expression, substrate specificity and molecular structure.

This Ph.D thesis describes two different approaches for studying the regulation of TK1.

In the first part I investigated the relation between TK1 mRNA and TK activity in normal quiescent human lymphocytes stimulated to enter the cell cycle. As different regulation mechanisms may cause development of malignant cells, I also investigated the relationship between TK1 mRNA level and TK enzyme activity in a quiescent malignant cell type, chronic lymphatic leukemia (CLL). To measure the level of TK1 mRNA a very sensitive method, Competitive PCR, was established.

In the second part I studied the structure-function relationship of TK1. TK1 purified from lymphocytes was expressed as a glutathione-S-transferase fusion protein in *E.coli*. Two TK1 mutants with deletions from the C-terminal were constructed by the Recombination PCR method. In one mutant, TK1-193, a stop codon was introduced at amino acid position 194, deleting 40 amino acids from the C-terminal. According to a hypothetical structure for TK1 no function-essential site is deleted in this mutant. In the other mutant TK1-176, 57 C-terminal residues were deleted. According to the hypothetical structure, two residues (Cys¹⁸⁶ and Arg¹⁸⁷) which may contribute to ATP and/or substrate binding were absent in this construction. To evaluate any structure-function relationship, the recombinant TK1 wildtype and TK1 mutants were characterized and compared with native lymphocyte TK1.

Background

Thymidine kinase

Thymidine kinase (TK) - a salvage pathway enzyme

The DNA precursors (dATP, dCTP, dGTP and dTTP) can be synthesised by two different pathways, the *de novo* pathway and/or the salvage pathway. In the *de novo* pathway the deoxyribonucleotides are synthesised from ribonucleoside diphosphate through reduction of the 2'-hydroxyl group. The key enzyme for this reaction is ribonucleotide reductase (Thelander and Reichard 1979). In the salvage pathway the DNA precursors are synthesised by phosphorylation of deoxyribonucleosides, originating from the breakdown of DNA in dying cells or from damaged DNA. Figure 1 shows a survey of the salvage pathway and the last steps in the *de novo* pathway leading to dTTP.

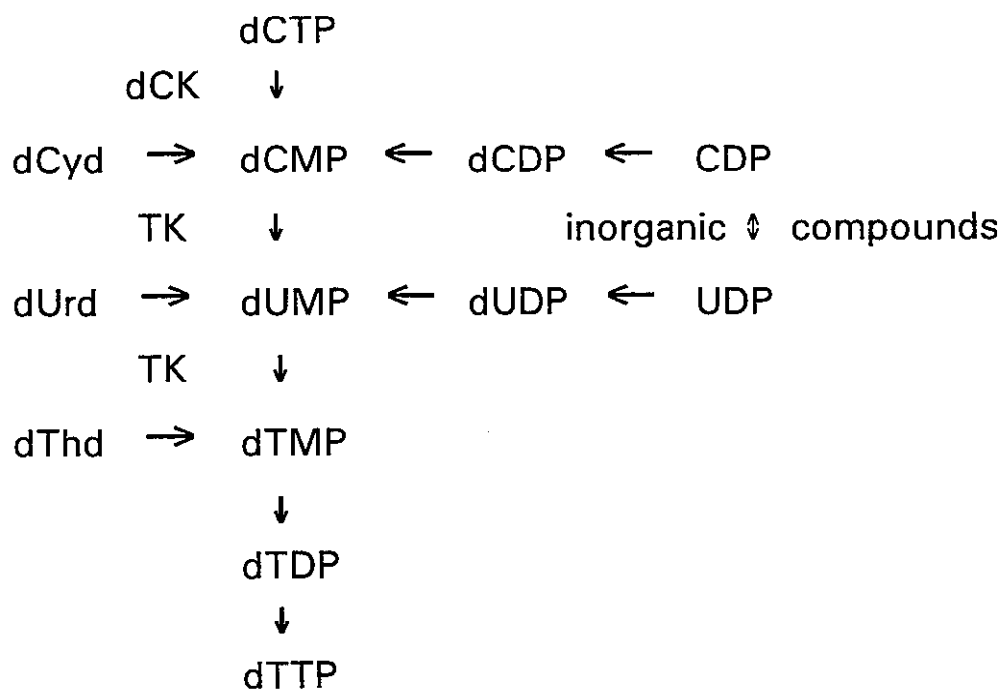


Figure 1: Synthesis of dTTP by salvage and *de novo* pathway. The right side represent the *de novo* synthesis, while the left side is salvage synthesis.

In the synthesis of dTTP, production of dTMP is the critical step. dTMP can be produced either by methylation of dUMP or by TK phosphorylation of dThd. dUMP can be produced by TK phosphorylation of dUrd or by deamination of dCMP. Both deoxyuridine and thymidine are phosphorylated by TK, so this gives TK a central position in the synthesis of the dNTP's.

As *de novo* synthesis can provide a dividing cell with all the necessary DNA precursors, it would be easy to regard the salvage pathway solely as a "cheaper" way to provide DNA precursors. However, there are important roles for the salvage pathway enzymes. Cohen et al (1983) have shown that in S-phase thymocytes¹, the *de novo* synthesis is responsible for the production of the purine DNA precursors whereas the pyrimidine DNA precursors are synthesised primarily by the salvage pathway. Likewise, in resting or G₁ thymocytes, where there is no ribonucleotide reductase activity the salvage pathway provides the cell with deoxyribonucleotides necessary for DNA repair (Cohen et al 1983).

dTTP as a regulator of the dNTP pool

An important role for the enzymes in the salvage pathway is also to maintain a balanced dNTP pool. It is well known that an imbalanced dNTP pool can result in cell killing, mutation induction, induction of chromosomal aberrations and carcinogenesis (Kunz 1988, Meuth 1989). As shown in Figure 1 TK plays an important role in the dTTP synthesis. dTTP, on the other hand has a major role in regulation of the dNTP pool. Ribonucleotide reductase catalyses the reduction of all the NDP to dNDP and the regulation of this reduction is controlled by the balance between the end products, the dNTP's. In the balance between the dNTP's, dTTP

¹ thymocytes are immature T lymphocytes

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is essential. An increase in the dTTP pool down-regulate reduction of CDP and up-regulate the reduction of GDP, which then up-regulate the reduction of ADP. So, an increase in the dTTP pool shifts the specificity of ribonucleotide reductase away from pyrimidine ribonucleotides and towards reduction of purine ribonucleotides (Thelander and Reichard 1979, Reichard 1987). As TK play an important role in dTTP synthesis, a deregulated TK will cause an imbalanced dTTP pool and that affect incorporation of the correct nucleotide into DNA.

TK as a tumour marker

As mention previously human cells have two TK's, TK1 and TK2. TK1 is activated at the G1/S phase of dividing cells whereafter the level of TK1 fluctuates with the progression through the cell cycle, being highest at DNA-synthesis (Bello 1974, Munch-Petersen and Tyrsted 1977). TK1 is therefore a good indicator of cell proliferation and is used as a tumour marker for a number of malignant diseases such as breast cancer (O'Neill et al 1992, Robertson et al 1990, Romain et al 1995) and non-Hodgkins lymphoma (Schwartz 1992, Hallek 1992). In a study of Romain et al (1995) 290 breast cancer patients were included in a 10-year follow-up study. Measurement of serum TK1 activity showed a good correlation between TK1 level and malignancy, and a high TK1 level was strongly associated with shorter overall survival.

Ellims et al (1981) have investigated the occurrence of TK1 and TK2 in patients with chronic lymphatic leukemia (CLL). The distinction between TK1 and TK2 activity were done, using the pronounced differences in substrate specificity. Both isoenzymes use ATP efficiently as phosphate donor, but TK2 can also use CTP efficiently, whereas it is a poor phosphate donor for TK1 (Ellims et al 1981, Ellims et al 1983, Sakamoto et al 1984, Sakamoto et al 1992). Ellims et al (1981) showed that in the indolent forms of CLL the dominating enzyme was TK2, but in the more aggressive forms of CLL the dominating enzyme was TK1. They suggested that the appearance and level of TK1 in CLL is coupled to aggressiveness of the disease.

Isoforms of TK1 and TK2 in leukemic cells

Enzymatic characterization of TK in several cancer types, has revealed TKs with enzymatic properties different from TK1 and TK2 (Munch-Petersen and Tyrsted 1985, Munch-Petersen and Tyrsted 1986, Munch-Petersen 1990). In these experiments the characterization was based on substrate kinetics with thymidine and ATP plus inhibition kinetics with dTTP. In three patients with AML (acute myelocytic leukemia) (Munch-Petersen and Tyrsted 1988) and in one patient with CLL (chronic lymphatic leukemia) (Munch-Petersen and Tyrsted 1986) a TK enzyme-form with thymidine substrate kinetics and TTP inhibition kinetics similarly to TK1 from dividing lymphocytes was observed. However, the enzyme, nominated TK1-*onc* (oncogenic form of TK1), had a lower molecular weight and an altered ATP kinetic than TK1 (Munch-Petersen and Tyrsted 1986). In a patient with AMOL (acute monocytic leukemia) yet two other forms of TK appeared. These two enzymes, called TK3-*onc* and TK4-*onc*, differed from TK1 and TK2 in kinetic properties and in molecular weight (Munch-Petersen and Tyrsted 1985). The three oncogenic forms of TK have also been observed in CML (chronic myelocytic leukemia), where three patients possess the TK3-*onc* form, two patients have the TK2-*onc* form and one patient has the TK1-*onc* form (Munch-Petersen 1990).

Apparently, leukemic cells express different forms of the normal counterpart, TK1 and TK2. Whether the altered TK enzymes are part of the development of a cancer cell or a consequence of it, is not known.

The cell cycle

TK1 is a cell cycle regulated enzyme tightly associated with DNA replication. Before explaining the several regulation mechanisms responsible for TK1 expression and regulation during the cell cycle, an overview of the mechanisms controlling the cell cycle will be presented.

The phases of the cell cycle

The proliferative cell cycle can be divided into four distinct phases; G_1 , S, G_2 and M.

The genome is replicated in S-phase and in the following G_2 phase the cell prepares for entry into M-phase, where the chromosomes condense and structural proteins important for mitosis are synthesized. After separation of the duplicated chromosomes and subsequently cell division the cells transverse into the G_1 phase. Here, the cell synthesizes many factors and enzymes needed for replication, and it is by far the phase where the cycling cell spend most of its time. In late G_1 a major control mechanism for cell proliferation is exerted. The mechanism, called the restriction-point (R-point) (Pardee 1974) controls the onset of a new cell cycle. Only cells that have a certain amount of growth factors can pass the R-point and start a new cell cycle. If conditions do not support a new cell cycle, a normal cell will not pass the R-point, but will instead enter a G_0 phase. Cells in G_0 phase are non-dividing but have still the potential to reenter the cell cycle. While the G_0 cells await a stimulation to reenter the cell cycle, proteins and RNA molecules are degraded and the rate of protein synthesis is markedly decreased (Furukawa et al 1990, Hofbauer and Denhardt 1991).

To be able to leave G_0 phase and enter G_1 phase the cell must respond to some "competence factors", which for lymphocytes may be an antigenic stimulus. The competence factors enhance expression of a set of genes, called early-response

genes, including the proto-oncogenes² *c-myc*, *c-fos* and *c-jun*³ (Hofbauer and Denhardt 1991). It has been shown that especially *c-myc* protein is important for the commitment to leave G₀ phase, as cells in which *c-myc* expression is prevented do not leave G₀ phase (Evan and Littlewood 1993). When the competent cells respond to a second group of growth factors, "the progression factors", e.g. lymphocytes respond to interleukin 2, they will progress into early G₁-phase. Further progression into late G₁ phase depends on the availability and abundance of a third set of growth factors, such as the insulin-like growth factors-1 (IGF-1) (Pardee 1989, Hofbauer and Denhardt 1991). In late G₁ phase another class of genes, the delayed-response genes, are transcribed. These include the genes for among others the cyclin-dependent kinases, the cyclins, thymidine kinase, dihydrofolate reductase and DNA polymerase α (Hofbauer and Denhardt 1991).

Transcription of some of these genes, e.g. the cyclins and the cyclin-dependent kinase are depending upon the product of the early-response *c-myc* gene (Furukawa et al 1990). At this point in G₁ the cell prepares for a new cell division, and after passage of the R-point the cell is independent of exogenous growth factors.

²proto-oncogenes are the normal counterparts in the eucaryotic genome to the oncogenes carried by some retroviruses. A single mutational event in a proto-oncogene is sufficient to activate it so the oncogene product can contribute to the abnormal growth of cells.

³*c-myc*, *c-fos* and *c-jun* encodes proteins that are implicated in transcription. The *c-myc* protein interact directly with DNA and influence gene expression and DNA repair. *c-fos* and *c-jun* proteins contributes to the formation of the AP1 complex (gene regulatory protein) that bind to specific sites in promoter/enhancer elements and augment transcription (Hofbauer and Denhardt 1991)

Control mechanisms in the cell cycle

More than 100 genes responsible for cell cycle regulation have been identified (Hofbauer and Denhardt 1991). It is out of the scope of this thesis to present a detailed elucidation of the mechanism controlling the cell cycle, but I will give an overview of the mechanisms taking place in the cycle.

Cyclins and cyclin-dependent protein kinases

Progression through the cell cycle is tightly linked to the activation of cyclin-dependent protein kinases (Cdk). Activation of a Cdk requires binding to a regulatory protein, a cyclin.

A general feature of the cyclin-Cdk complex is, that the concentrations of the Cdk stay constant through the cell cycle, whereas the concentrations of most of the cyclins fluctuate. This promotes a sequential activation and inactivation of the cyclin-Cdk complexes that control the progression through the cell cycle.

To transverse the cell cycle different cyclins are required at different stages (figure 2).

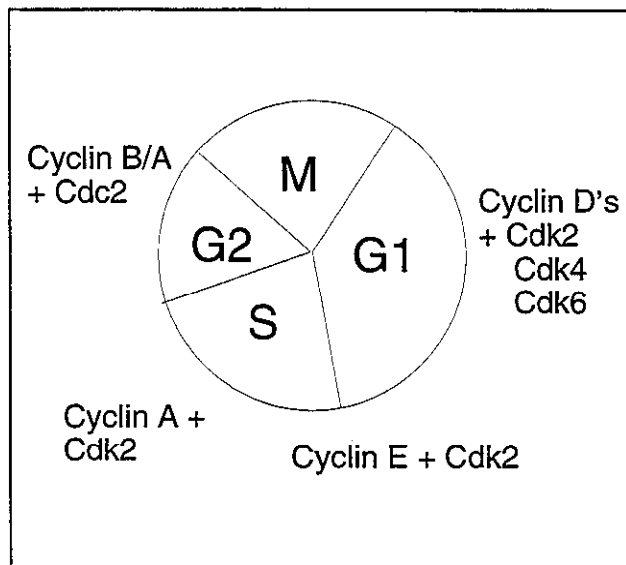


Figure 2: Cyclins through the cell cycle

In progression through G₁ phase two cyclins are the key regulators. The D-type cyclins (D1, D2, D3) which associate with either Cdk4 or Cdk6, drive the cell through the R-point (Lew et al 1991, Sherr 1994) by phosphorylation of the Rb (retinoblastoma) protein. From here, cyclin E in combination with Cdk2 takes over and controls the passage through the G₁/S transition (Lew et al 1991, Dulic et al 1992). After entrance into S-phase the cyclin E-Cdk2 complex dissociates and cyclin E is degraded (Sherr 1994). Cdk2 can now combine with cyclin A, into a complex which is believed to activate replication of DNA (Sherr 1993). During S and G₂ phase an inactive complex of cyclin B and Cdc2 (also called Cdk1) is accumulating. A dephosphorylation of Cdc2 in the cyclin B-Cdc2 complex at the end of G₂ activates Cdc2 and signals an entrance into M-phase (Hunter and Pines 1994). In M-phase there is an important checkpoint to ensure that the mitotic apparatus is correctly formed and the chromosomes are properly aligned. The cell can only pass this point if cyclin B is degraded, resulting in the release of inactive Cdc2.

There are more cyclins to control progression through the cell cycle than the above mentioned. These include cyclin C, F, G and H but their role is not fully elucidated (Hunter and Pines 1994).

Regulation by tumour suppressors

The Rb protein is a tumour suppressor⁴ which is an important regulator of cell proliferation. Through the cell cycle the Rb protein alternates between an inactive phosphorylated form and an active hypophosphorylated form. Rb is hypophosphorylated in G₁ phase, phosphorylated just before S phase and remains so until late M phase. Active, hypophosphorylated Rb binds a set of regulatory genes that favour cell proliferation. The binding inactivates the regulatory genes by preventing these from binding to the appropriate gene sequence. Hyperphosphorylated Rb protein is unable to bind regulatory genes, which then bind to the target genes and start transcription (Levine 1993).

One of the regulatory genes is probably the cellular transcription factor E2F. The promoters of several genes activated in late G₁ contain binding sites for E2F. These genes include dihydrofolate reductase, thymidine kinase and DNA polymerase α . But also genes which are critical for controlling the entrance into S-phase, such as Rb and cyclin A contains a site for E2F. It is therefore assumed that E2F is a critical regulator of activation of genes at the G₁/S-phase boundary (Nevins 1992a, Farnham et al 1993). For example, during G₁, hypophosphorylated Rb protein interacts with E2F and by that inhibits binding of E2F to the DNA (Chellappan et al 1991, Weintraub et al 1992). However, when the cyclin E-Cdk2 complex (which signals entrance into S-phase) has been assembled in late G₁, the Rb in the Rb-E2F complex is hyperphosphorylated (Cobrinik et al 1992, Hollingsworth et al 1993), thereby releasing "free" E2F, which can bind to the promoter and start transcription (Nevins 1992a, Nevins 1992b).

Another control mechanism is exerted by the tumour suppressor p53. p53 is a transcription factor but the gene or genes it regulates is not fully elucidated. Levine (1993) has shown that when normal cells are exposed to UV irradiation, the

⁴tumour suppressor genes encode proteins that suppress tumour formation. If both alleles of a tumour suppressor is lost by mutation, the lack of a functional suppressor can initiate a cancer

level of p53 protein increases due to a stabilization of the protein. The increased level of p53 protein blocks for progression through the cell cycle, allowing the cell to pause in G₁ while DNA repair proceeds. Cells with mutant p53 protein do not stop in G₁ and move into S-phase with damaged DNA (Levine 1993).

Cdk inhibitory proteins

The assembly of the cyclin-Cdk complex and their phosphorylation of other cell cycle regulatory proteins, e.g. Rb allows the cell to progress through the cell cycle. Besides this positive regulation, negative regulation occurs at check points. These regulation mechanisms are under control of CKI proteins (Cdk inhibitory proteins) that binds to the cyclin-Cdk complexes and inhibits their activity. It seems that inhibition of a cyclin-Cdk complex is a question of how many "CKIs" there are bound to the complex. During G₁ phase cyclin D-Cdk4 and cyclin E-Cdk2 complexes normally bind a single molecule of p21 (Hunter 1993, Sherr 1994) and the complex signals progression into S-phase. p21 is induced as part of the delayed early response to mitogen in cells entering the cycle from G₀ phase. However, the p21 gene expression is also under control of the tumour suppressor p53. Increased level of p53 increase the level of p21, which effect binding of more than one p21 molecule to the cyclin E-cdk2 or/and cyclin D-cdk4 complexes. This inactivates the cyclin-Cdk complex, thereby preventing progression into S-phase (Sherr 1994, Peter and Herskowitz 1994).

Two other negative regulators of G₁ progression are the tumour suppressor's p27 and p16. It has been shown that arrest of cells in late G₁ by addition of TGF β (transforming growth factor β) is associated with over expression of p27 (Peter and Herskowitz 1994). It is supposed that p27 inhibits cyclin E-Cdk2 activity in the same way as p21 (Sherr 1994). Recently it has been shown that Cdk4 and Cdk6 activity is under control of p16 (Lukas et al 1995). p16 is important as a negative regulator in late G₁ as it inhibits phosphorylation of Rb by binding to the Cdks, thereby preventing progression into S-phase.

Cell cycle and cancer

The R-point in late G₁ is an important control mechanism, to ensure that cells are properly "equipped" before they start a new cell division. The passage of the R-point and probably other minor checkpoints, are deregulated in malignant cells, due to deregulation of the cyclin-Cdk complexes (Hunter 1994). Lack of control can be due to aberrant expression of positive regulators, such as the cyclins and Rb, or lack of negative regulators, such as the CKI (p16, p21 and p53). Both types of changes have been documented in tumour cells (Hunter and Pines 1994).

Tumour cells do also display defects in a G₂/M checkpoint, allowing cells with damaged or incompletely replicated DNA to initiate mitosis. As mentioned previously, entrance into the M-phase is controlled by the cyclin B-Cdc2 complex. To enter M-phase the cyclin B-Cdc2 has to be activated, through a dephosphorylation of Cdc2. In normal cells DNA damaged by, e.g. radiation prevents dephosphorylation and arrests the cells in G₂, by a yet unknown mechanism. In several tumour cell lines, the cyclin B-Cdc2 complex is dephosphorylated regardless of the state of the DNA and therefore enters M-phase with damaged DNA (Hunter and Pines 1994).

TK1 as a model system for S-phase specific events

The combination of being cell cycle regulated and being involved in DNA synthesis have provided that TK1 is used as a model system for the regulatory events taking place at the G₁/S boundary of the cell cycle. An investigation of TK1 gene expression will not only provide insight into regulation mechanisms in normal cells but also into cancer cells.

A very good system for investigation of S-phase specific events is to use lymphocytes stimulated to cell division by mitogen. Peripheral blood lymphocytes are regarded as differentiated cells representing the end stage of lymphoid maturation. The cells are in a non-dividing stage and may have spent several years there without replicating DNA or undergoing mitosis. However, the cells have not

lost the ability to reenter the cell-cycle upon certain stimuli, e.g. by the mitogen PHA (phytohemagglutinin). PHA is an extract from the red kidney bean, *Phaseolus vulgaris*, (Nowell 1960). PHA has an agglutinating effect on the red and white blood cells, but is also able to trigger biochemical and cellular events that transform the non-dividing lymphocytes into a metabolically active cell. Initially after PHA addition, an increase in RNA, protein and DNA synthesis is observed (Hausen et al 1969). The increase in the rate of DNA synthesis is observed at ≈ 20 hours after PHA addition and is followed by a concomitant increase in the size of the dNTP pool (Munch-Petersen et al 1973, Tyrsted and Munch-Petersen 1977). At the onset of DNA synthesis several late-response genes, including DNA polymerase, thymidylate kinase, dihydrofolate reductase and thymidine kinase (Hofbauer and Denhardt 1991) show dramatically increased activities. Therefore PHA stimulation of lymphocytes is a good model system for studying changes and regulation of S-phase specific enzymes, e.g. thymidine kinase.

Regulation of TK1 expression

The human cytosolic thymidine kinase locus (TK1) is located on chromosome 17 q23-25 (Solomon et al 1993). The TK1 gene is 12.9 kb and consists of seven exons and intervening introns. The size of the exons is: 130, 32, 111, 94, 90, 120, 851 base pair (bp) for exons 1 through 7, respectively (Flemington et al 1987). Together they code for a 1430 bp mRNA with an open reading frame of 702 bp, specifying a protein of 25.5 kDa (Bradshaw 1983, Bradshaw and Deininger 1984).

Transcriptional regulation

Quiescent cells express very low levels of TK1 (Bello 1974, Johnson et al 1982) while at the onset of DNA synthesis, there is a severalfold increase in the rate of transcription, resulting in an increase in TK activity (Stuart et al 1985, Coppock and Pardee 1987, Stewart et al 1987). The very precise onset of transcription at the G₁-S border suggests that sequences in the TK1 promoter have an influence on the increase of transcription. That the TK1 promoter can confer cell cycle regulation is seen from experiments with chimeric constructs of the TK1 gene. When the non-cell cycle regulated genes, chloroamphenicol transferase (*CAT*) (Travali et al 1988) and neomycin resistance gene (*neo*) (Kim et al 1988), respectively, are under control of the TK1 promoter, both genes are maximally expressed in the S-phase, concomitant with the DNA synthesis.

The expression of eucaryotic genes is governed by a gene control region that consist of promoter and regulatory elements. Generally, most promoters for genes transcribed by RNA polymerase II contain three motifs for sequence-specific DNA binding proteins; a TATA-box, a CCAAT-box(es) and GC-rich elements. The TATA-box binds the general transcription factor, TFIID, and through a series of reactions RNA polymerase II is bounded. It is assumed that the CCAAT box play a strong role in determining the efficiency of the promoter, but the CCAAT box can also be a target for regulation. The GC-rich sequences (GGGCGG) bind the

transcription factor SP1. The promoter region responsible for transcription of the TK1 gene is located at region -456 to +32 bp. It contains a TATA-like sequence, two inverted CCAAT sequences and several GC-rich sequences. The two inverted CCAAT-boxes in the TK1 promoter is found at -40 (the proximal CCAAT) and at -70 (the distal CCAAT), see figure 3 (Kreidberg and Kelly 1986, Flemington et al 1987, Arcot et al 1989).

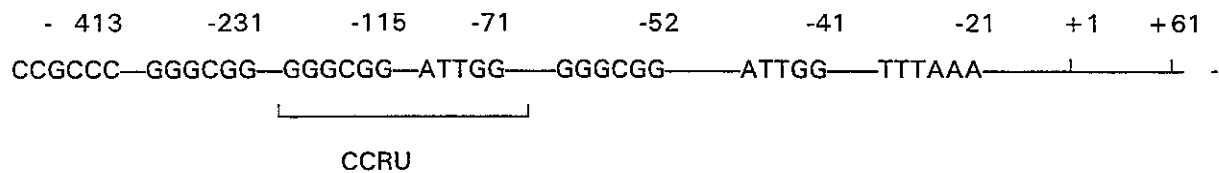


Figure 3: The human TK1 promoter. The numbers refer to the first nucleotide in the written sequence starting from +1 which is the transcription start. +61 is the translation start. CCRU is the cell cycle regulating unit, see text. The figure is based on Kreidberg and Kelly 1986.

Site-directed mutagenesis of the distal CCAAT-box shows that the box is important for TK1 expression. A TK1 minigene, which was under the control of a TK1 promoter with mutation in the distal CCAAT box, was transfected into TK⁻ts13⁵ cells. The mutation (deletion of the first C in CCAAT) resulted in a loss of promoter activity (Lipson et al 1989). Recently, it has been shown that both the distal and the proximal CCAAT box contribute to the promoter strength but the contribution is not equivalent. Excision of the proximal CCAAT-box did not alter expression from the TK1 promoter, whereas excision of the distal CCAAT-box reduced expression to 55% of that of the parental promoter (Mao et al 1995). Mutation of either the proximal or the distal CCAAT-box reduced the promoter strength to about 40% of that of the native TK1 promoter. Apparently, a mutation

⁵TK⁻ts13 is a temperature-sensitive TK deficient mutant, derived from hamster fibroblast. As HAT (hypoxanthine, aminopterin, thymidine) medium does not support the growth of TK⁻ts13, the transformants can be selected by their phenotype in HAT medium.

in a CCAAT-box is more fatal than a deletion, which can be explained by the fact that when a CCAAT box is removed, other protein binding sites can partially or completely substitute for the missing segment. It is suggested that the distal CCAAT box can substitute for the proximal CCAAT-box, but the reverse is not possible (Mao et al 1995).

To delimit the minimal promoter sequence that confers cell cycle regulation, Kim and Lee (1991) created a series of TK1 promoter subfragments with deletions from the 5' end. The TK1-promoter was fused to the *neo* gene and transfected into K12⁶ cells. They identified a 70 bp region spanning - 133 to -64, designed CCRU (cell cycle regulatory unit, figure 3 page 17) to be essential for cell cycle regulation. This region contains the distal inverted CCAAT-box, a GC-island and an area (-84 to -109) with E2F-like protein binding sites (the consensus sequence is 5' TTTSSCGC, where S is C or G, Farnham et al 1993). Mutations of the E2F-like sequences eliminated the S-phase specific transcription of TK1 (Kim and Lee 1992).

In investigations of the promoter elements responsible for TK1 regulation Arcot et al (1989) have shown that the distal and the proximal CCAAT-box bind the same cellular protein factor, NF-Y. It was shown that the proximal CCAAT-box has a higher affinity for NF-Y than the distal CCAAT-box. As the NF-Y protein normally binds to non cell cycle regulated promoters, the higher affinity to the proximal CCAAT-box may indicate an involvement in constitutive expression of TK1. Results from Pang and Chen (1993) support the idea of differential binding to the two CCAAT-boxes. They showed that the binding of a CCAAT-binding protein was growth specific. In quiescent young fibroblast (IMR-90, human embryonic lung fibroblast) no binding of nuclear protein could be detected, but in serum-stimulated fibroblast an increased level of binding was detectable. However, in old serum-stimulated fibroblast the binding activity was barely detectable. Competition experiments with other CCAAT binding proteins showed that the binding of the

⁶K12 is a hamster lung fibroblast cell line. It is temperature-sensitive. When shifted to the non-permissive temperature, 39°C, the cells synchronize in G₁.

nuclear protein was highly specific for the distal CCAAT-box of the human TK1 promoter. Pang and Chen (1993) suggested a unique identity for this binding protein, based on its physical properties. They named the protein, CBP/tk (CCAAT Binding Protein for TK gene). Recently, it has been shown by western blot that CBP/tk is identical to the NF-Y protein (Chang and Liu 1994). NF-Y consists of two subunits, NF-YA and NF-YB (Hooft van Huijsduijnen et al 1987). Both factors are required for DNA binding and both are present in the binding complex. Using the same cells as Pang and Chen (1993), Chang and Liu (1994) demonstrated that the A-subunit of NF-Y was expressed differently in serum-stimulated and quiescent cells. The level was high in serum-stimulated IMR-90 cells but absent in quiescent cells, whereas the level of NF-YB was unaltered. It was concluded that the altered expression of the A-subunit in quiescent and serum-stimulated IMR-90 cells accounts for the induction of TK1-mRNA in these cells (Chang and Liu 1994).

Post-transcriptional regulation

The TK1 promoter is activated at the G₁-S border, resulting in a S-phase specific increase in TK1-mRNA. However, the transcriptional regulation cannot fully account for the induction or increase in TK1 mRNA and enzyme activity. Nuclear *run on* experiment's reveal a 4-fold increase in transcription rate for serum stimulated cells followed by a 20-fold increase in TK1 mRNA (Stewart et al 1987, Coppock and Pardee 1987). These results show the presence of other regulation mechanism.

There are indications for post-transcriptional regulation through RNA processing. Investigations of BALB/c 3T3⁷ cells progressing from G₀ into S-phase have shown a dramatic change in the processing of TK1 hnRNA (heterogeneous nuclear RNA) when cells reach the G₁-S boundary. Cells were synchronised in G₀ by serum-starvation. In G₀ very little mature nuclear TK1 mRNA was detectable,

⁷BALB/c 3T3 are mouse cells.

while in G_1 a very high molecular weight TK1 hnRNA appeared. At the G_1 -S boundary the very high molecular TK hnRNA was processed to smaller molecular TK hnRNA and an accumulation of the mature 1.4 kb TK1 mRNA within the nucleus was observed. These results suggest that factor(s) required for processing of TK1 hnRNA is either synthesized or activated at the G_1 -S boundary, allowing for the accumulation of mature TK1 mRNA (Gudas et al 1988).

Gudas et al (1993) investigated the TK1-mRNA level in Chinese hamster embryo fibroblasts CHEF/18 cells synchronized in G_0 , early G_1 and mid G_1 . After serum stimulation, the steady state level of TK1 mRNA and transcription rate was investigated. They showed that the TK1 specific transcriptional and post-transcriptional mechanism could be uncoupled. It was concluded that a transcriptional mechanism was involved in the regulation of TK1 mRNA during progression from G_0 into the G_1 . For the progression from G_1 to the S-phase, primarily post-transcriptional mechanisms were involved.

Translational regulation

Studies of serum-stimulated rat cells transfected with different constructs of human TK1-cDNA, under control of a variety of heterogeneous promoters (Ito and Conrad 1990) indicate that TK1 protein and enzyme activity is regulated independent of TK1 mRNA. When TK1 cDNA was under the control of, e.g. the SV40 early promoter, TK1 mRNA level was high through G_1 phase, whereas the protein and enzyme level were low until ten hours after serum-stimulation. The induction of TK1 mRNA is therefore uncoupled from the induction of TK1 activity and TK1 protein.

In experiments with HeLa cells, Sherley and Kelly (1988) have revealed a different post-transcriptional regulation mechanism. Cycling HeLa cells synchronized by centrifugal elutriation and mitotic selection were used to investigate the relation between TK1 mRNA, TK1 activity and TK1 protein. They observed that during a cell cycle the increase in TK1 mRNA was less than 3-fold. Measurement of the TK1 protein with a specific antiserum raised against the purified HeLa enzyme, revealed that the increase in TK1 protein between G_1 and G_2 was 15-fold with a good

correlation to TK1 activity level. Pulse labelling experiments showed that cells in S and G₂ incorporate [³⁵S]methionine into thymidine kinase about 12-fold more efficient than cells in G₁. It was concluded that the increase in enzyme activity and protein were due to increased utilization of TK1 mRNA.

Post-translational regulation

Evidence for a post-translational regulation mechanism in cycling cells was found by Kauffman and Kelly (1991). Human minigenes were expressed in murine cells deficient in TK expression. The minigenes were under expression of the TK promoter, the heterologous SV40 early promoter or the HSV promoter, respectively, and all exhibit cell cycle regulation. By addition of nocodazole (NOC⁸) to the transfected cells, Kauffman and Kelly (1991) have delimited the timing of degradation of TK1 protein to occur between metaphase and cytokinesis. Here, the half-life of TK1 was less than 20 minutes, in comparison to 40 hours for the rest of the cell cycle. The specific degradation of TK1 protein before M/G₁ phase may be controlled by sequences in the C-terminal of the polypeptide. A construct where the last 40 amino acids were removed (TK1 Δ 40), was not cell cycle regulated and TK1 protein and TK activity was stabilized throughout the cell cycle. Results from Kauffman et al (1991) support the importance of C-terminal residues in serum-dependent regulation of thymidine kinase. Transfection of serum starved mouse cells with TK1 Δ 40 under control of an independent promoter resulted in expression of TK1 protein and activity in quiescent (G₀) cells (Kauffman et al 1991).

In serum-stimulated HL-60⁹ cells it was shown (Chang and Huang 1993)

⁸NOC is a microtubule inhibitor, that arrest cells between metaphase and anaphase.

⁹HL-60 is a human promyelocytic cell line. HL-60 cells are

that the TK1 protein was phosphorylated in a growth dependant manner, and the fluctuation of TK activity was correlated to the extent of phosphorylation of seryl residues (Chang and Huang 1993). Likewise, a growth dependent phosphorylation of TK1 protein has also been documented in proliferative and M-phase arrested HeLa cells (Chang et al 1994). The TK1 phosphorylation was 6 to 8-fold higher in cells arrested in M-phase than in proliferative cells and the phosphorylated TK1 had a 10-fold lower affinity for thymidine ($K_m = 15 \mu\text{m}$) than TK1 in proliferative cells ($K_m = 1.5 \mu\text{M}$). Chang et al (1994) suggested that the hyperphosphorylation observed in M-phase results from a M-phase specific phosphorylation.

Yet another regulation mechanism operating at the enzymatic level has been found by Munch-Petersen et al (1993). In normal human lymphocytes TK1 is functionally active as a dimer which in presence of ATP can form a tetramer (the so-called ATP effect). The ATP effect is reversible and the two forms of TK1 have different enzymatic properties. The dimer (-ATP) has a low affinity for thymidine ($K_m = 15 \mu\text{M}$), while the tetramer (+ATP) has a high affinity for thymidine ($K_m = 0.7 \mu\text{M}$).

The results of Munch-Petersen et al (1993) and Chang and Huang (1994) shows there are two forms of TK1. The investigations of Chang and Huang (1994) was performed with crude enzyme extracts and it is suggested that a mitotic kinase be responsible for the change between the two forms. The experiments of Munch-Petersen et al (1993) showed that the two forms of TK1 were enzymatically different and also had a different native molecular weight. As Munch-Petersen et al (1993) used pure enzyme and showed that the transition between the two TK1 forms occurred at 4°C, it is very unlikely that a protein kinase associated with the enzyme is responsible for the transition. Whether the work of Munch-Petersen et al (1993) and Chang and Huang (1994) complement each other or the results which look similar, are due to different factors is not known.

undifferentiated but can be induced to differentiation by TPA (12-O-tetradecanoyl-phorbol-13-acetate)

Structure of human TK1

In the treatment of virally-induced human disorders and malignant conditions nucleoside analogues are widely used. These analogues must be phosphorylated to exhibit their toxic effect on the DNA synthetic level. To ensure the development of selective analogs it is important that the phosphorylating enzyme is characterized with regard to its structure, activity and specificity.

X-ray crystallography or NMR (Nuclear Magnetic Resonance) can elucidate the 3D (three-dimensional) structure of a protein. Unfortunately, crystallization of proteins is time-consuming and may be difficult to succeed. Even through NMR-analyses of larger biomolecules are in rapid development, the size of the biomolecules is the biggest limitation. Molecules larger than 25-30 kDa cause problems due to the overlap of resonances and their increased line width. Such limitations make it difficult to resolve protein structure.

However, as the sequence of a protein contains some information to define the 3D structure, data about protein folding, conserved regions and active sites can be obtained from EDB technology. These include the use of database technology to store, retrieve and compare known sequences; computer graphics to display models and manipulate known 3D structures. Together these techniques in combination with site-directed mutagenesis studies can give information of the possible 3D structure of proteins. Nevertheless, it is important to specify that the 3D structure of a protein cannot solely be predicted from its amino acid sequence and therefore the computer models can only give a **proposed** structure. For an exact structure it is necessary to await a structure obtained by X-ray crystallography or NMR analyses.

Alignment of the primary structure of human TK1 with other TKs

The functional essential domains in a protein are most likely found in the highly conserved gene regions. By alignment of the primary structure of a protein from different species, the highly conserved regions can be identified.

From size, quaternary structure, substrate specificity and feedback inhibition by dTTP all TKs can be grouped into two separate classes (Black and Hruby 1990a). Class 1 comprises the *Herpesviridae* family, characterized by a subunit size of 49 kDa and functional active as a dimer. Class 1 TK is not feedback inhibited by dTTP and can use both dCyd and dThd as substrates. Some class 1 TKs are also able to phosphorylate dTMP to dTDP. The dTDP is produced by thymidine monophosphate kinase (dTMPK) (Gentry 1992). The best known member of class 1 is probably Herpes Simplex Virus type 1 (HSV 1).

Class 2 covers all the non-herpetic TKs, including human, chicken, hamster, mouse, *E. coli* and the members of the *Poxviridae* family. In this class the TK polypeptides have a subunit size of approximately 20-25 kDa, which assemble into an active tetramer. Class 2 members have a strict substrate specificity for thymidine and are feedback-inhibited by dTTP (Black and Hruby 1990b, 1992b).

Figure 4 shows the alignment of the primary structure of several class 2 TKs. The alignment is based on information from Gentry (1992) and Bockamp et al (1991). The figure shows seven regions of complete identity, nominated as domain 1 through 7, identified by Black and Hruby (1990b).

```

                                domain 1    domain 2
                                ┌──────────┬──────────┐
HUM:MSCINLPTVLPGPSKTRGQIQVIL|GPMFSGKSTEL|M|RRVRR|FQIAQYKCL51
HAM:--NY-----|-----|-----|-----N---
MOU:--Y-----S-----|-----|-----|-----
CHI:-N-LTV-G-H---GRP-----F|-----|-----|--L---R--
VAC:                MNG-H--L-I|-----|I|-----|Y-----V
                                └──────────┴──────────┘

```

```

                                ┌──────────┐
HUM:VIKYAKDTRYSSSFCTHDRNTMEALPACLLRDVAQEALGVA|VI94
HAM:-----S-----D-----A-|--
MOU:-----N--S-----D----M---T--L----|--
CHI:LV-----TTGVS-----R---A-Q--Y-----S-|--
VAC:T---SN-N--GTGLW---K-NF---E-TK-C--LESITDFS|--
                                └──────────┘

```

```

                                domain 3    domain 4
                                ┌──────────┬──────────┐
HUM:GIDEGQFFPDI|MEFCEAMANAGKT|VIVAALDGTQFQRK|PFGAILNLVPLAES145
HAM:-----|V---V-----|-----|A--S-----
MOU:-----|VD---M---E---|-----|A--S-----
CHI:-----|V---K--T---|-----|A--S---Y-----
VAC:-----|V---R---E--I|-----|--NN---I--S-M
                                └──────────┴──────────┘

```

```

                                domain 5    domain 6    domain 7
                                ┌──────────┬──────────┬──────────┐
HUM:|VVKLTAVCM|ECFREAAAYT|KRLG|TEKEVEVIGGADKYH|SVCR|LCY189
HAM:|-----|-----|----|L-----|----|V--
MOU:|-----|-----|----|L-----|----|---
CHI:|---N---|---Y---S---|----|A-R-----|----|A--
VAC:|-----|K--K--SFS|----|E-T-I-I---N-M-Q|----|K--
                                └──────────┴──────────┴──────────┘

```

```

HUM:FKKASGQPAGPDNKENCVPVGKPGEAVAARKLFAPQQILQCSPAN.234
HAM:---S-V-----L-Q---S-V-----V--HNST--
MOU:---S-A-T--S---L-L-Q---LVV-----S--V--YNS--
CHI:-Q-.RP-QL-SE---V-MGV-QLDMP-S--I--S
VAC:IDS

```

Figure 4: Multiple alignment of primary sequence of vertebrate TKs from: HUM; human, HAM; hamster, MOU; mouse, CHI; chicken. Poxviral thymidine kinase from VAC; vaccinia virus. Domain 1-7 represent sequences having complete identity with human TK1. Identical residues are indicated by '-'. Additional gaps are indicated by '.'.

As seen from figure 4 the vertebrate TKs are very closely related to each other. Further, a significant sequence homology between human TK1 and the vaccinia virus (a poxvirus) TK is observed, with 82% amino acid identity (Kauffman and Kelly 1991). The vertebrate TKs have additional 15 N-terminal and 42 C-terminal (human) residues, respectively (Boyle et al 1987). Whether, the vertebrate TK was the ancestor and lost its N and C terminals when it became incorporated into the virus genome, or a poxvirus-like enzyme was the ancestor, is an open question (Boyle et al 1987). Kauffman and Kelly (1991) have shown that the last 40 C-terminal residues in human TK1 are responsible for cell cycle regulation of human TK1, but not for TK activity. During evolution the viral TK may have lost the C-terminal region of the protein, because cell cycle regulation is not necessary for virus multiplication. Another possibility is that the C-terminal of vertebrate TKs has evolved to serve as a regulatory function, rather than having an enzymatic role. The homology of the vertebrate TKs with *E.coli* TK (not shown in the figure) is around 50% (Bockamp et al 1990). According to a genealogic tree relating prokaryotic, viral and vertebrate TK amino acid sequences (Bockamp et al 1990, Gentry et al 1992), *E.coli* does belong to a group separate from the groups of vertebrates and pox viruses, respectively. Herpes viral TKs are not included in Figure 4 because apart from three highly conserved areas (see next chapter) there is no apparent similarity between the sequence of herpesvirus TKs and vertebrate/poxviral TKs. As mentioned earlier most (if not all) herpes viral TKs have deoxycytidine kinase activity and it is suggested that herpes viral TKs are evolved from cellular deoxycytidine kinase (Harrison et al 1991).

Alignment of the primary structure of several TKs with isofunctional enzymes

In an attempt to address a function of the highly conserved regions, alignment with an isofunctional enzyme with resolved structure can reveal the function of the conserved regions. Proteins with the same function do often have a similar conformation. Human TK1 belongs to the family of nucleotide-binding enzymes, and

two isofunctional members of this family are adenylate kinase (ADK) and the protein elongation factor EF-Tu. ADK is a phosphate-transferring enzyme, catalysing the reversible monophosphorylation from adenosine monophosphate to the diphosphate ($\text{MgATP} + \text{AMP} \rightleftharpoons \text{MgADP} + \text{ADP}$). EF-Tu binds GTP and this complex binds to an amino-acyl tRNA molecule. The crystal structure for both ADK and EF-Tu has been resolved by X-ray crystallography (for ADK: Pai et al 1977 and for EF-Tu: Berchtold et al 1993). For ADK an NMR structure (Fry et al 1985, Yan and Tsai 1991) has also been solved.

Folkers et al (1991) have aligned some class 1 and class 2 thymidine kinases with ADK and EF-Tu. They have predicted three conserved regions to be essential for substrate binding and transfer of phosphate groups. The regions are the nucleotide binding site (A), the thymidine binding site (B) and a site which may be important for phosphate binding and transfer (C) (figure 5).

A:

The nucleotide binding site:

Mg^{2+} binding aspartic acid:

HSV1	48	TLLRVYID <u>GPHGMGKTTTT</u>	HSV1	
EBV	40	PACSLFLEGAPGV <u>GKTTML</u>	EBV	
HUM	18	RGQIQVIL <u>GPMFSGKSTEL</u>	HUM	93 VIGID <u>EGQF</u>
HAM	18	RGQIQVIL <u>GPMFSGKSTEL</u>	HAM	93 VIGID <u>EGQF</u>
MOU	18	RGQIQVIL <u>GPMFSGKSTEL</u>	MOU	93 VIGID <u>EGQF</u>
VAC	3	GGHIQLII <u>GPMFSGKSTEL</u>	VAC	78 VIGID <u>EGQF</u>
ADK	7	KSKIIFVV <u>GPGSGKGTQC</u>	ADK	89 GFLID <u>GYPR</u>
EFTU	10	PHVGVNIT <u>GHVDHGKTTLT</u>	EFTU	75 YAHVD <u>CPGH</u>

B:

The thymidine binding site:

HSV1	156	ALTLI	<u>FDRHP</u> IAAL
EBV	142	DCWIL	<u>MDRHLLS</u> AS
HUM	102	FPDIMEFCEAMANAGKTVIVAALD.	G <u>TFORK</u> PF GAI
HAM	102	FPDIVEFCEVMANAGKTVIVAALD.	G <u>TFORK</u> AF GSI
MOU	102	FPDIVDFCEMMANEGKTVIVAALD.	G <u>TFORK</u> AF GSI
VAC	87	FPDIVEFCERMANEGKIVIVAALD.	G <u>TFORK</u> PF FNNI
ADK	98	EVKQGEERFERKI..GQPTLLLYVDAGP-----	
EF-TU	84	AD...YVKNMITGAILILVVAATD-G-----	

C:

phosphate binding and transfer:

```

HSV1    317  KRLRPMHVF.I.LDYDQSPA.GCRDAL
EBV     314  -DLSE.....FQDDL...GCWTEI
HUM     164  KRLGTEKEVEVIGGADKYHS.VCRLCY
HAM     164  KRLGLEKEVEVIGGADKYHS.VCRVCY
MOU     164  KRLGLEKEVEVIGGADKYHS.VCRVCY
VAC     149  KRLGEETEIEIIGGNDMYQS.VCRKCY
ADK     148  KRLETYYKATEPVIAFYEKRGIVRKVN
EF-TU   ---  -----

```

Figure 5: Alignment of various TK sequences with the isofunctional ADK and EF-TU. HSV1: herpes simplex virus type 1 TK, EBV; epstein barr virus TK, HUM; human TK, HAM; hamster TK, MOU; mouse TK, VAC; vaccinia virus TK, ADK; adenylat kinase from rabbit muscle, EF-TU; elongation factor. Homologous sequences are denoted in dark and underlined. "." denotes gaps and "-" denotes that the homologous sequence could not be found. The figure is constructed after that of Folkers et al (1991).

The proposed secondary structure of TK1

Computer programs with the probability for an amino acid to promote α -helix, β -strand or β -turn, respectively, are used to predict the secondary structure of a protein. Further, comparisons with isofunctional enzymes are performed, as isofunctional enzymes may share some characteristic supersecondary structure. An example is the Rossman fold, a unit consisting of a $\beta\alpha\beta$ -unit. The Rossman fold is a very common feature in nucleotide-binding proteins (Bradley et al 1987).

The conserved domains for human TK1 and an alignment of the predicted secondary structure (Folkers and Trumpp 1987, Folkers et al 1991) led to a very high degree of similarity with ADK. This resulted in a hypothetical model for the human TK1 3D structure, see figure 6 and table 1.

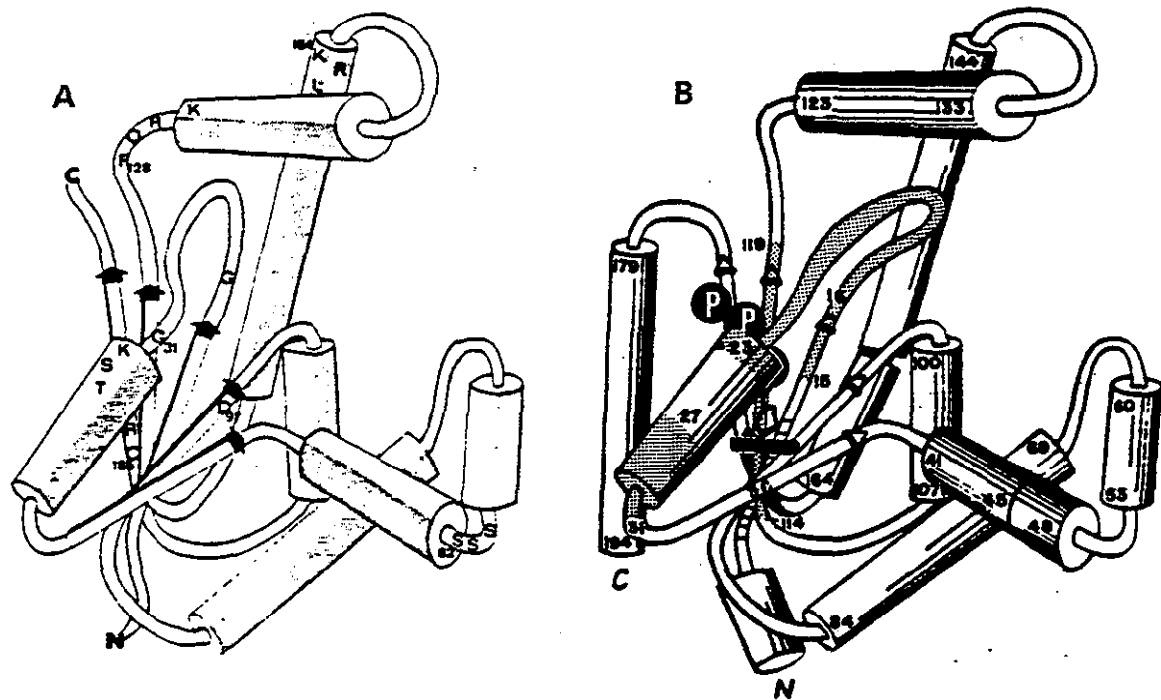


Figure 6: The proposed structure of A: human TK1 compared with the X-ray structure of B: ADK from rabbit muscle (Fry et al 1985). α -helixes are illustrated by bars and β -strands are illustrated by arrows. A is reprinted with permission from Folkers, G., Trumpp-Kallmeyer, S., Gutbrod, O., Krickl, S., Fetzer, J. and Keil, G.M. *J. Comput.-Aided Mol. Design*, 5 (1991) 385-404. Copyright 1991 ESCOM Science Publishers B.V..

Table 1: The hypothetical location of β -strands and α -helices in human TK1. The numbers refer to the β -strand and α -helix, starting from the N-terminal in figure 6. The table is based on information from Folkers et al (1987), Folkers and Trumpp (1987), Folkers et al (1989) and Folkers et al (1991).

β -strands in human TK1	
β 1	I ²¹ QVIL ²⁵
β 2	F ⁴³ QIAQ ⁹⁷
β 3	V ⁹⁴ IGI ⁹⁶
β 4	V ¹²⁰ IVALL ¹²⁵
β 5	V ¹⁸⁵ CRLC ¹⁸⁹
α -helices in human TK1	
α 1	K ³² STELMRRVRR ⁴²
α 2	see text
α 3	see text
α 4	see text
α 5	P ¹⁰³ DIMEFCEAMANA ¹¹⁵
α 6	K ¹³¹ PFGAI ¹³⁶
α 7	K ¹⁶⁴ RLGTEKEVEVIGGADKY ¹⁸¹

Comparison of figure 6 and table 1 reveals 3 α -helices (2, 3 and 4) depicted in the figure not accounted for in table 1. These α -helices are lying between amino acid 49-92 and secondary structure prediction state that it is an area with large helical character (Folkers et al 1991). Unfortunately, this area has a low primary structural homology to ADK, so alignment with ADK could not specify the precise location of the α -helices. As seen from figure 4 (page 25) it is also an area with very high variety through the various vertebrate TKs, so it is assumed that this region is located at the surface of the protein and does not play an essential role in substrate binding and conversion (Folkers et al 1991).

Folkers et al (1991) predict that human TK1 contains five parallel β -strands

and a very common structural feature for nucleotide binding enzymes. It is, as already mentioned, the mononucleotide binding domain also called the Rossmann fold. In figure 6 and table 1 it corresponds to $\beta_1\alpha_1\beta_2$.

In this theoretical model, the phosphate-binding loop, represented by the sequence G²⁶PMFSGK, appears after the first β -strand. This loop is followed by a very high conserved hydrophilic sequence, presumably an α -helix. The putative Mg-binding Asp⁹⁷ is located at the C-terminus of the third β -strand. The thymidine recognition site with the sequence F¹²⁸QRK is found in a loop region after the fourth β -strand, just above the nucleotide-binding site. Approximately 40 amino acids downstream of the supposed thymidine recognition site are the homologous sequence K¹⁶⁴RL, the phosphate binding site. Geometrical, it is assumed that the phosphate binding site is above the nucleotide binding site.

The hypothetical structure of human TK1 has been validated by a Ramachandran plot, a two-dimensional plot of the phi (φ) and psi (ψ) angles in the peptide bonding. Due to the partly double-bonding character of the peptide bond and sterical hindrance between the amino acid R-groups, the free rotation around the phi (-N-C ^{α} -) bond and the psi (-C ^{α} -C') bond is restricted. The Ramachandran plot gives the permitted values of phi and psi for different residues. A proposed structure is validated if less than 5-10% of the phi and psi coordinates are located in theoretical unfavored areas. The theoretical TK1 structure was also investigated for intramolecular hydrogen bonds and the appearance of charged amino acids in the core region. To achieve the most stable structure, the polar groups in the core region will form hydrogen bonds. In the model, this was the case for 90% of the internal polar groups. Normally, charged side chains will only appear in the core region, if they participate in the catalytical process. In the model, there were two charged amino acid in the core region, Lys³² and Asp⁹⁷. It is suggested that Lys³² participate in phosphate binding and Asp⁹⁷ in Mg²⁺-binding. It must be stated that this structure for human TK1 is purely hypothetical. To examine if and how the conserved regions are important for enzyme structure and function, site-directed mutagenesis must be performed. Up-to-date, there are no reports about site-directed mutagenesis of human TK1. As previous mentioned the homology of human TK1 with ADK and several other class 2 TKs is high (figure 4, page 25 and

5, page 27). Therefore, results from site-directed mutagenesis in ADK and class 2 TKs may provide an insight into the function essential sites in human TK1.

In the following I will describe the three conserved regions (figure 5) and the site-directed mutagenesis experiments performed in related TKs and ADK.

The nucleotide binding site

Many ATP-binding enzymes bind ATP as a complex with Mg^{2+} . The consensus sequence for the Mg-ATP binding site, consist of two motifs, nominated segments A and B. Segment A (figure 5.A), the ATP-binding motif consists of the consensus sequence GXYXGKZ, where X is any amino acid, Y is often a glycine or a proline and for ATP/ADP binding protein Z is usually a threonine (Rose et al 1985). This sequence is also called the glycine loop because of its relative high contain of glycine. Structurally this segment is a flexible loop, bounded by a β -sheet and an α -helix and is a part of the Rossmann fold. The importance of the glycine loop has been shown by Liu and Summers (1988) by site-directed mutagenesis. In HSV-1 the consensus sequence is GXXGXGKT and changes of any of the Gly to Val resulted in an inactive enzyme.

Segment B (figure 5.A) is the Mg^{2+} -binding motifs, which in the most simple form is represented by the consensus sequence Xh Xh Xh Xh D, where Xh is any hydrophobic amino acid (Myles et al 1991). The aspartic acid is the key residue in this segment and is normally found at the end of a β -strand (Black and Hruby 1992a). In ADK (from pig muscle) the ATP binding consensus sequence is located between amino acid Gly¹⁵ and Gly²² (figure 5.A and 6.B) and the Mg^{2+} binding amino acid is predicted to be Asp⁹³ (Yan and Tsai 1991). NMR-analyses of ADK from rabbit muscle (Fry et al 1986) show, that binding of Mg-ATP involves structural changes in several regions of the enzyme, with the largest displacement (6 Å) occurring at the glycine-rich loop. During binding of Mg-ATP the conformation of the protein change from an "open" to a more "close" conformation. This conformational change may affect: 1) The accessibility to the substrate binding site.

(2) Modification of binding-site affinity. (3) The conformational change may bring the catalytic groups towards the reaction centre and facilitate the transfer of a phosphoryl group (Fry et al 1986). The crystal structure of ADK was first resolved by Pai et al (1977). They suggested the ATP binding-site to be located between α -helix 69-84 and 100-107, and then according to figure 6.B, to be located on the right side of the glycine loop. The AMP binding-site was suggested to be located between the glycine loop 16-22, α -helix 23-30 and the C-terminal α -helix (figure 6.B). This prediction has caused debate and several reports suggest a rearrangement for the localisation of the two substrates. Kim et al (1990) have performed site-directed mutagenesis of several highly conserved arginine's, which were predicted to interact with phosphoryl groups of AMP and MgATP. They mutated Arg⁴⁴, Arg¹³², Arg¹³⁸ and Arg¹⁴⁹ to Ala and found that Arg¹³² interacted with MgATP and with AMP but with a decreased affinity. Arg¹³⁸ interacts only with AMP and Arg¹⁴⁹ interacts with AMP and to a lesser extent with MgATP. Arg⁴⁴ does not interact with either AMP or MgATP but may be located in the AMP binding-site. Based on these results they suggested placing ATP on the left side of the loop, with the α and β -phosphate groups of ATP around the glycine loop. The AMP binding site is placed on the right side. Binding of ATP to the glycine loop agrees with results from Yan and Tsai (1991). ²⁵Mg²⁺-NMR analyses on ADK (from chicken) reveal that Asp⁹³, which correspond to Asp⁹⁷ in human TK1, participates in binding of the co-substrate Mg-ATP. Substitution of Asp⁹³ → Ala resulted in a 650-fold decrease in V_{\max} (ATP). Asp⁹³ binds the Mg²⁺ which, in turn orients the polyphosphate chain so the phosphate groups are accessible for transfer (Yan and Tsai 1991). Interaction of the highly conserved glycine rich loop with ATP instead of AMP is more probable as ATP is generally required for most kinases, whereas AMP is not (Gentry 1992).

Black and Hruby (1992a) showed that site-directed mutagenesis of the Mg-binding Asp⁸² in vaccinia virus TK (which correspond to Asp⁹⁷ in human TK1) to hydrophobic residues; Asp⁸² → Leu, Asp⁸² → Ile, Asp⁸² → Val, respectively, resulted in an inactive enzyme. But, mutation of Asp⁸² → Asn resulted in a mutant with minor TK activity (9.4%). All the above mentioned mutants retained their tetramer-form. When Gly⁸⁴, which is part of the highly conserved region around the Mg²⁺-binding site, was mutated to a Val, the enzyme activity as well as the tetramer-

conformation was lost. This shows that Asp⁸² in vaccinia virus is involved in Mg²⁺ binding whereas Gly⁸⁴ has a structural role in the oligomerization of the enzyme.

Recently, a crystal structure of a truncated, but fully active HSV-1 TK was resolved (Wild et al 1995). HSV-1 TK was expressed in *E.coli* as a glutathione (GST)-fusion protein. Due to unspecific cleavage by thrombin, the 33 N-terminal amino acid was deleted (Michael et al 1995). From the crystal structure it appears that the phosphate-chain of ATP transverse the glycine loop but the Mg²⁺ binding amino acid is not revealed (Wild et al 1995).

For human TK1 segment A is predicted to span amino acid 26-34, while D⁹⁷ in the motif VIGID⁹⁷ may be the putative Mg²⁺-binding residue (these two sequences correspond to domains 1 and 3 from figure 4).

The thymidine binding site

As seen in figure 5, region B includes a sequence, for human TK1 represented by the consensus sequence F¹²⁸QRK and for HSV-1 TK represented by F¹⁶¹DRH. This sequence is highly conserved between class 1 and 2 TKs, but HSV-1 TK has an Asp¹⁶² corresponding to Gln¹¹⁴ in vaccinia virus TK. In the view of the different substrate specificity for the two enzymes, it was suggested that this domain may be involved in nucleoside binding (Folkers and Trumpp 1987). Experiments in favour of this proposal are seen with HSV-1 TK where mutation of Asp¹⁶² → Asn resulted in a completely inactive enzyme and mutation of Ala¹⁶⁸ → Thr resulted in decreased affinity of HSV-1 TK for the nucleoside analog BvdU (E-5-bromovinyl-2'-deoxyuridine) (Darby et al 1986). Another example is seen in the acyclovir (ACV)-resistant mutant of a varicella zoster virus, VZV, (a herpesvirus, not shown in the figure). In this TK mutant, in comparison with the wildtype VZV, is a change of the highly conserved Arg¹³⁰ in the F¹²⁸DRH motif to Gln. It was suggested that this amino acid change was responsible for the lack of ability to phosphorylate acyclovir (Sawyer et al 1988). The F¹²⁸DRH motif in wildtype VZV corresponds to F¹⁶¹DHR in HSV-1 TK so this indicated that the F¹⁶¹DRH motif is important for recognition of

acyclovir. However, HSV-1 TK accepts acyclovir as substrate whereas BHV1-TK (a bovine herpes virus) does not, and both TKs possess similar amino acids in the FDRH motif. Thus, additional amino acids may be part of the thymidine recognition site. For HSV1-TK two other highly conserved regions, D²¹⁵RL and K³¹⁷RL, have been suggested to be part of the active site. Michael et al (1994) have constructed the mutants Asp²¹⁵ → Arg and Lys³¹⁷ → Gly. Both mutants were enzymatically active, with the same K_m value as the wildtype, but a 4-fold lower V_{max} . These mutations are rather dramatic, with a change in polarity and an increased size. Therefore, Michael et al (1994) suggested a location for Asp²¹⁵ and Lys³¹⁷ on the surface of the protein instead of the core region. This contradicts the hypothetical model for HSV1-TK (Folkers et al 1991) but agrees with the preliminary X-ray structure of HSV1-TK (Wild et al 1995).

A phosphate binding site

Region C contains a sequence which is very highly conserved in many TKs. For human TK1 the sequence is represented by the amino acids: K¹⁶⁴RL and it is assumed that this region might play a role in phosphate binding and transfer of a phosphate group to thymidine (Folkers et al 1991).

Also, in region C and near the C-terminal are two highly conserved residues in nearly all TKs, Cys¹⁸⁶ and Arg¹⁸⁷, believed to have an influence on ATP and/or phosphate binding (Folkers et al 1991).

Experimental

Introduction

The regulation of TK1 gene expression is complex and differs from one cell type to another. To create selective nucleoside analogs, it is of high value with a detailed knowledge about the phosphorylation enzyme regarding substrate specificity, molecular structure and expression.

In the first part of my Ph.D thesis I wanted to investigate the relation between expression of TK1 mRNA and TK1 activity in quiescent human lymphocytes stimulated to enter the cell cycle by PHA stimulation. As different regulation mechanisms may cause development of malignant cells, I also wanted to investigate the TK1 mRNA and TK1 activity relationship in a quiescent malignant cell type, chronic lymphatic leukemia (CLL).

As mentioned earlier (page 14), lymphocytes stimulated to growth by PHA are a very used system for investigations of S-phase specific events and the system is well characterized with respect to TK activity, DNA synthesis and cell division (Barlow and Ord 1975, Munch-Petersen and Tyrsted 1977, Tyrsted and Munch-Petersen 1977). However, the level of TK1 mRNA in quiescent and PHA stimulated lymphocytes has not been determined.

The standard assays for detection and quantification of RNA include Northern blot hybridization and RNase protection assays. Nevertheless, beside being very time-consuming, the quantity of RNA required and the efficiency of RNA binding to the hybridization membranes limits the method. Especially, when low copy mRNA as TK1 mRNA is used. As the material from patients with CLL was restricted, a more sensitive method was necessary. The competitive PCR method (Gilliand et al 1990) can provide a much more sensitive method of detection and quantification. The method includes purification of total RNA and reverse transcription of RNA to cDNA. The quantification is achieved by co-amplification of

a competitive template that uses the same primers as those of the target cDNA, but the competitive templates can be distinguished from the target cDNA after amplification. The relative amounts of target cDNA versus a competitor can be revealed by ethidium-stained gel. Because the starting concentration of the competitive template is known, the initial concentration of the target cDNA, which is taken as representative for the mRNA can be determined.

Comparing the ratio of TK1 mRNA and TK1 activity in healthy lymphocytes and in CLL cells, respectively, may reveal differences in the regulation of TK1.

I also wanted to study the structure-function relationship of TK1. As described, the 3D structure of human TK1 (figure 6.A, page 29) is only a hypothetical structure based on computer models and alignment studies.

Structural elucidation of proteins by NMR-analyses or X-ray crystallography, demand amounts in the range of mg. The purification of TK1 from human sources is a very time-consuming task, as the cellular amount of TK1 is very low. From 1.5×10^{10} PHA stimulated lymphocytes (isolated from 12 l of donor blood) $\sim 30 \mu\text{g}$ pure TK1 can be isolated (Munch-Petersen et al 1991). Therefore, having an easily available source of pure TK1 is important. This can be achieved by expressing eukaryotic genes in procaryotes such as *E.coli*.

In 1994, Jensen constructed an expression system for direct expression of human TK1 in *E.coli*. The amino acid coding sequence of TK1 was cloned from pTK11 (Bradshaw and Deininger 1984) into a pET3a vector and transformed *E.coli* BL21(DE3)lysS. With this system the unmodified TK1 protein was expressed but the yield was only 1 mg TK1 protein/l bacterial culture, before purification. For this reason, I have chosen to work with a different expression system (pGEX2T-TK1) where the human TK1 gene is expressed as a fusion protein with glutathione S-transferase (GST). Fusion proteins have the advantage that the bacterial part of the fusion protein "masks" the eucaryotic gene. This normally allows for expression of larger amounts (Marston 1986).

To achieve information about the structure-function relationship I have constructed two TK1 mutants. As the long term perspective is to perform NMR-analyses on TK1 polypeptide, this had an influence on the type of mutants. The

subunit molecular size of TK1 is 24 kDa (234 amino acids) and in absence of ATP it will appear as a dimer (48 kDa), which is above the limit of what can be analysed by NMR. Therefore, deletion's mutants were constructed. In one mutant, TK1-193, a stop codon is introduced at amino acid position 194, deleting 40 amino acids from the C-terminal. According to the hypothetical structure, no function-essential site is deleted in this mutant. The other mutant, TK1-176, contains a stop codon at amino acid position 177. According to the hypothetical structure, the two residues Cys¹⁸⁶ and Arg¹⁸⁷, which may contribute to ATP and/or substrate binding are removed. Cys¹⁸⁶ and Arg¹⁸⁷ are in a supposed β -strand region, covering amino acid 185-189 (table 1, page 30). Beside deleting the last β -strand, a part of the last putative α -helix (number 7) are also deleted.

The stop codon was introduced by site-directed mutagenesis. The PCR method is best known as a method for detection and amplification of a specific DNA sequence, but PCR is also an excellent method for site-directed mutagenesis of a DNA sequence. Modifications can be introduced, because the primer sequence is incorporated into the amplification products. Substituting one or a few nucleotides in the primer sequence will cause alterations of the DNA sequence. The PCR-product can then be used in cloning procedures. One strategy for subcloning of PCR fragments is to take advantages of *E.coli*'s ability to recircularize linear plasmid molecules by a recombinational process. The recombination PCR (Jones and Howard 1991, Jones and Winistorfer 1992, Jones 1994) is a method for making DNA joints *in vivo* by the recombination of PCR-generated homologous DNA ends in *E.coli*. The basis for this event is that DNA ends containing short regions of homology can undergo intramolecular recombination *in vivo* in *E.coli* (Conley et al 1986a, Conley et al 1986b). Even recombination-deficiency (*recA*⁻) cells can produce transformants from linear DNA, although with an approximately 40-fold lower frequency (Conley and Saunders 1984).

The mutants and the recombinant wildtype proteins are expressed and purified. Pure enzyme is used for enzymatic characterizations. To evaluate the effect of deleting 40 and 57 amino acids, respectively, comparisons with the recombinant wildtype and the native lymphocyte TK1 are performed.

Methods for quantification of TK1 mRNA in healthy lymphocytes and in lymphocytes from patients with CLL.

Cells

Peripheral blood from six control persons was collected in Heparin vacuum tubes. Peripheral blood, from five patients with untreated chronic lymphatic leukemia (CLL) was similarly collected at Roskilde Hospital.

Isolation of mononuclear white blood cells

The lymphocytes were isolated by the Isopaque-Ficoll gradient centrifugation (Bøyum 1976). Peripheral blood (20 ml) was transferred to 50 ml centrifuge tubes and centrifuged 10 min at 900 RPM. The plasma layer was discarded and an equal amount of PBS (phosphate buffered saline) was added. The plasma/PBS solution was placed on top of 2/3 volume of Isopaque-Ficoll. After centrifugation for 30 min at 2500 RPM, the lymphocyte layer was washed three times with repeated suspension with PBS/5% FCS (fetal calf serum) and centrifuged for 5 min at 1600 RPM. Finally, the cells were resuspended in 8 ml PBS/5% FCS and the cell numbers were determined by coulter counting. After counting, the cells were pelleted and either stimulated with PHA or stored at -80°C (in portions of 5×10^6 cells).

PHA stimulation of lymphocytes from healthy persons

Lymphocytes from control persons were suspended in RPMI-1640 medium supplemented with 10% FCS and 20 $\mu\text{g/ml}$ penicillin/streptomycin, at a concentration of 10^6 cells per ml in 5% CO_2 at 37°C . The cells were stimulated to growth by 20 $\mu\text{g/ml}$ PHA for 48, 72, 96 and 168 h, respectively. The cells were harvested, counted and stored similarly to unstimulated lymphocytes.

Enzyme extract

The isolated cells from CLL patients and control persons were suspended in Loeb's buffer (20 mM K-phosphate buffer (pH 7.4), 15% glycerol, 1 mM K-EDTA, 10 mM DTT (dithiotreitol)), lysed by sonication (40W, 3 x 1-2 sec) and centrifugated at 20,000g for 30 min. The supernatant (the enzyme extract) was used for TK activity

measurement and determination of total protein.

TK activity assays

The radioactive assay for measuring TK activity is based on the conversion of the substrate [^3H]dThd to [^3H]dTMP and removal of the unphosphorylated substrate on anion exchange filters (DEAE-cellulose 81 paper) by three times washing in 5 mM ammoniumformiate and one times in H_2O for 5 min. The nucleotides were eluted from the filters with 0.2 M KCl/0.1 M HCl and the radioactivity determined by scintillation counting. Activity is measured as initial velocities (Munch-Petersen and Tyrsted 1977, Munch-Petersen et al 1991) by applying samples of 13 μl on DEAE filters 5, 10 and 15 min after starting the reaction by addition of enzyme extract to the assay mixture. The reaction temperature is 37°C. One unit of enzyme activity is the amount of enzyme catalysing the formation of one nmol dTMP per min. The standard assay mixture contained: 50 mM Tris-HCl (pH 7.5), 10 mM DTT, 2.5 mM ATP, 2.5 mM MgCl_2 , 3 mM NaF, 0.5 mM CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 3 mg/ml BSA (bovine serum albumin) and 10 μM ^3H -thymidine (2 Ci/mmol), in a total volume of 50 μl . In assays with CTP as phosphate donor ATP was substituted with equimolar CTP.

Protein determination

The protein content was measured by Coomassie brilliant blue as described by Bradford (1976).

RNA purification

Total RNA was purified by the method of Chromczynski and Sacchi (1987). Use of guanidinium thiocyanate, which is a strong inhibitor of ribonucleases provides a preparation of non-degraded RNA. The purity of the RNA preparation was improved by applying an extra phenol extraction and alcohol precipitation. 5×10^6 cells were centrifuged 14,000g for 10 min and the supernatant discarded. 500 μl of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) was added. Sequential 50 μl sodium acetate (2 M, pH 4), 500 μl phenol and 100 μl chloroform:isoamylalcohol (49:1) was added. The samples

were mixed thoroughly by inversion after the addition of each reagent. The final suspension was cooled on ice for 15 min and centrifuged 20,000g for 10 min at 4°C. The aqueous phase (RNA) was transferred to a fresh tube and 500 μ l isopropanol was added. The samples were mixed and placed at -20°C for at least 1 h to precipitate RNA. To sediment RNA the samples were centrifuged 10,000g for 20 min at 4°C. The supernatant was discarded and the RNA pellet was dissolved in 150 μ l solution D and afterwards precipitated by 200 μ l isopropanol and incubation at -20°C for at least 1 h. After centrifugation at 10,000g for 10 min at 4°C, the pellet was dissolved in 100 μ l DEPC (diethyl pyrocarbonate)-treated EDTA-buffer (0.5 mM, pH 8.0). The purity was improved by adding 50 μ l phenol:chloroform:isoamylalcohol (25:24:1) and the samples centrifuged 5,000g for 1 min at room temperature. The liquid phase was transferred to a fresh tube and 0.1 volume of 3 M NaAc and 3 volume of 96% EtOH was added. Before precipitation the samples were placed at -20°C for at least 1 hour and then centrifuged at 10,000g for 10 min at 4°C. The RNA pellet was dissolved in 50 μ l DEPC-treated EDTA-buffer. The RNA quality was examined by agarose gel electrophoresis and the concentration estimated from optical density at 260 nm. The purity was measured by the 260 nm/280 nm ratio.

Estimation of RNA recovery

In two experiments ^3H -uridine (5 μ Ci/ml, 5 Ci/mmol) was added to the growth medium during PHA-stimulation of the lymphocytes. A total of 5×10^6 labelled cells was harvested on 3 MM filters and non-incorporated ^3H -uridine was washed away. From an equal number of cells, RNA was isolated and applied to 3 MM filters. The radioactivity on the filters was determined by scintillation counting. The amount of the isotope in RNA was compared with the amount of the isotope in the cells. The RNA recovery estimated from these comparisons was between 70-90%.

Northern Blot

The RNA preparations were fractionated under denaturing conditions on a 1.8% agarose gel containing 2.2 M formaldehyde and transferred to a Hybond N⁺ membrane. The probes were human TK1 cDNA from plasmid pTK11 (Bradshaw and

Deininger 1984) and human β -actin cDNA labelled with ^{32}P -dCTP. Hybridisation was done with a high stringency wash.

Reverse transcription

Total RNA was converted to cDNA using the enzyme reverse transcriptase (RT), random oligonucleotides (hexamer) and dNTPs. RNA (0.67 μg) was transcribed to cDNA in a 50 μl volume of 1x PCR-buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.015% gelatine, 0.1% Tween 20), 7.5 mM MgCl_2 , 1mM of each of the dNTP's, 40 units RNasin, 7 μM random hexamers and 250 units of M-MLV reverse transcriptase RNase H minus. The reaction was ended after 2 h at 37°C. The extent of reverse transcription was controlled by a parallel reaction where ^3H -TTP, instead of TTP was added. Aliquot of the reaction mixture was applied on 3 MM filters. The non-incorporated ^3H -TTP was removed from the filter by washing 3x10 min in 1 M HCl containing 0.6 mM $\text{Na}_5\text{P}_3\text{O}_{10}$, 10 min in 0.26 M NaAc/EtOH and finally in EtOH. The radioactivity was measured by scintillation counting.

Competitive PCR

Quantification of TK1 mRNA is performed by the competitive PCR method as described by Gilliland et al (1990).

For quantification of TK1 mRNA exon 1 and exon 2 with intron 1 from the TK gene is used as internal standard and exon 1 and 2 as the target cDNA fragment. The primer pair used for amplification of both the genomic DNA and the cDNA are identical. The sizes of the resulting fragments were 138 bp with cDNA as template and 248 bp with genomic DNA as template. The internal standard of 248 bp genomic DNA was prepared by PCR with DNA as template and using the primers mentioned above. The product was quantified by agarose gel electrophoresis with different amounts of DNA. The unknown amount of cDNA was estimated from a set of PCR reactions performed in a dilution series with known amounts of the genomic DNA. The PCR products were separated by agarose gel electrophoresis. The amount of cDNA (g) in the sample was estimated as that amount (g) of genomic DNA giving equal intensity of the two amplification products (figure 7). The number of TK1 cDNA copies was calculated from the amount of

cDNA, by division with the average molecular weight of the 138 bp cDNA fragment (average molecular weight/base = 308). The number of copies TK1 cDNA was taken as representative for the number of TK1 mRNA.

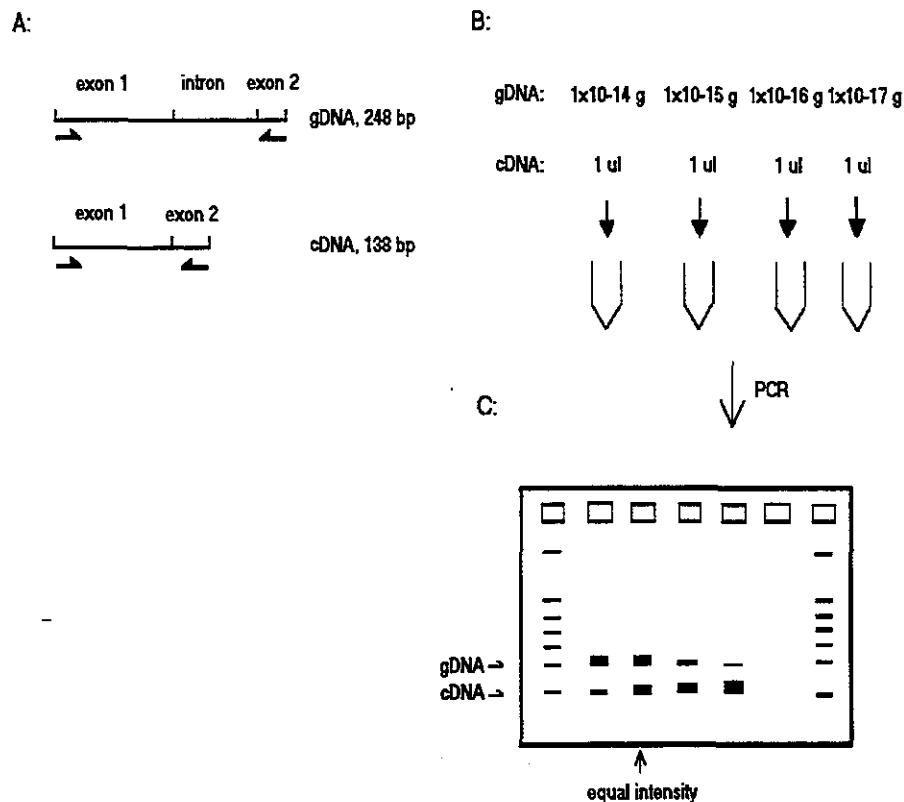


Figure 7: A schematic example of the Competitive PCR. A: The same primer pair is used to amplify genomic DNA and cDNA resulting in a 248 bp fragment for genomic DNA and a 138 bp fragment for cDNA. B: titration with dilutions of TK1 genomic DNA competing with 1 μ l of cDNA (138 bp). C: Analysis of the amount of cDNA. From the sample with equal intensity the amount of cDNA is calculated. The number of TK1 cDNA was taken as representative for the amount of TK1 mRNA.

The competitive PCR analyses were performed in a volume of 25 μ l containing 1x PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.015% gelatine, 0.1% Tween 20), 0,28 nM of each primer (Sense primer: TK1: 5'CTT GGA GAG TAC TCG GGT TCG TG 3', Anti-sense primer: TK2: 5'CCT TTT CCT GAG AAC

ATC GGC 3'), 200 μ M of the dNTP's, 1.5 mM MgCl₂ and 0.5 units of *Thermus aquaticus* DNA polymerase. Heat-denatured cDNA (100°C, 2 min) and internal standard were added to the reaction mixture, with a layer of mineral oil to avoid evaporation. The amplification was performed in a Perkin-Elmer/Cetus Thermal Cycler according to the following program: denaturation for 1 min at 95°C, annealing for 1 min at 60°C and polymerisation for 1 min at 72°C, for 35 cycles.

Methods for investigations of TK1 structure-function relationship.

Plasmid

The plasmid, pGEX2T-TK1, was constructed by the groups of Dr. Hofbauer and Dr. Folkers. As it is not published, I will present a short description of the cloning procedure (figure 8).

The amino acid coding region of TK1 from human lymphocytes was PCR-amplified. The two primers used to amplify TK1 are designed with restriction sites in the 5'ends (BamHI and Sph1, respectively). The resulting PCR product was subcloned into the expression vector pGEM. In pGEM, the BamHI-TK1-Sph1 fragment is surrounded by a KphI and Hind III restriction site. This fragment is subcloned into the pBluescript II KS +/- vector. pBluescript II KS +/- is digested with BamH1 and EcoRI and the BamH1-TK1-EcoR1 fragment is then subcloned into the expression vector pGEX2T, resulting in the pGEX2T-TK1 plasmid (figure 8). In pGEX2T, the fusion protein is cleaved with the restriction protease thrombin. Thrombin recognises the sequence LVPRGS (single letter code) and cleaves after the arginine (R). To construct the thrombin cleavage site in pGEX2T-TK1 modifications of the N-terminal in TK1 was necessary. MCS of the N-terminal of the native TK1 was changed to GSMCS. Likewise, to reconstruct the EcoRI site the C-terminal was modified. ILQCSPAN of the native TK1 was changed to ILQCMQA.

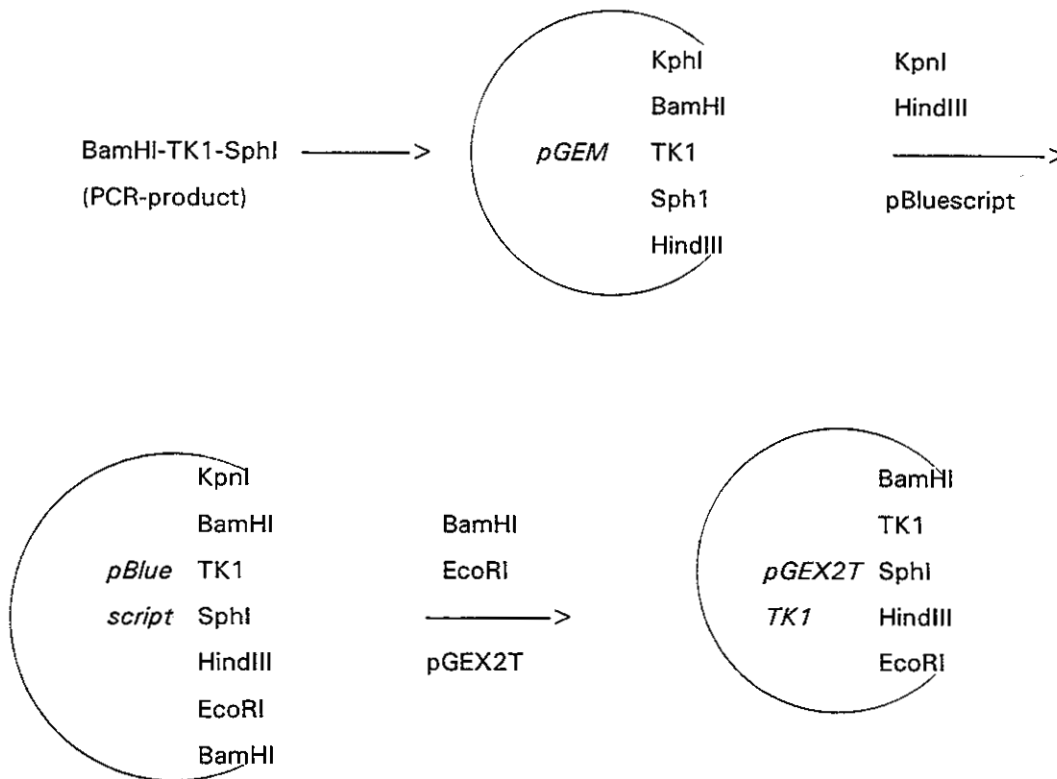


Figure 8: Cloning of pGEX2T-TK1 by the groups of Dr. Hofbauer and Dr. Folkers.

The pGEX2T vector contains the *lacI^q* gene region coding for the lac repressor, so expression of the GST fusion protein is independent of the *E. coli* host *lacI* status. The expression of GST fusion proteins is under control of the *tac* promoter and is efficiently repressed until induction with the lactose analog isopropyl β -D-thiogalactoside (IPTG).

***E. coli* strains**

KY895: a TK-deficient strain (F^- , *tdk-1*, *ilv-276*) (Hiraga et al 1967).

Max efficiency DH5 α competent cells: (F^- , $\phi 80d/lacZ\Delta M15 \Delta(lacZYA-argF)$ *recA1 endA1 thi-1*) (Life Technologies).

Recombination PCR

In the recombination PCR technique two separate PCR-reactions are performed. Each reaction contains the pGEX2T-TK1 plasmid, carrying the TK1 insert that is to be mutated. Prior to each PCR-reaction, pGEX2T-TK1 is linearized by digestion with

a restriction enzyme. The linearization serves two purposes. First, any circular plasmid products, which otherwise will be favoured in the transformation procedure are eliminated. Secondly, amplification of large fragments from linear templates is easier than from circular, supercoiled structures. In reaction 1 pGEX2T-TK1 is linearized by Aat II and in reaction 2 pGEX2T-TK1 is linearized by Hpa I. The restriction sites are located outside the fragment to be amplified.

The PCR-reaction is performed with a primer containing the mismatch bases, designated the mutagenic primer. The second primer is non-mutagenic and is used to delimit the amplified fragment. A sense and an anti-sense version of both the mutating and non-mutating primers are constructed. Each PCR-reaction is then performed with a mutagenic and a non-mutagenic primer, resulting in two PCR-fragments with homologous ends. Combining the two PCR fragments and transforming them into high competent DH5 α *E.coli* cells results in an *in vivo* recombination. The product is a mutant with the desired mutation (figure 9).

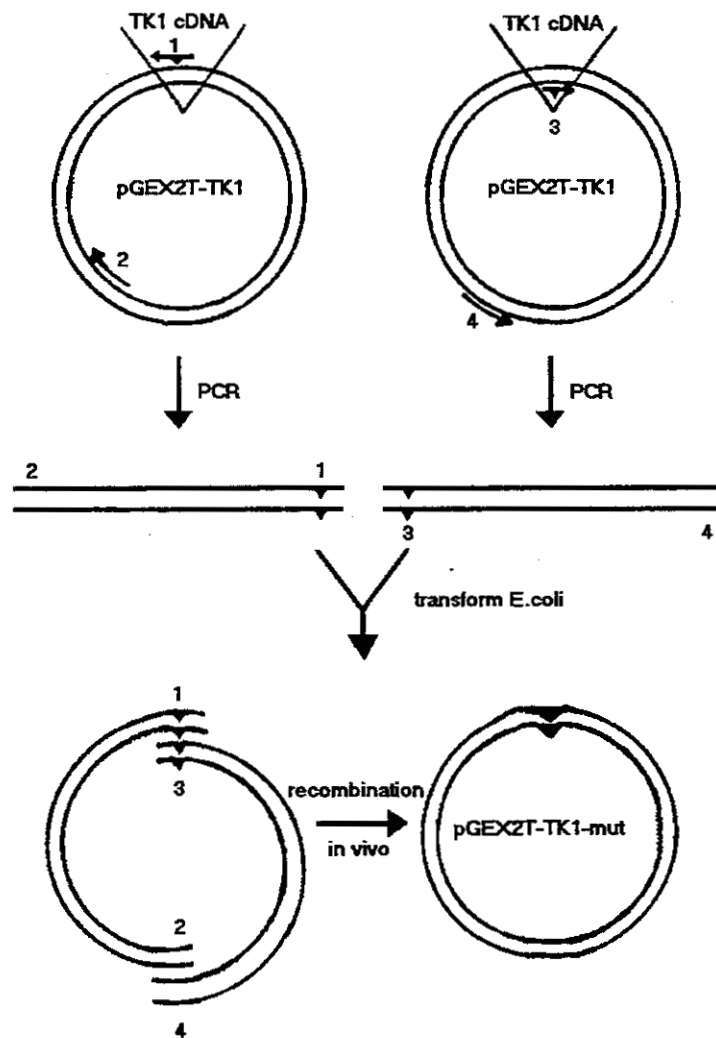


Figure 9: Recombination PCR. X shows the location of the desired mutation. Primer 1 and 3 are the mutagenic primers with the mismatch base(s) indicated by: -v-. The non-mutagenic primers are nominated 2 and 4. The two PCR reactions result in two fragments with homologous ends: where end 1 and 3 are homologous to each other and end 2 and 4 are homologous to each other. pGEX2T-TK1-mut contains the site-specific mutation.

As the only change in the mutants is the introduction of a stop codon, this cannot be used for differentiation between the parent and the mutant clone. Therefore, in these experiments the mutagenic primers were designed so they contained a unique restriction site overlapping the stop codon. This allowed for identification of the transformants and served as a control for the occurrence of the desired mutation.

Primers used for recombination PCR

Mutagenic primers:

TK1-193-1, sense:

189 194 200
 5'T⁵⁶⁵AC TTC AAG AAG GCC TGA GCT CAG CCT GCC GGG CCG⁶⁰⁰3'
 TGA
 Stop codon
 GA GCT C
 Sac1 site

TK1-193-2, anti-sense:

200 194 189
 5'CGG CCC GGC AGG CTG AGC TCA GGC CTT CTT GAA GTA 3'

TK1-176-1, sense:

172 177 183
 5'G⁵¹⁴TC GAG GTG AAT GGG TGA GCT CAC AAG TAC CAC TCC⁵⁴⁹3'
 TGA
 Stop codon
 GA GCT C
 Sac1 site

TK1-176-2, anti-sense:

183 177 172
 5'GGA GTG GTA CTT GTG AGC TCA CCC AAT CAC CTC GAC 3'

The non-mutagenic primers:

Lac1-1: 5'C³⁹⁴⁷CA CGC GGG AAA CGG TCT GAT AAG 3'

Lac1-2: 5'C³⁹⁷⁰TT ATC AGA CCG TTT CCC GCG TGG 3'

Figure 10: Primer sequence for the mutagenic and non-mutagenic primers. Altered bases are shown in dark and underlined. The numbers above the primer refer to amino acid position whereas the number in the primer refer to the position of binding in TK1. 193 and 176 refer to the last coding amino acid in the pGEX2T-TK1-193 and pGEX2T-TK1-176 construct, respectively. Lac refers to the location of the primers in the LacI gene region.

Location of the non-mutagenic primers to the lacI gene region ensures that the two PCR fragments have similar size.

The PCR-reactions contained in a total volume of 50 μ l: 0.24 μ g/ml linearized plasmid, 0.36 μ M of each primer, 200 μ M of each dNTP, 1.5 mM MgCl₂, 1xPCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.015% gelatine, 0.1% Tween 20), and 1.5 units of *Thermus aquaticus* DNA polymerase. A drop of mineral oil was

placed on top of each reaction mix before amplification. The amplification was performed according to the following program: denaturation for 1 min at 95°C, annealing for 1 min at 58°C and polymerization for 3.5 min at 72°C, for 30 cycles.

Purification of PCR-products from low-melting agarose-gel

The PCR product was analysed and purified from a 1% low melting agarose gel. The PCR-fragment was excised under UV-light at 360 nm. 5 volume of TE-buffer (10 mM Tris-HCl pH 8,3, 1 mM EDTA pH 8,0) was added and the agarose was melted at 65°C in a water bath. After cooling to room-temperature, 1 volume of phenol was added and the mixed solution was centrifuged 14,000g for 5 min. The liquid phase was transferred to a fresh tube and the phenol extraction was repeated 3 times. To precipitate the nucleic acids, 1/10 volume of 3 M NaAc and 3 volume 96% EtOH was added and the tubes were kept at -20°C for at least 1 h before centrifugation of the nucleic acids was performed at 20,000g for 15 min at 4°C. The pellet was dissolved in E-buffer. The product was visualized by electrophoresis on a 1% agarose gel. If 5 µl of the ethidium bromide stained PCR-product could be clearly seen, the amount of products was sufficient for transformation of *E.coli*.

Transformation procedure

Approximately 30 ng (2.5 µl) of each PCR-product was mixed and transformed directly into MAX Efficiency DH5α competent *E.coli* ($> 1 \times 10^9$ transformants/µg of pBR322; Gibco BRL/Life technologies). In prechilled 10 ml Ole Dich tubes, the following reactions are performed:

- 1: 50 µl competent cells + 5 µl of premixed PCR-products from reaction 1 and 2 (ratio 1:1).
- 2: 25 µl competent cells + 2.5 µl of control DNA (stock pUC 19 solution (0.01 µg/ml).
- 3: 25 µl competent cells + 5.0 µl of PCR-products from reaction 1 and 5.0 µl TE-buffer.
- 4: 25 µl competent cells + 5.0 µl of PCR-products from reaction 2 and 5.0 µl TE-buffer.
- 5: 25 µl competent cells + 10 µl TE-buffer.

After addition of the DNA, the solutions were mixed carefully. The cells were incubated on ice for 30 min, whereafter they were heat-shocked for 45 sec at 42°C. The cells were cooled on ice for 2 min whereafter 0.9 ml of room-tempered S.O.C (see appendix 1) medium were added. After incubation with shaking for 1

h at 37°C the cells were dispensed onto LB plates.

Reaction 5, containing only competent cells was plated onto LB plates - ampicillin. Reaction 2, containing the control DNA was diluted 1:100 with S.O.C medium and 100 μ l was plated onto LB plates containing 100 μ g/ml ampicillin. The other reactions were used undiluted and 100-400 μ l was spread onto LB plates containing 100 μ g/ml ampicillin.

Calculation of the transformation efficiency

To calculate the transformation efficiency, the number of colony forming units (CFU) with the positive control (pUC19) was calculated.

$$\text{CFU}/\mu\text{g} = \frac{\text{CFU on the control plate}}{\mu\text{g pUC19 used for the transformation}} \times \text{the dilution factor}$$

According to the manufactures (Gibco BRL Life Technologies) the transformation efficiency should be higher than 1×10^6 CFU/ μ g pUC19.

Individual colonies were grown overnight at 37°C in TB medium (see appendix 1) containing 100 μ g/ml ampicillin. Plasmid DNA was isolated (Alkaline miniprep, Sambrook et al 1989) and the clone of interest was selected by Sac1 digestion.

Sequence analysis

The wild-type (pGEX2T-TK1) and mutant (pGEX2T-TK1-193 and pGEX2T-TK1-176) plasmids were used to transform the TK⁻ deficient *E.coli* strain, KY895. KY895 was made competent according to the CaCl₂ method (Sambrook et al 1989). Ampicillin resistant bacteria were grown overnight at 37°C in LB medium containing 100 μ g/ml ampicillin. Plasmid DNA was isolated with the PEG (polyethylene glycol)-precipitation method (Sambrook et al 1989). Alkaline denaturation and preparation of samples for sequencing were done according to "Step-by-step protocols for DNA sequencing with sequenase version 2.0 T7 DNA polymerase" (8th edition, United states biochemical). The clones were sequenced on both strands by the dideoxy

method with the Sequenase version 2.0 DNA sequencing kit (United states Biochemical), using the following primers.

Sense primers:

pGEX-Seq-1 : 5'CTATCCCACAAATTGATAAGT 3'
pBAF3 : 5'CCAAAGACACTCGCTACAGC 3'
pTK6 : 5'CCATTTGGGGCCATCCTGAACCTG 3'
TK1-176-1 : 5'GTCGAGGTGATTGGGTGAGCTCACAAGTACCACTCC 3'

Antisense primers:

pGEX-Seq-2 : 5'ACGTGACTGGGTCATGGCTGC 3'
TK1-205-2 : 5'CCTTCCTGGCACTAGTCAGTTCTCTTTGTT 3'
pBAF2 : 5'GTCAGCTTCACCACGCTCTC 3'
pBAF1 : 5'CGGTCATGTGTGCAGAAGCT 3'

The sequence strategy is shown in figure 11

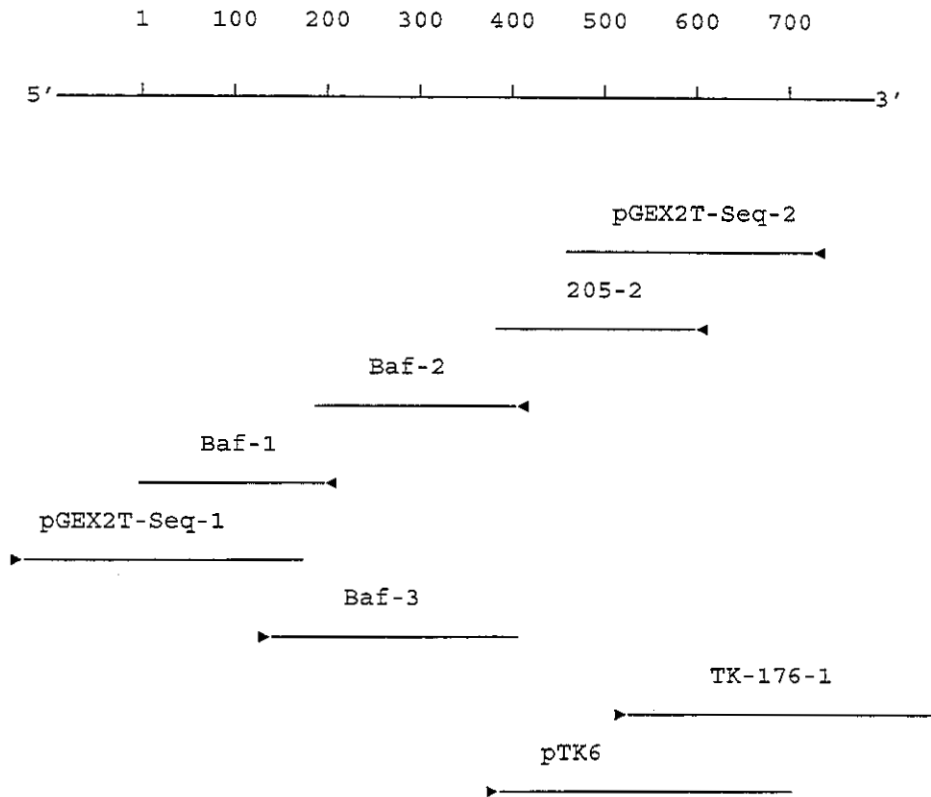


Figure 11: Sequence strategy for the human lymphocyte's TK1 cDNA insert in pGEX2T. The numbers refer to bases in TK1 cDNA. The first base in the start codon is numbered 1. The surrounding part is pGEX2T cDNA. The arrowhead signifies the location of primer binding. — shows the area sequenced with that primer.

Protein expression

Ampicillin resistant KY895/pGEX2T-TK1, KY895/pGEX2T-TK1-193 and KY895/pGEX2T-TK1-176 were grown overnight at 37°C in LB medium (see appendix 1) containing 100 µg/ml ampicillin. The overnight culture was diluted 1:10 in LB medium containing 100 µg/ml ampicillin or in ABTG medium supplemented with amino acid mix (see appendix 1). The culture was grown to $OD_{600} \approx 0.5$ at 25°C. The production of the glutathione S-transferase-thymidine kinase 1 (GST-TK1) fusion protein was induced by addition of the lactose analog IPTG at a final concentration of 0.1 mM. After 15-17 hours of growth, the cells were harvested at 4000 RPM for 15 min at 4°C.

Preparation of bacterial extracts

The cells were resuspended in 1/20 culture volume of lysis buffer 1 (50 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1mM DTT, 10% glycerol, 1% Triton X-100, 0.1 mM PMSF (phenylmethylsulfonyl flouride), and 150 μ g/ml lysozyme). Portions of 3-4 ml were lysed on ice by sonication (50 W, 3x10 sec). The sonicate was centrifugated at 20000g for 20 min at 4°C and filtered through a 0.45 μ m sterile filter.

Induction of the fusion protein was monitored by measurements of the TK activity and the GST activity was monitored with the substrate 1-chloro-2,4-dinitrobenzene (CDNB).

Detection of GST-fusion protein

The induction of the fusion protein was monitored by the GST substrate CDNB. The GST-catalyzed reaction of CDNB with glutathione produces a conjugate that can be measured by absorbance at 340 nm (GST gene fusion system, Pharmacia Biotech 1994).

50 μ l of crude extract was added to 1 ml of CDNB solution (1mM CDNB, 1 mM reduced Glutathione, 100 mM K-phosphate buffer pH 6.5) in a quartz cuvette and the absorbance at 340 nm was recorded at one-minute intervals for 5 min.

Glutathione affinity chromatography

Glutathione agarose is an affinity matrix, where the glutathione is coupled to epoxy activated agarose through the oxirane group. The glutathione agarose binds the GST-part of the fusion protein. Glutathione agarose (750mg/10ml bed volume) was swelled overnight at 4°C in buffer 2 (50 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1mM DTT, 10% glycerol, 1% Triton X-100) and packed into a 25 mm x 200 mm disposable column. The following steps were performed at 4°C. The column was washed and equilibrated with 10-15 bed volume of buffer 2. Crude extract (\approx 500 mg) was applied and recirculated over the column. Unbound proteins were removed by washing with 5-8 bed volume of buffer 2. The column was equilibrated with 5 bed volume of buffer 3 (20 mM Tris/HCl, pH 8.4, 150 mM NaCl, 2.5 mM CaCl₂,

0.1% Triton X-100, 1 mM DTT). Cleavage of TK1 protein from the GST-partner was performed with thrombin at room temperature. Three vials of thrombin (Sigma) were resuspended in 1 bed volume of buffer 3, loaded onto the column and slowly recycled over the column for 1-1.5 h. The eluate was continuously kept at 4°C. The column was further washed three times with 1 bed volume of buffer 3. To elute remains of fusion proteins the column was washed with ~ 4 bed volume of buffer 4 (5 mM glutathione in lysis buffer 2). To stabilize the TK1 enzyme during freezing and thawing 10% glycerol was added to the samples.

Sephadex G-25 chromatography

The eluate from the glutathione agarose column was desalted by G-25 sephadex chromatography.

The Sephadex G-25 column (500 ml) was equilibrated with 3 bed volume of buffer 5 (20 mM K-phosphate pH 6.0, 5 mM MgCl₂, 10% glycerol, 2 mM DTT). Fractions containing TK1 protein from glutathione agarose column were applied to G-25 column and eluted with the buffer 5. Protein content and conductivity were continuously measured.

CM-sepharose column

The column (10 mm X 20mm) was equilibrated with buffer 6 (10 mM K-phosphate buffer pH 7.0, 5 mM MgCl₂, 10% glycerol, 5 mM DTT). The desalted fractions were applied and unbound material washed away. The TK1 protein was eluted with buffer 7 (100 mM K-phosphate buffer, pH 8.0, 5 mM MgCl₂, 10% glycerol, 5 mM DTT, 0.5 mM CHAPS, 0.1 M KCl).

SDS-polyacrylamide gel (SDS-PAGE)

The subunit molecular weight was determined by SDS-PAGE with a 4.5% stacking gel and a 12 or 15% separation gel prepared by standard methods (Sambrook et al 1989). The samples were denatured at 95°C for 2 min in protein loading buffer (125 mM Tris-HCl, 10 mM DTT, 1% SDS, 0.1% Bromphenol Blue and 25% glycerol) before loading onto the gel. Proteins were visualized by silver staining and the gel was dried on a slab gel drier for 2 h at 60°C.

Determination of native molecular weight by gel filtration chromatography

The native molecular weight was estimated on Sephadex G-200 (5 mm x 20 mm) or Superose 12 (10 mm x 300 mm column connected to a Gradifrac, Pharmacia). In both cases, the columns were equilibrated with buffer 8 (50 mM Tris, pH 7.4, 5 mM MgCl₂, 0.1 M KCl, 5 mM DTT). When determining the molecular weight in presence of ATP the enzymes were preincubated with 2.5 mM ATP and the column was pre-equilibrated with buffer 8 containing 2.5 mM ATP. The molecular weight was estimated by comparing the retention times for the sample with the retention times for five marker proteins: beta-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa).

Kinetics

The substrate kinetics was analysed by Hofstee plots (v versus v/s) and Wilkinson plots (s/v versus s). v is the initial velocity and s is the substrate concentration. V_{\max} was calculated using nonlinear regression analysis to obtain the best fit between the experimental data and the expression, $v = As^2 + Bs/(s^2 + Cs + D)$, where A , B , C , D are constants and s is the concentration of the varied substrate. For $s \rightarrow \infty$, A is used as an estimate of V_{\max} . K_m values and the Hill coefficient were determined by Hill plots of $\log(v/(V_{\max} - v)) = n \log s - n \log S_{0.5}$. n is the Hill coefficient and $S_{0.5}$ is the substrate concentration at half-maximal velocity ($= K_m$).

Results

Results for quantification of TK1 mRNA in healthy lymphocytes and in lymphocytes from patients with CLL.

TK1 is a strictly cell cycle regulated enzyme with a close correlation between the TK activity and the proliferative state of the cell. In a variety of cancer it has been shown that the expression of TK1 is deregulated. Generally, the TK activity is higher in patients with cancer than in control persons (Ellims et al 1981, Hallek 1992, O'Neill et al 1992, Robertson et al 1990) and in several cases TK1 activity has been observed in non-dividing cells (Ellims et al 1981, Munch-Petersen and Tyrsted 1986, Russo et al 1987). Likewise, TK enzymes with enzymatic properties different from TK1 and TK2 in normal cells, have been found (Munch-Petersen and Tyrsted 1986, Munch-Petersen and Tyrsted 1988). As different regulation mechanism may cause the development of malignancy, the expression of TK1 mRNA in healthy cells and in CLL cells was investigated. CLL cells were chosen for my investigation because, despite that they are characterized as non-dividing cells TK1 activity has been observed (Ellims et al 1981, Ellims et al 1983, Munch-Petersen and Tyrsted 1986).

By comparing the level of TK1 mRNA with the TK activity in normal cells and in CLL cells it can be investigated if the regulation mechanisms(s) are different in these cells.

Determination of TK activity in control persons

The expression of TK activity in quiescent and PHA stimulated lymphocytes were measured for six control persons. The lymphocytes were stimulated with PHA for 48, 72, 96 and 168 hours, respectively. Crude extracts were prepared from 5×10^6

cells and TK activity and total protein was measured. Figure 12 shows the amount of TK activity expressed in relation to the total protein content.

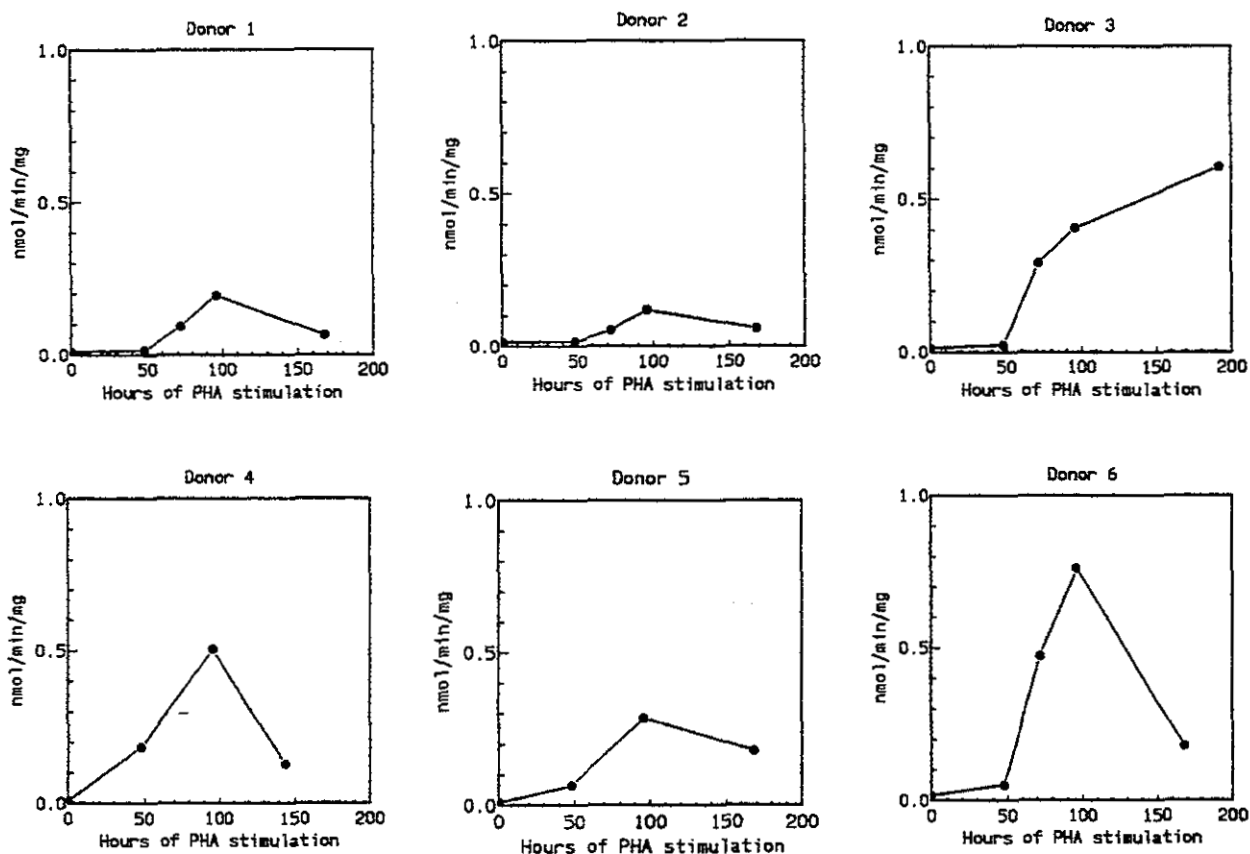


Figure 12: PHA induced variation of TK activity ($\text{nmol min}^{-1} \text{mg}^{-1} \text{ protein}$) for control person 1 - 6. The lymphocytes were stimulated with PHA for the indicated periods. TK activity was measured at standard conditions as described in "Methods".

The TK activity in the quiescent lymphocytes from the six control donors was between 0.009 to $0.016 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ with a coefficient of variation (CV) of 25%¹⁰. The TK activity increases as cells are stimulated with PHA, resulting in a peak level for five of six control persons after 96 hours. After PHA stimulation

¹⁰In the thymidine kinase activity assay the coefficient of variation (CV) of triplicate samples are below 5%.

the TK activity in the cells for the six control donors was between 0.12 and 0.76 nmol min⁻¹ mg⁻¹ protein, corresponding to a 9 to 62-fold increase in TK activity. For the phytohemagglutinin stimulated lymphocytes the CV was 60%. A high individual variation has earlier been reported by Koefoed et al (1986) and Munch-Petersen et al (1985) in experiments where the UVR-induced DNA synthesis in phytohemagglutinin lymphocytes was measured with incorporation of [³H]-thymidine. The variations probably reflect individual variations in the response to PHA among the donors or the immune response in some donors has been activated.

Distinction of TK1 and TK2

Investigations by Munch-Petersen and Tyrsted (1977) have shown that in dividing lymphocytes 1-2% of the TK activity is due to TK2, whereas in quiescent lymphocytes the TK activity exclusively is due to TK2. Both TK1 and TK2 use ATP as phosphate donor. Thus, the standard enzyme assay performed in this work reflects the total TK activity. However, it is possible to distinct between TK1 and TK2 by the different substrate specificity for the two enzymes. TK2 can use CTP efficiently as a phosphate donor, whereas it is a poor substrate for TK1 (Adler and McAusian 1974, Ellims et al 1981). Figure 13 shows the ratio of CTP- and ATP-mediated TK activity in the six control persons. The CTP phosphate donor capacity is most pronounced with crude extract from quiescent cells, showing that the dominating enzyme is TK2. The ability to use CTP as phosphate donor decrease as the cells are stimulated to enter the cell cycle. After 96 hours of PHA stimulation the dominating enzyme is TK1.

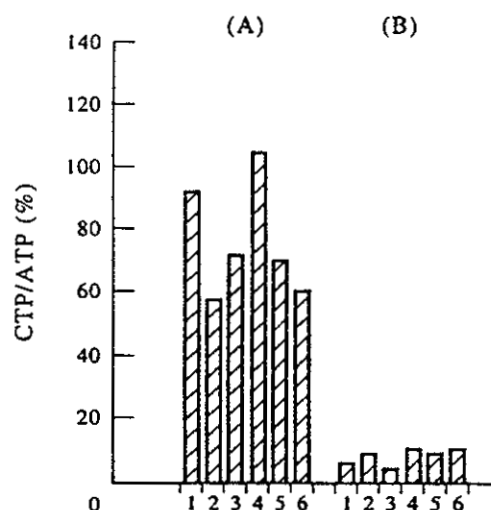


Figure 13: The ratio between phosphate donor capacity of CTP and ATP. In each experiment the enzyme activity is normalized to 100% with ATP as phosphate donor. (A) Quiescent lymphocytes; (B) lymphocytes after 96 hours of PHA stimulation. The numbers on the x-axis refer to the individual donors. Reprinted from *Leukemia Res.* vol. 18, Kristensen, T., Jensen, H.K and Munch-Petersen, B.: Overexpression of human thymidine kinase mRNA without corresponding enzymatic activity in patients with chronic lymphatic leukemia, 861-886., copyright (1994), with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX5 1GB, UK.

The reverse transcriptase reaction

As described in "Methods" the steps in the competitive PCR (Gilliand et al 1990) procedure is purification of total RNA from lymphocytes, reverse transcription of RNA to cDNA and quantification of TK1-cDNA exon 1 and 2, simultaneously with the corresponding genomic DNA serving as a competitive internal standard in the PCR-reaction. To achieve the best yield of the reverse transcription of RNA to cDNA (the RT-reaction), optimization with respect to $[MgCl_2]$, $[RNA]$ and $[dNTP]$ was performed. The extent of the RT-reaction was followed by applying 3H -TTP instead of TTP to the reaction mixture and measuring the amount of incorporated radioactivity by scintillation counting.

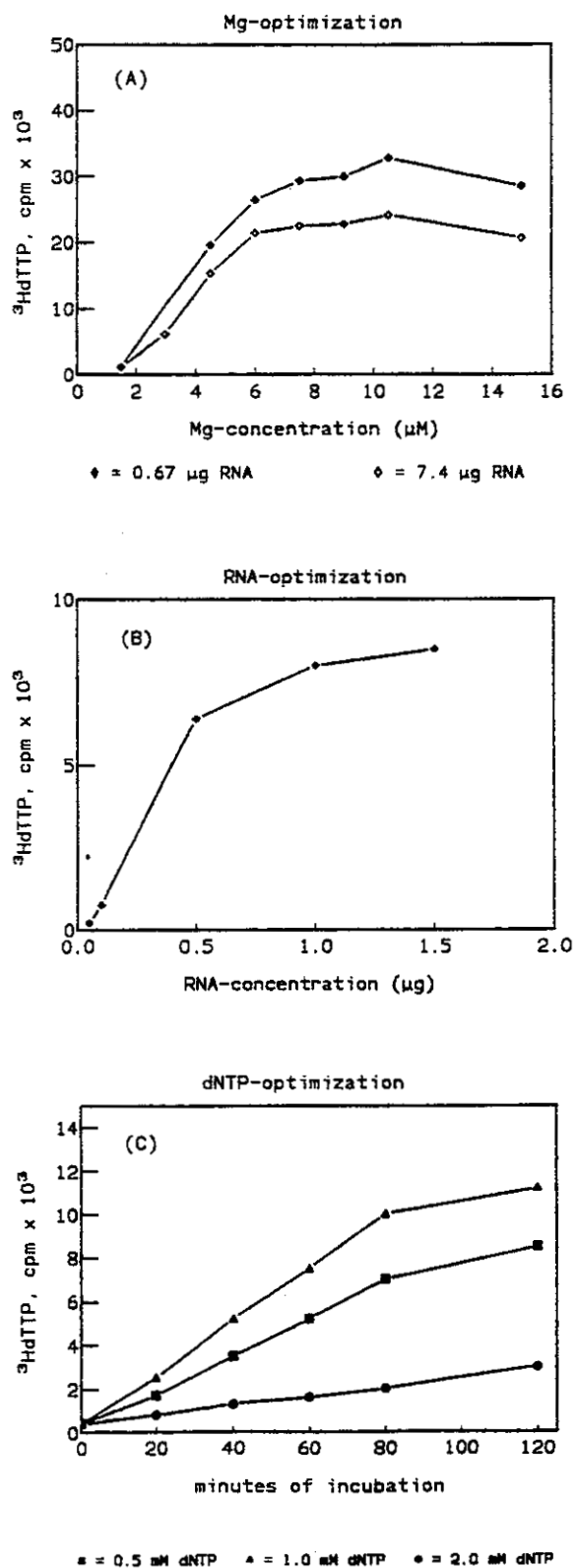


Figure 14: Optimization of the reverse transcription (RT) reaction.

(A) MgCl_2 -titration of 0.67 μg total RNA and 7.4 μg total RNA

(B) RNA-titration, (C) dNTP-titration.

When the MgCl_2 -concentration was optimized, the RT-reaction was performed with two different RNA concentrations, 0.67 μg and 7.4 μg , respectively, in a 50 μl RT-reaction (figure 14.A). For both RT-reactions the MgCl_2 concentration was varied from 1.5 mM to 16 mM. As seen from figure 14.A the optimal MgCl_2 concentration for both reactions is in the range of 9-12 mM. However, the RT-reaction with 7.4 μg RNA had a lower ^3H -TTP incorporation than the RT-reaction with the lower concentration of RNA.

Figure 14.B shows a closer optimization of the RNA concentration with 10.5 mM MgCl_2 . On behalf of this RNA titration it is not recommended to use less than 0.2 μg total RNA/50 μl RT-reaction since no incorporation of ^3H -TTP could be measured for RT-reactions with RNA below this limit. The last titration, shown in figure 14.C was with different dNTP concentrations. The reactions contained 0.67 μg total RNA, 10.5 mM MgCl_2 and 0.5, 1.0 or 2.0 mM dNTP, respectively. The RT-reaction with 1 mM dNTP gave the best result. Increasing the dNTP concentration to 2 mM gave a negative effect.

When PCR-reactions were performed with cDNA products from the RT-reactions in figure 14, there was a clear relation between a non-optimal RT-reaction and the presence of many unspecific amplification products in the PCR-reaction (results not shown).

Based on these experiments the reverse transcription was performed with 0.5-1.0 μg total RNA, 10.5 mM MgCl_2 and 1 mM dNTP.

Quantification of TK1 mRNA in control persons

The TK1 mRNA level was quantified in quiescent and in PHA-stimulated lymphocytes isolated from the six control persons. The TK1 mRNA level was quantified by competitive PCR. In the competitive PCR reaction TK1-cDNA is amplified with a primer pair enclosing exon 1 and 2, resulting in a 138 bp fragment. With genomic DNA this primer pair amplify a 248 bp fragment. Initially, the PCR-reaction was performed with a broad range of genomic DNA dilutions to titrate the "competition region". Afterwards, a fine-adjustment of the internal standard was

performed (figure 15.A and 15.B).

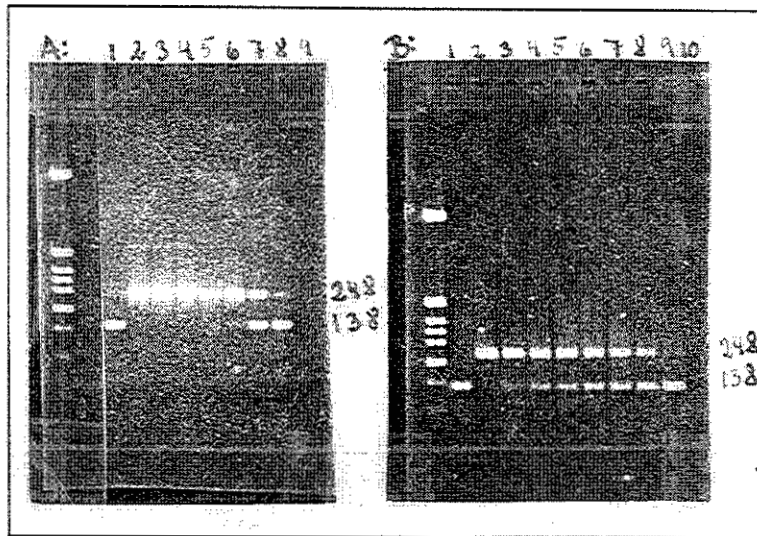


Figure 15: TK1 genomic DNA versus TK1 cDNA for control person 4 stimulated with PHA for 94 hours. A: Titration with a broad range of dilutions of TK1 genomic DNA (248 bp), competing with 1 μ l of TK1-cDNA (138 bp). Lane 1 is without genomic DNA. Lane 2 - 8 contain various amounts of genomic DNA (10^{10} g - 10^{16} g), decreasing with a factor 10 for each lane. Lane 9 is a PCR-reaction without DNA (negative control). B: Closer titration based on the competition range (about 10^{15} g) determined in A. Lane 2-9 represent titration from 10^{14} to 10^{16} g. Lane 1 is cDNA only and lane 10 is a negative control.

The amount (gram) of cDNA in the sample was estimated as that amount (gram) of genomic DNA giving equal intensity of the two amplification products (15.B, lane 8). From this quantity, the number of TK1 cDNA copies were calculated by division with the average molecular weight of the 138 bp cDNA fragment (average molecular weight/base = 308). The number of TK1 cDNA copies was regarded as representative for the number of TK1 mRNA's. The PCR reactions were always performed with a positive (TK1 cDNA) and a negative control (no DNA).

Figure 16 shows the increase in the TK1 mRNA level expressed in relation to the protein content for lymphocytes stimulated to growth with PHA.

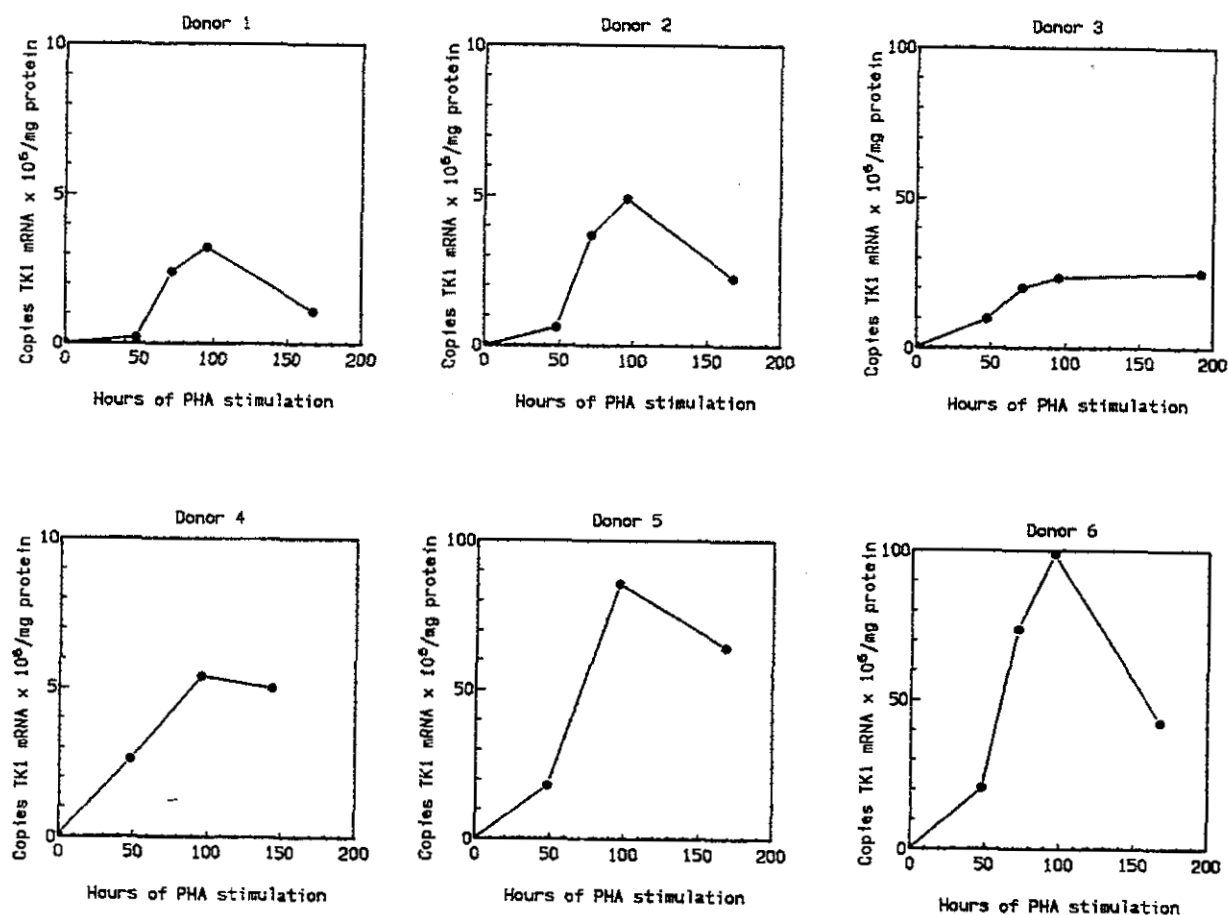


Figure 16: PHA induced variation of TK1 mRNA (copies/mg protein) for control person 1-6. The lymphocytes were stimulated with PHA for the indicated periods. TK1 mRNA was estimated by competitive PCR. The ordinate scale for donor 3, 5 and 6, differs from the ordinate scale for donor 1, 2 and 4.

As seen in figure 16 the level of TK1 mRNA in quiescent cells is very low and in four of the six control persons below the limit that is detectable with the competitive PCR method. The limit of detection in the PCR reactions with cDNA from 1×10^5 cells is 6 copies of TK1 mRNA/1000 cells. Below this level an amplification product of 248 bp appears. The 248 bp product is probably traces of DNA or non-spliced RNA in the RNA preparation.

After PHA stimulation the level of TK1 mRNA increase about 100-fold, reaching a peak level after 96 hours. Figure 17.A (page 65) shows that after PHA-

stimulation of lymphocytes the amount of TK1 mRNA and TK activity increases concomitantly.

TK1 mRNA and TK activity in lymphocytes from patients with CLL

Chronic lymphatic leukemia (CLL) cells are non-dividing cells, and therefore it is plausible that the low TK activity in these cells almost exclusively is due to TK2. Ellims et al (1981) showed that in eight patients out of 12 patients, the dominating enzyme was TK2, as evaluated of the TK activity from the CTP/ATP ratio. However, with four of the 12 patients the ability of the TK activity to use CTP as phosphate donor was low, indicating that the dominating TK was TK1. As the four patients with TK1 suffered from a more aggressive form of CLL, it was suggested that TK1 was an indicator of a change to a more aggressive form.

Occurrence of a TK1 isoenzyme in quiescent CLL cells may be due to a change in the control of the cell-cycle regulated expression of the TK1 gene. Therefore, it was investigated if any difference in expression of TK1 mRNA in normal and malignant cells could be detected.

In the five CLL patients used in these experiments, the TK activity was low and at the same level as that in quiescent lymphocytes. Investigation of the enzyme activity by the CTP/ATP ratio showed that the TK activity from the five CLL patients behaved like TK2 in quiescent lymphocytes. Due to the quiescent stage of CLL cells and the dominance of the TK2 isoenzyme, a low level of TK1 mRNA was expected. However, when TK1 mRNA in CLL cells was quantified the level was about 30 to 300-fold higher than the level in quiescent lymphocytes. In fact, the level was in the same range as PHA stimulated lymphocytes. Duplicate determination of TK1 mRNA showed a CV of ~20%. Figure 17.A and 17.B show the amount of TK1 mRNA and TK activity in one control donor and in the five CLL patients.

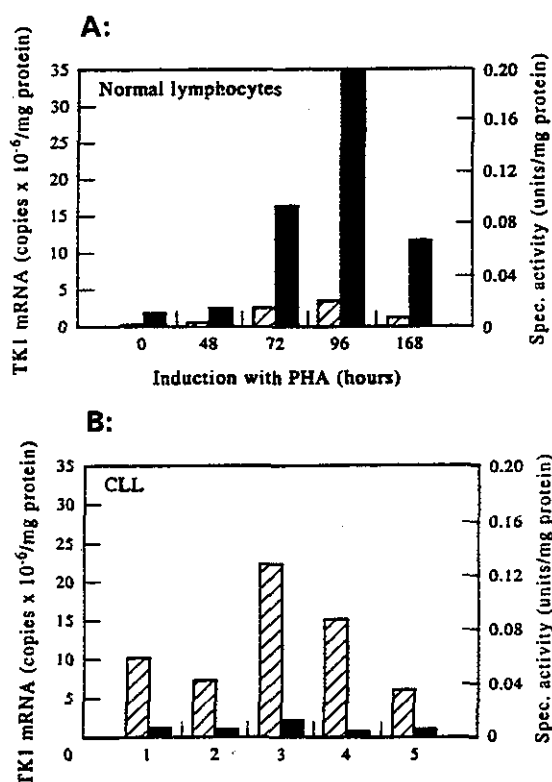


Figure 17: A: The PHA induced variation of TK1 mRNA (▨) and TK activity (■) for control donor 1. B: The amounts of TK1 mRNA in CLL cells. The number on the x-axis refer to the individual CLL patient. Reprinted from *Leukemia Res.* vol. 18, Kristensen, T., Jensen, H.K and Munch-Petersen, B.: Overexpression of human thymidine kinase mRNA without corresponding enzymatic activity in patients with chronic lymphatic leukemia, 861-886., copyright (1994), with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX5 1GB, UK.

Resumé of quantification of TK1 mRNA in healthy lymphocytes and in lymphocytes from patients with CLL

The relation between TK1 mRNA and TK enzyme activity has been investigated in control persons and in patients with CLL. Human lymphocytes, which are truly G₀ cells, can be stimulated to growth by PHA and these experiments have been used as a model system for expression of TK1 mRNA and TK activity in normal cells. PHA stimulation of lymphocytes from control persons results in an about 100-fold increase in TK1 mRNA copies' mg⁻¹ protein and is followed by a concomitant increase in TK activity.

In CLL cells which are quiescent cells the TK activity level was low and in

the same range as quiescent lymphocytes from control persons. The dominating enzyme in the CLL cells was TK2. However, the level of TK1 mRNA copies mg^{-1} protein was 30 to 300-fold higher than the level found in quiescent lymphocytes from control persons and actually the level was in the same range as in PHA stimulated lymphocytes.

Results from structure-function relationship of human TK1

Recombination PCR

Two deletion mutants were constructed for the investigation of the structure-function relationship of human TK1. In one mutant, TK1-193, a stop codon is introduced at amino acid position 194, deleting 40 amino acids from the C-terminal. In the other mutant, TK1-176, a stop codon was introduced at amino acid position 177, deleting 57 amino acids from the C-terminal. The mutants were constructed by the recombination PCR method, where two separate PCR-reactions were performed. Each reaction contained the pGEX2T-TK1 plasmid carrying the amino acid coding sequence for human TK1. By changing a few nucleotides in the primer sequence used for the PCR-amplifications a new stop codon as well as a restriction site was introduced into the TK1 cDNA. Each PCR-reaction was performed with a mutagenic and a non-mutagenic primer. A sense and an antisense version of both primers were constructed. The PCR-reactions result in a PCR-fragment with homologous ends and transforming PCR-fragments from each PCR-reaction into *E. coli* will result in an *in vivo* recombination.

pGEX2T-TK1 linearized with HpaI and AatII, respectively, was used in the PCR-reactions. For construction of the TK1-193 clone, the "right side" (figure 9, page 47) of pGEX2T-TK1 was amplified with the LacI-1/TK1-193-1 primer pair, resulting in a fragment of 2563 bp. Amplification of the "left side" of pGEX2T-TK1 was performed with the LacI-2/TK1-193-2 primer pair, resulting in a 3138 bp fragment. For construction of the TK1-176 mutant, similar reactions are set up, but with TK1-176 primers instead of TK1-193 primers. These reactions result in PCR

fragments of 2612 bp for amplification with the *LacI*-1/*TK1*-176-1 primer pair and of 3089 bp with the *LacI*-2/*TK1*-176-2 primer pair. Figure 18 shows an agarose gel electrophoresis of the PCR-reactions.

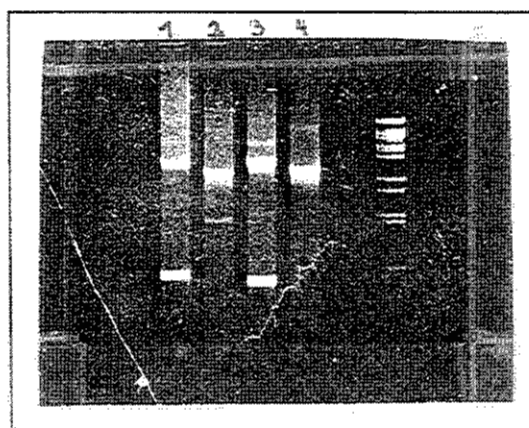


Figure 18: PCR-amplification of *pGEX2T-TK1*. Lane 1: *pGEX2T-TK1/AatII* with primers *LacI*-2 + *TK1*-193-2. Lane 2: *pGEX2T-TK1/HpaI* with primers *LacI*-1 + *TK1*93-1. Lane 3: *pGEX2T-TK1/AatII* with *LacI*-2 + *TK1*-176-2. Lane 4: *pGEX2T-TK1/HpaI* with *LacI*-1 + *TK1*-176-1. Lane 6: Marker λ /*BstEII*: from bottom; 700, 1264, 1371, 1929, 2323, 3675, 4824 bp.

The PCR-fragments were cut out of the gel, purified and transformed into MAX Efficiency *DH5 α* *E.coli*. Table 2 shows the number of transformants.

	No. of colonies	Transformation efficiency (CFU ^a /μg pUC)	No. of clones with <i>SacI</i> site
<i>pGEX2T-TK1</i> -193	10		5/5
<i>pGEX2T-TK1</i> -176	33		17/17
pUC-control/193		4x10 ⁷	
pUC-control/176		1x10 ⁸	

Table 2: Number of transformants of *pGEX2T-TK1*-193 and *pGEX2T-TK1*-176 after transformation and recombination of the PCR fragments from the PCR reactions in figure 18. a: CFU, colony forming units. pUC-control refers to the transformation efficiency for that particular experiment, as controlled by transformation with pUC DNA.

As it appears from table 2 both transformation experiments with pGEX2T-TK1-193 and pGEX2T-TK1-176 plasmid resulted in transformed colonies, 10 and 33, respectively. The frequency of the site-specific mutagenesis in these experiments is 100%, as the plasmid DNA from all the tested clones could be digested with Sac1. As the only Sac1 site in the pGEX2T plasmid is part of the inserted stop codon, a digest is obtained only if the site-specific mutagenesis has been performed.

Considering the amount of purified PCR products (≈ 30 ng of each PCR-product) transformed into MAX Efficiency DH5 α *E. coli* the number of transformants may seem low. The control experiments, with transformation of pUC plasmid shows that the transformation efficiency in each experiment is acceptable, as the efficiency is higher than 1×10^6 CFU/ μ g pUC. There are several reasons for the low number of transformants with the PCR fragments. First, the transformation frequencies with linear plasmid molecules are several orders of magnitude (10^2 - 10^3) lower than those obtained with equivalently closed circular molecules and of the transformed fragments only a small part ($< 10^3$ per 10^{11} added molecules) will survive the nucleases in *E. coli* (Conley et al 1986a). Therefore it is important to use high competent *E. coli* strains in the recombination PCR procedure. I tried to transform PCR products into competent Library DH5 α *E. coli* and *E. coli* cells made competent by the CaCl₂ method (Sambrook et al 1989), respectively, but without success. This was probably due to a lower transformation efficiency of 1×10^7 transformants/ μ g of monomer pUC19 for both strains (Sambrook et al 1989). Secondly, the low number of transformants is due to the *recA*⁻ genotype of the DH5 α *E. coli* host. The numbers of transformants in *recA*⁻ *E. coli* strains are about 40-fold lower than in *recA*⁺ *E. coli* strains (Conley and Saunders 1984). Conley and Saunders (1984) and Conley et al (1986b) have shown that transformation of blunt-ended pBR322 into a *recA*⁺ *E. coli* strain result in a mutation frequency (not to be confused with site-specific mutagenesis frequency) of 80%. The types of mutation are mainly deletions of bases and Conley et al (1986b) propose that exonucleolytic processing of the terminus of linear plasmid DNA generates fragments suitable for recombinational recyclization and deletion. Actually, transformation of *E. coli* with linearized plasmid DNA molecules is used deliberately as a method of obtaining

deletions of cloned DNA sequences in vivo (Sambrook et al 1989). However, Jones and Howard (1991) have shown that a *recA*⁻ *E. coli* strain can recombine DNA with minimal stretches of homology. The transformation efficiency is decreased, but likewise is the mutation frequency. In a series of recombination PCR experiments Jones and Howard (1991) found that the highest mutation frequency was 50% and in several experiments the mutation frequency was 0%. The best results (0% mutation frequency) were obtained when the region of homology between the mutating ends (corresponding to end 1 and 3 in figure 9, page 47) and the non-mutating ends (corresponding to end 2 and 4 in figure 9), respectively, was delimited to ~ 30 bases. Increasing the area of homology also increased the mutation frequency. It is not known how the recombination between very short regions of homology in a *recA*⁻ host proceeds (Jones and Howard 1991).

Induction of wild type and mutants GST-TK1 fusion proteins

An *E. coli* strain defective in TK expression (KY895, Hiraga et al 1967) was transformed with pGEX2T-TK1, pGEX2T-TK1-193 and pGEX2T-TK1-176, respectively. Due to observations by Fetzer and Folkers (1992) and Fetzer et al (1994) the fusion proteins were expressed at 25°C. They found that expression of Herpes simplex virus 1-thymidine kinase (HSV-1 TK) as a GST-fusion protein in *E. coli* KY895 resulted in GST-TK_{herpes} inclusion bodies at 37°C but not at 25°C. The GST-TK_{herpes} inclusion bodies, which are aggregates of insoluble proteins, could be solubilized in 6 M guanidin HCL, but then the ability to bind to glutathione was lost, making the purification by glutathione affinity chromatography impossible. Why eukaryotic polypeptides are sequestered into inclusion bodies in *E. coli* is not fully understood. It is not simply a response by *E. coli* to "foreign" proteins, since normal *E. coli* proteins synthesized to high levels using recombinant DNA techniques can also accumulate in insoluble forms (Marston 1986).

Figure 19 shows the time course of expression of the GST-TK1 (wild type) fusion protein at 25°C. The expression is measured by the TK1 activity. The expression of GST-TK1 is optimal after four hours, whereafter the level slightly

decreases.

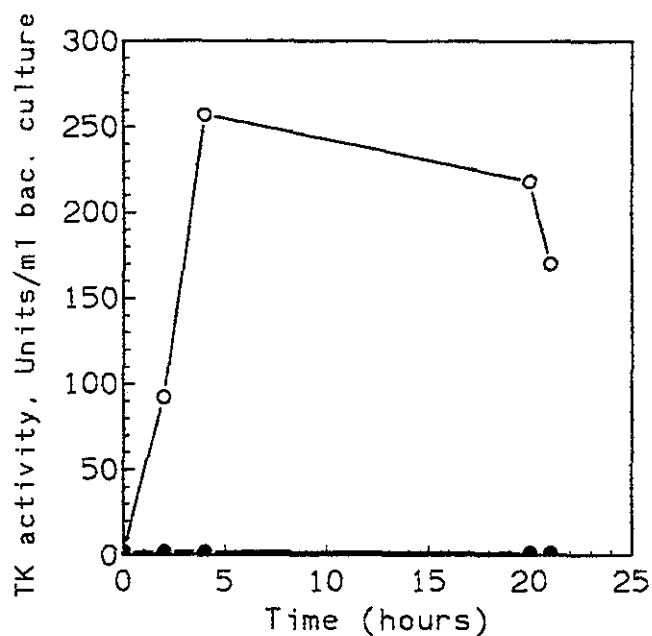


Figure 19: Expression of the GST-TK1 fusion protein measured by the TK1 activity. Open circles are induction of GST-TK1 with IPTG. Filled circles are control: GST-TK1 without IPTG. Units: nmol min^{-1} .

Table 3 shows the induction of TK1 activity with IPTG. The results shown here are for induction in LB medium, but expression in minimal ABTG medium, supplemented with an FN18 amino acid mix (see appendix 1) gives the same result. The purpose of using minimal medium is to obtain isotope labelling of the expressed protein with an amino acid. This may be useful for the NMR analyses.

Table 3: The yield of wild type and mutants GST-TK1 fusion proteins, measured by TK assay. U: unit (nmol min^{-1}).

	- IPTG U/ml bac.culture	+ IPTG U/ml bac.culture
GST-TK1	1-10	100-250
GST-TK1-193	0.1-1.0	20-40
GST-TK1-176	0.1-1.0	0.1-1.0

As seen from Table 3 no increase of TK1 activity was found for GST-TK1-176 after induction of pGEX2T-TK1-176. It was investigated if the GST-part of the fusion protein was expressed, with the GST substrate CDNB (1-chloro-2,4-dinitrobenzene). GST mediates a reaction between CDNB and glutathione, and the product of the enzyme reaction is measured at 340 nm. The increase in the absorbance at 340 nm, as shown in figure 20 shows that the GST-TK1-176 is expressed.

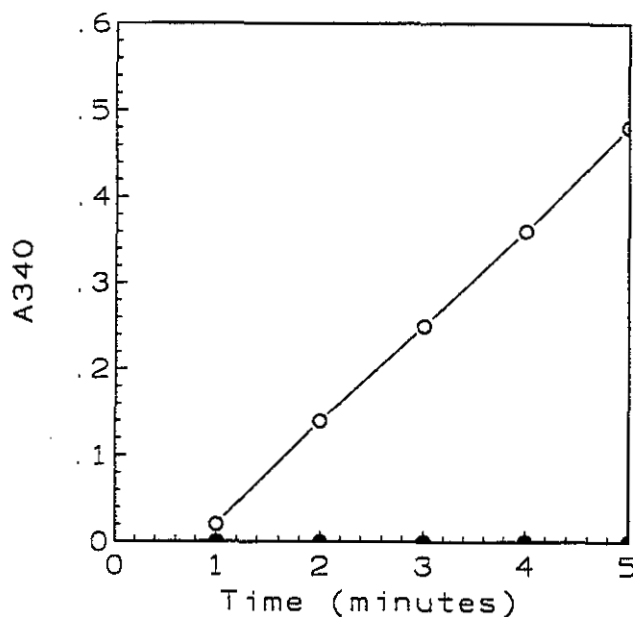


Figure 20: CDNB assay for GST-TK1-176 fusion protein. Open circles: 50 μ l of total protein from *E. coli* KY895/pGEX2T-TK1-176 sonicate harvest 15 hours after IPTG induction. Filled circles: 50 μ l of total protein from *E. coli* KY 895/pGEX2T-TK1-176 sonicate harvest before IPTG induction.

Sequence analysis

The sequence of TK1 cDNA in pGEX2T-TK1-193 and pGEX2T-TK1-176 was determined to ensure that no mutation, except the site-directed mutagenesis, had occurred during the PCR-amplification and the recombination process. The wildtype pGEX2T-TK1 that has not been subjected to PCR-amplification or recombination was used as a "control." The sequence strategy was shown on page 52.

Apart from the introduced mutations, the pGEX2T-TK1-193 and pGEX2T-TK1-176

cDNA sequences were identical to pGEX2T-TK1 and thus no mutations had occurred during the PCR-reactions or the recombination processes. However, when comparing the published sequence for human TK1 (pTK11, based on DNA from HeLa cells, Bradshaw and Deininger 1984) and the lymphocyte TK1 cDNA sequence in pGEX2T-TK1 two base changes was observed: base 316, A, in pTK11 is changed to a G in pGEX2T-TK1. This results in an amino acid change from Met¹⁰⁶ in pTK11 to Val in lymphocytes. The other change is base 632, where A in pTK11 is changed to a G in pGEX2T-TK1. The amino acid change is from Lys²¹¹ in pTK11 to Arg in lymphocytes.

pTK11		
A (nucleotide 316)	ATG	Met ¹⁰⁶
A (nucleotide 632)	AAG	Lys ²¹¹
pGEX2T-TK1		
G (nucleotide 316)	<u>G</u> TG	Val ¹⁰⁶
G (nucleotide 632)	<u>A</u> GG	Arg ²¹¹

Table 4: Differences between TK1 cDNA sequence in pTK11 and pGEX2T-TK1. The specific bases are numbered according to the first base in the start codon. The codon with the changed base and the corresponding amino acid are shown in column 2 and 3. The altered bases in pGEX2T-TK1 are shown in dark and underlined.

As the observed changes are identical in pGEX2T-TK1-176, pGEX2T-TK1-193 and pGEX2T-TK1, it is very unlikely that they arise from PCR-amplification or recombination.

Purification of recombinant TK1 protein

Enzymatic characterization of the recombinantly expressed enzymes is performed on pure enzymes. Cleavage of the TK1 protein from the GST fusion partner is

therefore necessary. In these experiments it is performed with thrombin, after immobilization of the fusion protein to a glutathione agarose column. The glutathione agarose chromatography results in an approximately 80% pure preparation. Remains of the fusion protein (50 kDa for GST-TK1 and 46 kDa for GST-TK1-193), the GST-part of the fusion protein (26 kDa) and a 70 kDa protein band was present in the gel (lane 2 and 5 in figure 21). GST-TK1-176 was purified as well to ensure that a subunit molecular size of 18.1 kDa was expressed, but as the mutant is without TK1 activity the results from the purification are omitted.

Fractions containing TK1 protein from glutathione agarose columns were applied to a G-25 column and the desalted protein were then applied on a CM-sepharose. TK1 has a positive netto-charge and will therefore bind to the negatively charged carboxymethyl-groups in CM-sepharose. This procedure gave a preparation of approximately 99% pure (lane 3 and 6 in figure 21). For recombinant TK1 the expected submolecular size is 24 kDa and for TK1-193 the expected size is 20,1 kDa. Figure 21 shows that the subunit molecular weights were as expected.

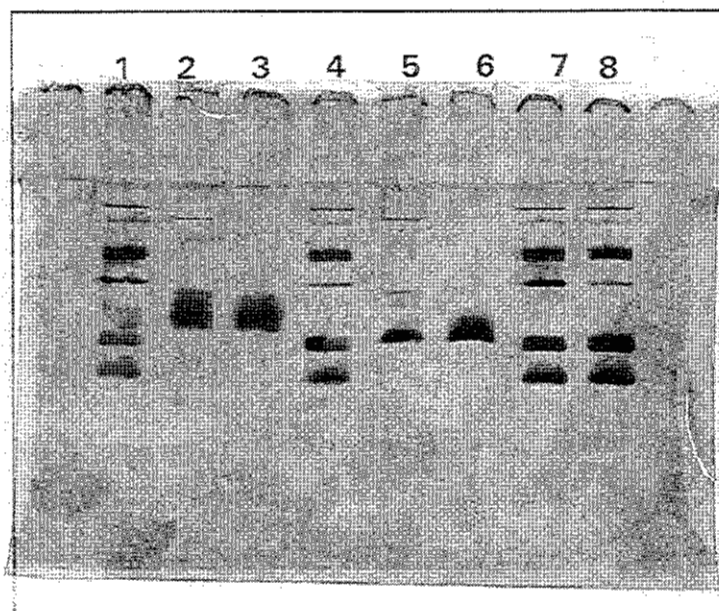


Figure 21: Silver-stained SDS-PAGE (15% separation gel) of pure recombinant TK1 and TK1-193. Lane 1, 4, 7 and 8 are markers: 97, 66, 45, 31, 21 and 14 kDa. Lane 1 and 4 contains 40 ng of each protein, lane 7 and 8 contains 60 ng of each protein. Lane 2: 0.3 μ g recombinant TK1 from glutathione agarose chromatography, Lane 3: 0.3 μ g recombinant TK1 after CM-column, Lane 5: 0.3 μ g TK1-193 from glutathione agarose chromatography, Lane 6: 0.3 μ g TK1-193 after CM-column.

In table 5 the yield and purification degree of recombinant TK1 protein corresponding to crude extract from 1 litre bacteria culture is shown. The yield is based on the total enzyme activity and protein in the crude extract. From 1 litre of bacteria culture 0.8-1.0 mg pure recombinant TK1 protein can be purified.

Table 5: Purification of recombinant TK1

	Volume (ml)	Protein (mg)	Units/ml	Units/mg	Total units	Yield %
Crude extract						
	50	490	1567	159	78350	100
Glutathione agarose chromatography						
	30	9.43	1511	4812	45330	58
Sephadex G-25 chromatography						
top#	71.5	3.43	261	5437	18661	23
CM-sepharose chromatography						
top #	14.3	0.86	590	9833*	8437	11

* The TK activity has been measured on enzyme stored with 2.5 mM ATP. Units: nmol min⁻¹.

Kinetic studies

Munch-Petersen et al (1993) have shown that pure lymphocyte TK1 has different enzymatic properties when stored with or without ATP. When stored without ATP the lymphocyte enzyme is a dimer with low affinity for thymidine ($K_m = 15 \mu\text{M}$). Stored with ATP, the enzyme is a tetramer with high affinity for thymidine ($K_m = 0.5 \mu\text{M}$) as described on page 22.

As TK1-176 is without activity, only the enzymatic properties of recombinant TK1 and TK1-193 enzymes were examined. The top-fraction from CM-sepharose was divided into two aliquots. 2.5 mM ATP was added to one fraction and this fraction is referred to as the + ATP form, while the other fraction without

ATP is referred as the -ATP form.

Reaction mechanisms

The effect of ATP on the thymidine substrate kinetics of recombinant TK1 and TK1-193 appears from the Hofstee plots in figure 22 A and B. The initial velocities were measured at various concentrations of thymidine. The effect of ATP on the thymidine substrate kinetics of recombinant TK1 and TK1-193 is similar to that observed with lymphocyte TK1 (Munch-Petersen et al 1993).

In the Hofstee plot for the +ATP forms of recombinant TK1 and TK1-193 the points have been fitted to a straight line, but a curved tendency may indicate positive cooperativity. Positive cooperativity is obtained when binding of one substrate molecule enhance binding of the next molecule. All the +ATP samples of TK1 (n=3) and TK1-193 (n=3) display the same curved tendency. This phenomenon is also observed for the native lymphocyte TK1 (Munch-Petersen et al 1993). For the -ATP forms the curve has a clear biphasic shape that may indicate negative cooperativity, e.g. binding of one substrate molecule decrease binding of the next to the neighbour subunit. The degree of apparent cooperativity has been analysed from Hill plots of $\log v/(V_{\max}-v)$ versus $\log [dThd]$ (μM), where v is the initial velocity. The slope of the curve, the Hill coefficient (n), gives a measure of the apparent cooperativity. Values of n below one indicate negative cooperativity while n values above one indicate positive cooperativity. The Hill coefficient for the +ATP forms was 1.38 ± 0.05 (mean \pm s.d, $n = 3$) for recombinant TK1 and 1.25 ± 0.15 ($n = 3$) for TK1-193. For the -ATP forms the Hill coefficient was 0.46 ± 0.08 ($n = 3$) for recombinant TK1 and 0.5 ± 0.1 ($n = 3$) for TK1-193.

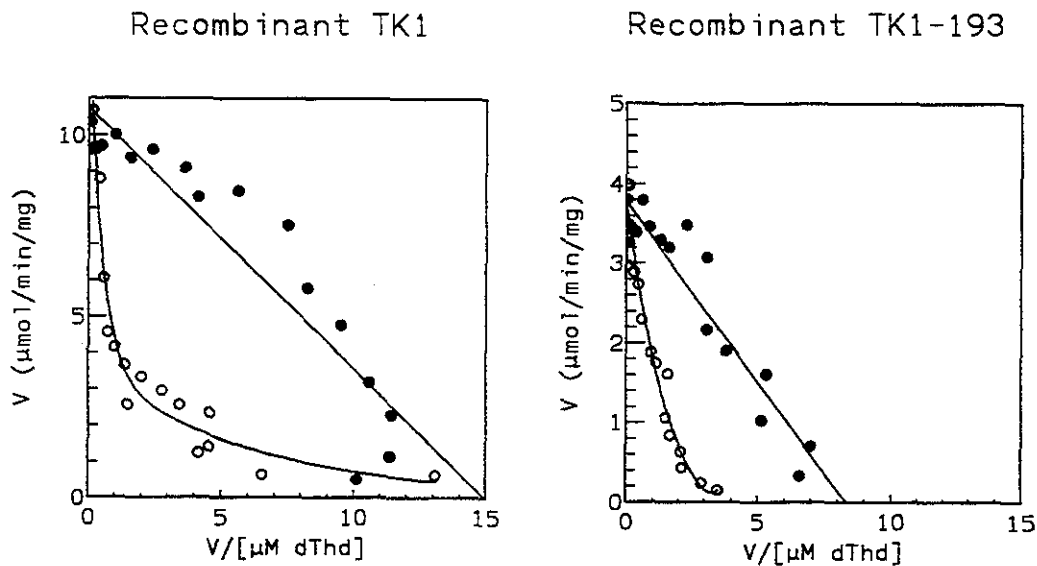


Figure 22: Hofstee plots of (A) recombinant TK1 and (B) TK1-193. The filled circle represent the +ATP form of the enzyme and the unfilled circles represent the -ATP form.

K_m

The -ATP form has a low affinity for thymidine with a K_m value of 14.08 ± 0.68 (mean \pm s.d., $n = 3$) μM for TK1-ATP and 12.8 ± 0.65 ($n = 3$) μM for TK1-193-ATP. Incubation of the enzymes with ATP gave a K_m value of 0.5 ± 0.1 ($n = 3$) μM for recombinant TK1 +ATP and for TK1-193 +ATP 0.5 ± 0.15 ($n = 3$) μM . This indicates that the affinity of the +ATP forms is about 25-fold higher for thymidine than that of the -ATP forms.

V_{\max}

Determination of the maximal velocity, V_{\max} , revealed a difference between recombinant TK1 and TK1-193. For recombinant TK1 the V_{\max} value was 9700 units/mg but for TK1-193 a 2.5-fold decrease was observed ($V_{\max} = 3800$ units/mg).

k_{cat}

k_{cat} indicates the maximum number of substrate molecules that can be converted to product per active site per unit time, and is therefore also called the "turn-over number" of the enzyme.

k_{cat} is calculated from V_{max} and the total enzyme concentration ($V_{max} = k_3 \times [E_T]$), presuming a molecular weight of 4 x 24,000 for recombinant TK1 and 4 x 20,000 for TK1-193.

k_{cat} with dThd as substrate is 16 s⁻¹ for recombinant TK1 but 5 s⁻¹ for TK1-193.

Stability

Investigations of the effect on the stability of the deletion of 40 amino acids from the C-terminal.

The investigations of stability were performed on the +ATP and -ATP forms of recombinant TK1 and TK1-193. The -ATP forms was incubated in 50 mM Tris and 10 mM DTT whereas for the +ATP form ATP to a final concentration of 2.5 mM was added. The TK activity was measured after incubation of the enzymes for 0, 4, 8, 12, 16 and 20 minutes at 37°C. For each measurement the TK activity was normalised to the TK enzyme level at 0 minutes of incubation. Figure 23 shows the stability for recombinant TK1 and TK1-193.

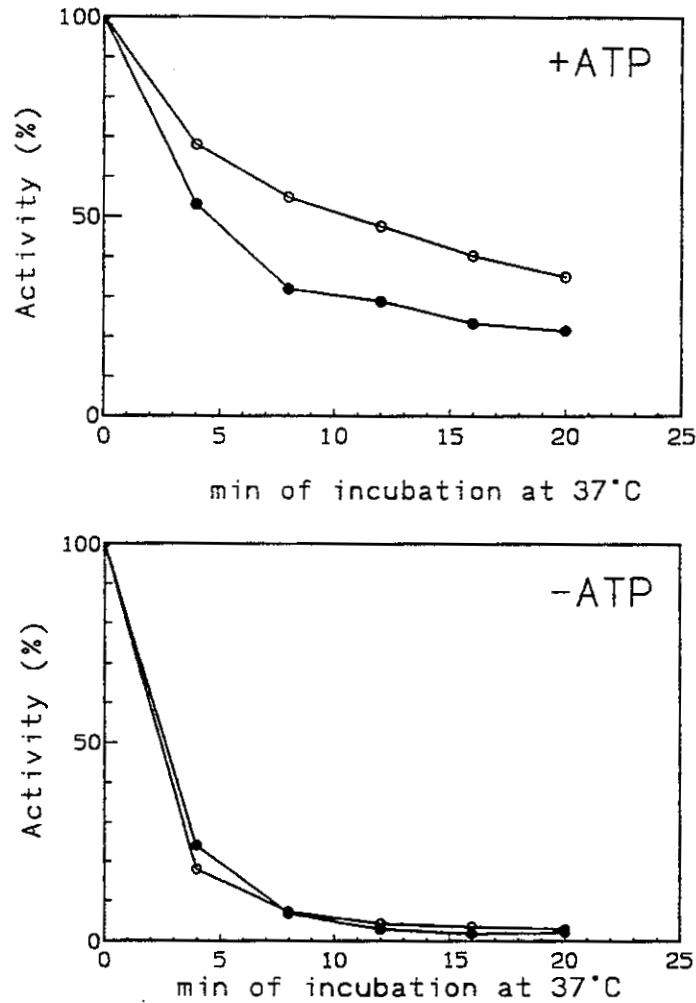


Figure 23: Stability for recombinant TK1 and TK1-193. Unfilled circles are TK1 and filled circles are TK1-193.

Comparison of the +ATP forms of recombinant TK1 and TK1-193 indicates that the mutant is less stable. Measurement of the TK activity for TK1 + ATP ($n=4$) and TK1-193 + ATP ($n=4$) at 0 minutes and 4 minutes revealed that the difference in stability was significant ($P<0.005$, t-test).

The stabilising effect of ATP is clear from these experiments, as for the -ATP forms only ~20% of the activity is preserved after 4 minutes of incubation whereas for the +ATP forms 50-70% activity remains.

Molecular weight

The native molecular weight for recombinant TK1 and TK1-193 was estimated by gelfiltration in absence and presence of ATP on a Superose 12 column. In presence of ATP the column was pre-equilibrated with buffer containing 2.5 mM ATP. The molecular weight was estimated by comparing the retention times for the sample with the retention times for the following marker proteins: beta-amylase 200 kDa, alcohol dehydrogenase 150 kDa, bovine serum albumin 66 kDa, carbonic anhydrase 29 kDa, cytochrome C 12.4 kDa.

Table 6: Native molecular weight determination. n = number of experiments

	SDS	molecular size mean ±s.d (kDa)	n
TK1-ATP	24	55 ±3.3	4
TK1 + ATP	24	116 ±4.7	3
TK1-193-ATP	20	44 ±2.8	5
TK1-193 + ATP	20	92 ±2.5	4

The effect of ATP on the native molecular weight of TK1 and TK1-193 is seen in Table 6. Without ATP the enzymes appeared as a dimer, with sizes of ~55 kDa and ~44 kDa for recombinant TK1 and TK1-193, respectively. In the presence of ATP during chromatography the enzymes eluted as tetramers, of 116 and 92 kDa, respectively. This indicates that ATP induces a tetramerization of the recombinantly expressed enzymes similarly to what observed with the native lymphocyte TK1.

Determination of the native molecular weight was quite difficult as the recovery of the enzyme was very low. The experiments were repeated several times with high levels of enzyme. Normally 0.003 units of lymphocyte TK1 activity gives a recovery of about 70-80% on superose 12 column. For the recombinantly expressed enzymes the applied amounts were 2-3 units and the recovery was 1-5

%. The reason for the low recovery may be that the recombinantly expressed enzymes are more hydrophobic due to amino acid changes or that they are more unstable and therefore monomerize.

Table 7: The kinetic data

	Native TK1 *	Recombinant TK1	TK1-193
Subunit molecular mass, kDa	24	24	20.1
Molecular mass + ATP	110	116 ± 4.7	92 ± 2.5
kDa -ATP	55	55 ± 3.3	44 ± 2.8
Thymidine Specific activity, Units/mg	9500	9700	3800
k_{cat} , s ⁻¹	15.1	16	5
K_m , μ M	+ATP	0.5-0.7	0.5 ± 0.15
	-ATP	12-15	14.1 ± 0.68
Hill coef- ficient, n	+ATP	1.2	1.25 ± 0.15
	-ATP	0.7	0.46 ± 0.08

*The results for the native TK1 are from Munch-Petersen et al 1993.

Resumé for investigations of TK1 structure-function relationship

For the investigation of the structure-function relationship of human TK1, two deletion mutants were constructed. Deletion of 57 amino acids from the C-terminal (TK1-176) resulted in an inactive protein. Deletion of 40 C-terminal amino acids reduced V_{max} 2.5 fold the level of recombinant TK1, but did not affect the K_m value or the Hill constant.

Discussion

The principal subject of my work was to study various aspect of the regulation of human TK1 at the cellular and the enzymatic level.

In the first part of these investigations I have quantified the level of TK1 mRNA in quiescent and PHA stimulated lymphocytes from control persons and in lymphocytes from patients with chronic lymphatic leukemia (CLL). Comparing the level of TK1 mRNA and the TK activity can reveal if these cells have any differences in the regulation mechanism(s) of TK1. The TK1 mRNA was quantified with the competitive PCR method. There are two advantages of the competitive PCR method: the method is more sensitive than Northern blot techniques and the quantisation are independent of the many variables that affect PCR amplification (different templates are amplified with different efficiency, the primers have different efficiency, intrinsic variability of PCR reactions). With competitive PCR it is possible to detect a very low amount of TK1 mRNA in quiescent lymphocytes from control persons. The detection limit was 6 copies per 1000 cells. Below this level, a 248 bp amplification product interferes with the competitive PCR. This is probably a result of traces of DNA or non-spliced RNA in the RNA preparation. However, the competitive PCR method has some limitations, it quantifies the amount of cDNA in a given sample and if the efficiency of reverse transcription is less than 100%, the method will underestimate the actual amount of mRNA. As the same protocols have been used for all donors, I presume that the underestimation is in the same range for all donors.

Lymphocytes stimulated to growth by PHA are used as a model-system for studying S-phase specific events. The system is well characterized with respect to TK activity, DNA synthesis and cell division (Loeb et al 1970, Munch-Petersen and Tyrsted 1977, Tyrsted and Munch-Petersen 1977). In my experiments a very low level of TK1 mRNA quiescent cells was estimated. After 96 h of PHA stimulation the TK1 mRNA level reached a maximum, with a 100-fold higher level than in

quiescent lymphocytes. Based on the ability to use CTP as phosphate donor it was established that the dominating enzyme in quiescent lymphocytes was TK2, while the dominating enzyme in dividing lymphocytes was TK1. It was demonstrated that the amounts of TK1 mRNA and TK activity increase concomitantly during incubation of the lymphocytes with PHA. This pattern correlates well with serum-starved cells stimulated to reenter the cell cycle by serum (Stuart et al 1985, Stewart et al 1987). In these cells the increase in TK1 mRNA is accompanied by a corresponding increase in TK activity, and both transcription and post-transcription mechanisms account for the induction of TK1 mRNA (Coppock and Pardee 1987, Stuart et al 1985, Stewart et al 1987). PHA-stimulated lymphocytes probably display the same cell cycle regulated patterns as serum-stimulated cells. However, there are some differences. In the experiments by Stewart et al (1987) a low level of TK1 mRNA, as measured by Northern blot analysis, was detected in the serum-stimulated cells. The TK1 mRNA level was not quantified but after 12 h of serum stimulation the level of TK1 mRNA reached a peak level (Stuart et al 1985, Stewart et al 1987). For the PHA stimulated lymphocytes, my experiments show that TK1 mRNA does not reach a peak level until 96 h after stimulation and with the Northern blot technique it was not possible to detect any transcription of TK1 mRNA in quiescent lymphocytes. The explanation for this difference is probably that peripheral blood lymphocytes are truly G_0 cells and may have spent several years in a non-dividing stage. The length of the first G_1 period (after stimulation) depends on how long time the cells were in G_0 . In experiments with WI38 cells (a nontransformed mortal human diploid fibroblast) two distinct stages of quiescence have been identified. Cells that were in G_0 for 1-10 days were minimally affected, in contrast to cells that were in G_0 for 10-20 days. In the last case a decrease in overall protein and RNA content was observed. When the cells were stimulated to reenter the cell cycle, expression of several late response genes, e.g. TK1 was considerably retarded (Hofbauer and Denhardt 1991). The serum-starved cells are stopped while they progress through the cell cycle, so when they are stimulated to reenter the cell cycle their "machinery" for replication is still active and therefore provide a quicker entry into the cell cycle.

CLL cells are in a quiescent stage, so I expected to obtain similar results as with the lymphocytes from control persons, e.i. a low TK1 mRNA level and a corresponding low TK activity. The TK activity was low, in the same range as in quiescent lymphocytes, and when investigated for phosphate donor specificity, it was established that the enzyme activity was TK2. It was therefore unexpected to find a high level of TK1 mRNA, about 100-fold higher than the TK1 mRNA level in quiescent lymphocytes. This shows that CLL cells have an abnormal regulation of the S-phase regulated TK1. TK1 mRNA level is high and is prevented from translation into an active enzyme. It is generally accepted that the R-point mechanism in late G₁ controls normal cell proliferation and this mechanism are deregulated in several cancer cells. The mechanisms to control the passage through the R-point are among others: cyclins, Cdk, Rb and p53. An altered regulation and increased level of cyclins, particularly cyclin D (Musgrove et al 1994), E and A (Dou et al 1993, Keyomarsi and Pardee 1993), have been shown in several transformed cell lines and in breast cancer tumours. As mentioned on page 12, TK1 carries an E2F (a cellular transcription factor) binding site in its promoter. An intact E2F binding site is required for transactivation of TK1 expression by polyoma large T antigen, as well as for serum stimulation. Ogris et al (1993) have shown by mobility shift assays that an increase in free E2F coincided in time with the appearance of TK1 mRNA. It could be, that the level of free E2F in CLL cells is increased and thereby provides an over-expression of TK1. The level of free E2F can increase if the Cdk2-cyclin E complex phosphorylates Rb in the E2F-Rb complex and thereby release "free" E2F. An over-expression of cyclin E mRNA and a deranged expression of cyclin E protein has actually been measured in proliferating breast tumour cell lines (Keyomarsi and Pardee 1993). These mechanisms could explain the occurrence of a high level of TK1 mRNA in the CLL cells. However, they do not explain why an increase in TK activity is not observed, either do they explain why the CLL cells remain quiescent and do not initiate cell division as a respond to the high TK1 mRNA level. It is possible, that the TK1 mRNA is defect, preventing it from being translated into an active enzyme. For the quantification of TK1 mRNA only exon 1 and 2 out of total 7 exons have been PCR amplified, so a truncation could not be excluded. However, in experiments by Laursen et al (1994), TK1

mRNA from normal persons and CLL patients were investigated to detect possible sequence differences. They used the SSCP analysis (Single Stranded Conformational Polymorphism, Orita et al 1989), which can detect down to single nucleotide deletions or substitutions in PCR fragments of 300-400 bases. The PCR-fragments were denatured and electrophoresed on a polyacrylamid gel. These experiments were performed on five normal donors and four CLL patients but did not reveal any sequence differences in the TK1 mRNA.

Another purpose of my work was to investigate the relationship between structure and function of TK1. Two mutant, TK1-193 and TK1-176 were constructed. In TK1-193 40 C-terminal amino acids were deleted whereas in TK1-176 57 amino acids from the C-terminal were deleted. According to the hypothetical structure for TK1 (Folkers et al 1991) no functional-essential site has been deleted in TK1-193. In TK1-176 a site including the residues Cys¹⁸⁶ and Arg¹⁸⁷, which may contribute to binding and transfer of a phosphate group from ATP to thymidine, was deleted. The mutants were constructed by the recombination PCR method. With its high site-specific mutagenesis frequency this method is a very efficient method for site-directed mutagenesis. In my experiments, where I have introduced both a Sac1 restriction site and a stop codon in the primer used for PCR-amplification, it was shown that the plasmid DNA from all the tested clones (17) could be digested with Sac1. This shows that all clones possess the desired mutation.

An expression system (pGEX2T-TK1) constructed by the groups of R. Hofbauer, Institut für Molekularbiologie, Universität für Vienna, and G. Folkers, Institut für Pharmazie, ETH Zürich, Switzerland, was chosen. In this construct TK1 purified from lymphocytes is expressed as a glutathione-S-transferase (GST)-fusion protein in *E.coli*. Purification of the recombinant TK1 mutant proteins from glutathione agarose columns resulted in 5-10 mg ~ 80% pure TK1 mutant protein pr. litre bacterial culture. The SDS PAGE shows remains of the fusion protein and a 70 kDa protein. The 70 kDa protein is probably an *E.coli* protein produced by the gene dnaK. This gene product is involved in degradation of "abnormal" proteins in *E.coli* (Yu-Sherman and Goldberg 1992). Further purification to 99% purity by CM-

affinity column resulted in 0.8 - 1.0 mg protein per litre of bacteria culture. For NMR-analyses 1-2 mg should be sufficient, so with this expression and purification system it is possible to achieve sufficient TK1 protein for structure elucidation by NMR-analyses.

By measuring the glutathione transferase activity and SDS-PAGE it is shown that the fusion protein is expressed in both mutants, but TK1-176 is devoid of TK activity. Sequence analysis revealed that no base mutations had occurred during the PCR and recombination procedure. Therefore, it can be concluded that deletions of 57 amino acids from the C-terminal completely destroy the TK activity. The mutants were constructed by a site-directed mutagenesis-based method. However, to state that the mutants are performed by site-directed mutagenesis are an incorrect term, in the sense that site-directed mutagenesis refers to change of one (or a few) residues. My mutants have "severe" alterations as 40 and 57 amino acids have been deleted. For TK1-176 it is not possible to establish, if the lack of activity is due to removal of the putative phosphate binding residues (Cys¹⁸⁶ and Arg¹⁸⁷) or more likely that deletions of the 57 amino acids destroy the protein structure. In TK1-176 both a putative β -strand and part of a putative α -helix is removed. Another mutant constructed in the laboratory recently (Larsen and Sønnichsen 1995) is TK1-184, where 50 amino acids from the C-terminal were deleted. In this mutant the residues for the putative phosphate binding Cys¹⁸⁶ and Arg¹⁸⁷ are deleted as well as the last putative β -strand. This mutant has no TK activity either, but as TK1-184 has not yet been sequenced entirely, the interpretation that the activity is abolished due to lost of 50 amino acids must be regarded with precautions. As TK1-193 posses TK activity these results indicate that structural elements between residue 193 and 176 are important for TK activity. Kaufmann and Kelly (1991) have shown, that the last 40 amino acids of HeLa TK1 are not necessary for TK activity, but do have a regulatory role. In experiments with TK1 minigenes expressed in TK deficient mouse cells they have shown that there is a mitosis specific degradation of TK1 protein. When the cells were transfected with a TK1-minigene with deletion of 40 C-terminal amino acids the M-phase specific degradation of TK1 protein was abolished and TK1 protein and activity were stabilized throughout the cell cycle. Likewise, investigations of primary amino acid alignments through several

vertebrates TK1 and *E.coli* TK shows that exon 7, which codes for the 40 C-terminal amino acids, has a very high degree of variation and some TK'S, e.g. vaccinia virus TK do not have the last 40 amino acids.

My results establish that the last 40 amino acids are not necessary for TK activity but may possess a structural role. Removal of more than 40 amino acids from the C-terminal is not possible without destroying the protein structure.

Investigations of the kinetics for recombinant TK1 and TK1-193 showed that the recombinant enzymes display the same kinetic properties as the native lymphocyte TK1. Thus, without ATP, recombinant TK1 and TK1-193 appear as dimers with apparent negative co-operativity and low affinity for thymidine (Hill coefficient = 0.4, $K_m = 12 \mu\text{M}$). With ATP, TK1 and TK1-193 appears as tetramers with positive co-operativity and high affinity for thymidine (Hill coefficient = 1.3, $K_m = 0.4 \mu\text{M}$). Although, these results agree with those obtained with TK1 purified from human lymphocytes (Munch-Petersen et al 1991, Munch-Petersen et al 1993) they conflict with results obtained by Jensen (1994). Here, an expression system in *E.coli* for direct expression of unmodified HeLa TK1 was established. The recombinant HeLa TK1 stored both without and with ATP behaved as the native lymphocyte TK1 stored with ATP with respect to size, specific activity, substrate specificity and K_m value for the two substrates, thymidine and ATP. Thus, the recombinant HeLa TK1 was a tetramer with high affinity for thymidine also in the absence of ATP. It has been shown by Chang et al (1994) that TK1 is differentially phosphorylated through the cell cycle (see post-translation regulation mechanisms). Jensen (1994) suggested that a post-translational mechanism responsible for the ATP-shift in human cells was lacking in *E.coli* cells. However as the ATP-shift is observed in my experiments where recombinant lymphocyte TK1 and TK1-193 are expressed in *E.coli* as well, the post-translational explanation does not seem correct. Regrettably, at the moment I cannot give a satisfactory explanation.

For the recombinant TK1 the V_{max} value was determined to 9700 units/mg. This value is in correspondence to V_{max} values for TK1 purified from lymphocytes, being 9600 units/mg (Munch-Petersen et al 1991, Jensen 1994). However, the

mutant TK1-193 has a 2.5-fold lower V_{\max} value (3800 units/mg) than recombinant TK1. An unchanged affinity for the natural substrate, thymidine, combined with a lower V_{\max} value suggest that the last 40 amino acids have a structural role, or that the ATP binding is disturbed. The last 40 amino acids can be of importance for stabilising the active site conformation or for the flexibility of the protein during the induced-fit movement of the phosphate transfer. It is possible that, due to the lack of the 40 residues, the two phosphate binding residues Cys¹⁸⁶ and Arg¹⁸⁷ are not able to "get close enough" to the ATP. This may inhibit the transfer of a phosphate group from ATP to thymidine. Another possibility is that the lower V_{\max} value is due to a disturbed binding of ATP. The stability experiments showed that TK1-193 is less stable ($p < 0.005$, $n = 4$) than recombinant TK1. This may favour the hypothesis that there is a disturbed binding of ATP.

The kinetic analyses showed that recombinant TK1 behave similarly as lymphocyte TK1, but there are some differences. Determination of the native molecular weight for recombinant TK1 and TK1-193, respectively, did cause some problems, as the recovery of enzyme activity was very low ($\approx 5\%$). A possible explanation is that the recombinantly expressed enzymes are more hydrophobic than the native enzyme. The enzyme may therefore adhere to the chromatography tubes before and after the column. The reason for such a higher hydrophobicity is not known, but there are amino acid changes in recombinant TK1, which may confer the higher hydrophobicity. The recombinant TK1 and TK1-193 have two additional residues in the N-terminal; Gly and Ser, due to reconstruction of the thrombin cleavage sequence LVPRGS (single letter code). Thrombin cleaves after the arginine (Chang 1985) and therefore all the cleavage products start with the amino acids Gly and Ser at their amino terminus. The sequencing experiments revealed a difference between the sequence in wildtype pGEX2T-TK1 and the published sequence of TK1 cDNA purified from HeLa cells. Examinations of the two mutations Met¹⁰⁶ \rightarrow Val and Lys²¹¹ \rightarrow Arg, shows that Met¹⁰⁶ \rightarrow Val is located in the putative fifth α -helix, shortly after the putative Mg²⁺-binding Asp⁸⁷. Comparison with several other mammalian TKs shows that they also possess a Val and not a Met. As seen on figure 4 (page 25) Met¹⁰⁶ is located in an area that is highly homologous. According to Dr. Hofbauer (personal communication) who has sequenced several TK1 genes,

there is in fact a difference between HeLa and lymphocyte cDNA. Lymphocytes possess a valine at position 106 while HeLa cells possess a methionine. The other mutation, Lys²¹¹ → Arg, is currently under investigation by Dr. Hofbauers group in Vienna. However, if the Lys → Arg mutation has arisen during the cloning procedures, it is according to Bordo and Argos (1991) a so-called "safe" mutation. In this context safe means that the alteration provides a very small conformational change of the protein. That the Lys²¹¹ → Arg alteration/mutation in recombinant TK1 is not of importance can also be concluded from the kinetic data. Recombinant TK1 has the same specific activity and enzyme kinetics as the native lymphocyte TK1.

English summary

Thymidine kinase (TK) catalyses the ATP-dependent phosphorylation of thymidine to thymidine monophosphate, which is subsequently phosphorylated to thymidine triphosphate and utilized for DNA synthesis. Human cytosolic TK (TK1) is cell cycle regulated, e.g. the TK1 activity increases sharply at the G₁-S phase transition and remains elevated throughout S-phase. The regulation of TK1 involves transcriptional, post-transcriptional, translational and post-translational regulation mechanisms. In a variety of cancers the regulation mechanisms(s) are changed and TK isoforms with altered biochemical properties have been observed. An investigation of TK1 gene expression will not only provide insight into the regulation mechanism in normal cells but also in cancer cells. Besides, differences in expression, substrate specificity and molecular structure of TKs in healthy and malignant cells can be used for construction of selective nucleoside analogs, only used by cancer TK isoenzymes.

In this Ph.D thesis the cell cycle regulated TK1 has been subject for two different approaches.

- 1: Investigation of the relationship between TK1 mRNA level and TK activity in lymphocytes from healthy donors and in lymphocytes from patients with chronic lymphatic leukemia (CLL).
- 2: Structure-function relationship of recombinant TK1.

In the first part a sensitive method (competitive PCR) for quantification of TK1 mRNA was established. The TK1 mRNA level was quantified in quiescent lymphocytes from control donors (n = 6) and in lymphocytes stimulated to growth by the mitogen phytohemagglutinin. The expression in normal cells was compared with the level of TK1 mRNA level in patients with chronic lymphatic leukemia (n = 5).

The results for the six control donors show a very low level of TK1 mRNA (below 0.006×10^6 copies mg^{-1} protein) and TK activity (0.009 to $0.016 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein) in quiescent lymphocytes. In dividing lymphocytes the TK1 mRNA level increases 50 to 5000-fold (3 to 98×10^6 copies mg^{-1} protein) with a concomitant increase in TK activity (0.12 to $0.76 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein). In CLL cells which are characterized as being quiescent, the TK activity was in the same range as in quiescent lymphocytes from control donors. However, quantification of the TK1 mRNA level shows that all five CLL patients had a very high level (6 to 22×10^6 copies mg^{-1} protein) of TK1 mRNA, corresponding to the level in dividing lymphocytes. As the high TK1 mRNA level is not translated into an active enzyme, these results indicate a defect in the regulation of TK1 in CLL cells.

For the studies of the structure-function relationship of TK1 a recombinant TK1 protein, which is expressed as a glutathione-S-transferase (GST) fusion protein was used. TK1 protein is cleaved from the GST-part with thrombin. Two TK1 mutants, TK1-193 and TK1-176, with deletions from the C-terminal were constructed by the recombinant PCR method. Deletion of 57 amino acids from the C-terminal (TK1-176) results in an inactive enzyme. Deletion of 40 amino acid from the C-terminal decreases V_{max} 2.5-fold ($3800 \text{ nmol min}^{-1} \text{ mg}^{-1}$) than the level of the recombinant wildtype (recombinant TK1) which has a V_{max} value of $9700 \text{ nmol min}^{-1} \text{ mg}^{-1}$. Except for the V_{max} value the recombinant TK1 and TK1-193 behave similarly as the native lymphocyte TK1. When ATP is absent from the enzyme, the enzyme appears as a dimer with low affinity for thymidine and when ATP is present the enzyme appears as a tetramer with high affinity for thymidine. K_m for thymidine for recombinant TK1 and TK1-193 incubated with ATP is 0.5 ± 0.1 (mean \pm s.d., $n = 3$) μM , while enzyme incubated without ATP has a K_m of 14.08 ± 0.68 ($n = 3$) μM for TK1 and 12.8 ± 0.65 ($n = 3$) μM for TK1-193. The Hill coefficient for enzyme incubated with ATP is 1.38 ± 0.05 ($n = 3$) for recombinant TK1 and 1.25 ± 0.15 ($n = 3$) for TK1-193. For the -ATP forms the Hill coefficient is 0.46 ± 0.08 ($n = 3$) for recombinant TK1 and 0.5 ± 0.1 ($n = 3$) for TK1-193.

An unchanged affinity for the natural substrate, thymidine, combined with a lower V_{max} value suggest that the last 40 amino acids have a structural role or

that the ATP binding is disturbed.

Danish summary

Thymidin kinase (TK) er et salvage pathway enzym, der katalyserer fosforylering af thymidin til thymidin monofosfat, som derefter fosforyleres videre til thymidin trifosfat og indgår i DNA syntesen. Human cytoplasmatisk TK (TK1) er celle cyklus reguleret og følger DNA syntesen, d.v.s høj aktivitet i delende og maligne celler og lav eller ingen aktivitet i hvilende celler. Celle cyklus regulering af TK1 involverer et sammenspil mellem transkriptionel, post-transkriptionel, translationel og post-translationel reguleringsmekanismer. I nogle cancer celler er reguleringsmekanismerne ændret og der er observeret forskellige isoformer af TK. Undersøgelse af TK1 gen ekspresion vil give informationer om reguleringsmekanismer ikke kun i normale celler men også i maligne celler. Desuden kan eventuelle forskelle i ekspresion, substrat specificitet og struktur mellem TK's i raske og maligne celler udnyttes til konstruktion af nukleosid analoger som virker selektivt på maligne celler.

Afhandlingen omfatter:

- 1: Bestemmelse af TK1 mRNA og TK aktivitet i lymfocytter fra raske donorer og i lymfocytter fra patienter med kronisk lymfatisk leukemi (CLL).
- 2: Struktur-funktions undersøgelser af rekombinant TK1.

I første del af afhandlingen er en følsom metode (competitive PCR) til kvantitering af TK1 mRNA blevet etableret. TK1 mRNA niveauet er kvantiteret i hvilende lymfocytter fra raske donorer ($n = 6$) samt i lymfocytter der er stimuleret til vækst med phytohemagglutinin. Expressionen i normale celler er sammenholdt med niveauet af TK1 mRNA i patienter med kronisk lymfatisk leukæmi ($n = 5$).

Resultaterne fra de 6 normale donorer viser, at der er et meget lavt niveau af TK1 mRNA (under 0.06×10^6 kopier mg^{-1} protein) og TK aktivitet (0.009 til 0.016

nmol min⁻¹ mg⁻¹ protein) i hvilende celler. I delende lymfocytter stiger mængden af TK1 mRNA 50 til 5000 gange (3 til 98 × 10⁶ kopier mg⁻¹ protein) og bliver efterfulgt af en stigning i TK aktiviteten (0.12 til 0.76 nmol min⁻¹ mg⁻¹). I CLL celler, der er karakteriseret ved at være hvilende celler, er TK aktiviteten af samme størrelsesorden som i hvilende lymfocytter fra raske donorer. Kvantitering af TK1 mRNA viser overraskende at der i alle 5 CLL patienter er et meget højt TK1 mRNA (6 til 22 × 10⁶ kopier mg⁻¹ protein) niveau, svarende til niveauet i delende lymfocytter. TK1 mRNA niveauet er højt men bliver ikke udtrykt som aktivt enzym. Dette indikerer at der er en defekt i reguleringen af det S-fase specifikke TK1 enzym i CLL celler.

Til struktur-funktions undersøgelserne af TK1 anvendes et rekombinant TK1 protein der udtrykkes som et glutathion-S-transferase-(GST) fusions protein. TK1 protein kløves fra GST-delen med thrombin. To deletions mutanter, TK1-193 og TK1-176, er konstrueret v.h.a. recombination PCR metoden. Fjernelse af 57 amino syrer fra C-terminalen (TK1-176) resulterer i et inaktivt enzym. Deletion af 40 amino syrer (TK1-193) fra proteinets C-terminal medfører at V_{max} formindskes 2.5-gange (3800 nmol min⁻¹ mg⁻¹) i forhold til den rekombinante vildtype (rekombinant TK1) der har en V_{max} på 9700 nmol min⁻¹ mg⁻¹. Bortset fra V_{max} værdien opfører rekombinant TK1 og TK1-193 sig som den native lymfocyt TK1, d.v.s når ATP er fjernet fra enzymet, optræder det som en dimer med lav affinitet for thymidin og når ATP er tilstede optræder enzymet som en tetramer med høj affinitet for thymidin. K_m for thymidin for rekombinant TK1 og TK1-193 inkuberet med ATP er 0.5 ± 0.1 (middel \pm s.d, $n = 3$) μ M, mens enzym inkuberet uden ATP har K_m på 14.08 ± 0.68 ($n = 3$) μ M for TK1 og 12.8 ± 0.65 ($n = 3$) μ M for TK1-193. Hill koefficienten for enzym inkuberet med ATP er 1.38 ± 0.05 ($n = 3$) for rekombinant TK1 og 1.25 ± 0.15 ($n = 3$) for TK1-193. For -ATP formerne er Hill koefficienten 0.46 ± 0.08 ($n = 3$) for rekombinant TK1 og 0.5 ± 0.1 ($n = 3$) for TK1-193.

En uændret affinitet for thymidin kombineret med en lavere V_{max} indikerer at de 40 C-terminal amino syrer har en strukturel rolle og/eller at bindingen af ATP er ændret.

Abbreviations

a.a	Amino acids
ADK	Adenylate kinase
ATP	Adenosine triphosphate
AZT	Azidothymidine, 3'-Azido-2',3'-dideoxythymidine
Cdk	Cyclin-dependent protein kinase
CDNB	1-chloro-2,4-dinitrobenzene
CDP	Cytidine diphosphate
CFU	Colony forming units
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CKI	Cdk inhibitory proteins
CLL	Chronic lymphatic leukemia
CML	Chronic myelocytic leukemia
C-point	Competence point, located in G ₀ phase
Comp-PCR	Competitive reverse transcriptase-PCR
dATP	Deoxyadenosine triphosphate
dCDP	Deoxycytidine diphosphate
dCK	Deoxycytidine kinase
dCMP	Deoxycytidine monophosphate
dCTP	Deoxycytidine triphosphate
dCyd	Deoxycytidine
dGDP	Deoxyguanosine diphosphate
dGTP	Deoxyguanosine triphosphate
dNDP	Deoxyribonucleoside diphosphates
dNTP	Deoxyribonucleoside triphosphates
dThd	Thymidine
dTMP	Thymidine monophosphate
dTTP	Thymidine triphosphate
dUDP	Deoxyuridine diphosphate

dUMP	Deoxyuridine monophosphate
EFTU	Elongation factor
E2F	Cellular transcription factor
FCS	Fetal calf serum
G ₁	G ₁ -phase. The period in the cell cycle where the cells prepares for DNA synthesis
GST	Glutathione-S-transferase
GST-TK1	Glutathione-S-transferase-thymidine kinase fusion protein
HeLa	Human cervix cancer derived cell line
HIV	Human immunodeficiency virus
HL-60	Human promyelocytic cell line
HSV	Herpes simplex virus
IPTG	Isopropyl β -D-thiogalactoside
K _m	Michaelis constant
KY895	A TK ⁻ <i>E.coli</i> strain
n	Hill constant
NF-Y	DNA binding nuclear factor Y
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction
pGEX2T	Expression vector for GST fusion proteins
pGEX2T-TK1	TK1 cloned into pGEX2T
pGEX2T-TK1-193	Mutant clone. Amino acid 193 is the last amino acid in TK1
pGEX2T-TK1-176	Mutant clone. Amino acid 176 is the last amino acid in TK1
PHA	Phytohemagglutinin
PMSF	Phenylmethylsulfonyl flouride
Rb	Retinoblastoma protein
R-point	Restriction-point, a control mechanism located in G ₁
RT	Reverse transcriptase
S-phase	Period in the cell cycle where DNA is duplicated
TK	Thymidine kinase
TK1	The cytosolic thymidine kinase, it is cell cycle regulated
TK2	The mitochondrial thymidine kinase, it is constitutively expressed

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TK1-193	Mutant protein
TK1-176	Mutant protein
V_{\max}	The maximal velocity

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Appendix 1

ABTG medium:

A10 salt: 100 g $(\text{NH}_4)_2\text{SO}_4$, 300 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 150 g KH_2PO_4 , 150 NaCl, H_2O ad 5 litre. pH = 7.1, autoclave.

B salt: 1 ml 1 M MgCl_2 , 1 ml 0.1 M CaCl_2 and 0.3 ml 0.01 M FeCl_3 per litre medium.

T(thiamin): 0.5 ml 4 mg/ml thiamin per litre medium.

G(glucose) 10 ml 20% glucose per litre medium.

LB medium:

10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, 950 ml H_2O , pH 7.0, adjust with 5 M NaOH. H_2O add 1000 ml. Autoclave the solution.

S.O.C medium:

2 g bacto-tryptone, 0.5 g bacto-yeast extract, 1 ml 1M NaCl, 0.25 ml 1 M KCl, H_2O to 100 ml. Autoclave and after cooling add 1 ml 2 M Mg^{2+} stock (1 M $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 1 M $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$) and 1 ml 2 M glucose. Sterile filter the solution.

TB medium:

12 g bacto-tryptone, 24 g bacto-yeast extract, 4 ml glycerol, H_2O add 900 ml. Autoclave and add 100 ml 0.17 M KH_2PO_4 , 0.72 M K_2HPO_4)

FN18 amino acid mix (-met, -leu):

L-alanine 0.710 g, L-arginine 0.700 g, L-histidine 0.310 g, L-lysine/HCl 0.580 g, L-proline 0.460 g, L-threonine 0.480 g, glycine 0.600 g, L-asparagine 0.530 g, L-glutamine 0.270 g, L-isoleucine 0.520 g, L-phenylalanine 0.240 g, L-tyrosine 0.360 g, L-valine 0.700 g, L-aspartate 0.530 g, L-glutamate 0.880 g, L-cysteine 0.120 g, L-serine 10.00 g, L-tryptophan 0.200 g. Dissolve in 200 ml H_2O , sterile filter. Dilute 1:40 when use).

PAPER 1

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OVEREXPRESSION OF HUMAN THYMIDINE KINASE mRNA WITHOUT CORRESPONDING ENZYMATIC ACTIVITY IN PATIENTS WITH CHRONIC LYMPHATIC LEUKEMIA

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Abstract—The level of cytosolic thymidine kinase (TK1) mRNA in lymphocytes from six healthy people and in lymphocytes from five patients with untreated chronic lymphatic leukemia (CLL) was determined with competitive polymerase chain reaction (competitive PCR). Using this procedure we have shown that in patients with CLL, there is an overexpression of TK1 mRNA without corresponding enzymatic activity. The TK1 mRNA level is approximately 100-fold higher in lymphocytes from CLL patients than in lymphocytes from healthy persons. A high level of TK1 mRNA without corresponding enzyme activity may indicate a defect in the processing of the enzyme. This may disturb the cells' normal feedback system and thereby influence the development of malignant conditions.

Key words: Thymidine kinase, mRNA, chronic lymphatic leukemia, competitive PCR, quantification, CLL.

Introduction

Thymidine kinase (ATP: thymidine 5'phosphotransferase E.C. 2.7.1.21) is a pyrimidine nucleoside salvage pathway enzyme with two isoenzymes: TK1 and TK2, also called cytosolic and mitochondrial TK, respectively. Both enzymes catalyse the phosphorylation of thymidine to TMP which is subsequently converted to TTP and utilized in DNA synthesis. TK1 is the dominating form in dividing lymphocytes and TK2 is the only form present in non-dividing lymphocytes, but in low amounts [1]. The two isoenzymes have characteristic differences in their enzyme kinetic pattern and subunit molecular weights [2, 3]. TK1 is cell-cycle regulated and the enzyme level is low or undetectable in quiescent (G_0) cells, but increases dramatically when the cells enter S-phase [4-7]. It is generally accepted that there is a close correlation between TK1 activity and the proliferative state of the cell [1, 6, 8-10].

Abbreviations: CLL, chronic lymphatic leukemia; TK, thymidine kinase; TK1, thymidine kinase characteristic for dividing cells; TK2, thymidine kinase characteristic for non-dividing cells; PCR, polymerase chain reaction; PHA, phytohemagglutinin; FCS, fetal calf serum; ATP, adenosine triphosphate; CTP, cytidine triphosphate.

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* The two first authors made equal contribution to this paper.

Lymphocytes from patients with chronic lymphatic leukemia (CLL) are non-dividing and it is, therefore, plausible that the low TK activity in these cells almost exclusively is due to TK2. However, Munch-Petersen and Tyrsted found that the dominating TK activity isolated from lymphocytes from a CLL patient displayed an enzyme kinetic pattern similar to that observed with TK1 [11].

The occurrence of TK1 in quiescent CLL cells may be due to a change in the control of the cell-cycle regulated expression of the TK1 gene. The cell-cycle regulation of TK1 is a very complex system involving transcriptional, post-transcriptional [4, 12, 13], translational and post-translational regulation mechanisms [14, 15]. To investigate the expression of TK1 mRNA in CLL cells at the transcriptional level we have measured the level of TK1 mRNA by the very sensitive competitive polymerase chain reaction (competitive PCR), and compared this with the TK enzyme activity. Surprisingly, we have found that the level of TK1 mRNA in lymphocytes from CLL patients was about 100-fold higher than in lymphocytes from healthy persons.

Materials and Methods

Materials

^3H -thymidine (2 Ci/mmol), [$\alpha^{32}\text{P}$]dCTP (3000 Ci/mmol), Megaprime DNA labeling systems (RPN 1604) and

Hybond N⁺ membrane were from Amersham Denmark ApS. Isopaque-Ficoll was from Nycomed. RPMI-1640, fetal calf serum (FCS), phytohemagglutinin (PHA) and bovine serum albumin (BSA) were from Gibco. All nucleosides were from Boehringer and Mannheim, RNasin, random hexamer and M-MLV reverse transcriptase RNase H minus were from Promega. *Thermus aquaticus* DNA polymerase (AmpliTaq) was from Perkin Elmer/Cetus. The primers used were synthesized at the Department of Microbiology at the Technical University of Denmark. SpinBind DNA extraction Unit was from FMC Bio-products Europe. DEAE and 3MM filters were from Whatman. All other reagents were of the highest quality generally available.

Cells

Peripheral blood from six healthy persons was collected in heparin vacuum tubes. Peripheral blood, similarly collected from five patients with untreated CLL was kindly provided by Sven Erik Nielsen (M.D.), Roskilde Hospital. The lymphocytes were isolated by Isopaque-Ficoll gradient centrifugation [16] and washed in RPMI-1640 containing 10% heat-inactivated FCS. The cell number was estimated in a Coulter counter and cell pellets were stored at -80°C.

Phytohemagglutinin (PHA) stimulation of lymphocytes from healthy persons

The lymphocytes were stimulated by PHA in RPMI-1640 medium supplemented with 10% FCS, 20 µg/ml PHA and 20 µg/ml penicillin/streptomycin, at a concentration of 10⁶ cells per ml in 5% CO₂ at 37°C. For flow cytometry analysis aliquots of 10⁶ cells were centrifuged at 50 g for 10 min and fixed in 250 µl buffer (6.1 mM glucose, 140 mM NaCl, 5 mM KCl, 2.7 mM Na₂HPO₄, 1.1 mM KH₂PO₄, 0.5 mM EDTA) and 750 µl EtOH. The DNA was stained with 20 µg/ml EtBr and 10 µg/ml mithramycin and monitored by flow cytometry. In quiescent lymphocytes the S-fraction was about 8%. In lymphocytes cultured for 48 h with PHA the S-fraction was in the range of 30%. These data were in agreement with previous observations [17].

Extraction of lymphocytes for TK activity measurement

The cells were suspended in Loeb's buffer (20 mM K-phosphate buffer (pH 7.4), 15% glycerol, 1 mM K-EDTA, 10 mM dithiothreitol (DTT)), lysed by sonication (40 W, 3 × 1–2 s) and centrifuged at 20,000 g for 30 min. The supernatant (enzyme extract) was used for TK activity measurement and total protein measurement.

TK activity assay

TK activities were determined as initial velocities using the DEAE-cellulose 81 paper square method as previously described [2, 3]. The standard assay mixture contained: 50 mM Tris-HCl (pH 7.5), 10 mM DTT, 2.5 mM ATP, 2.5 mM MgCl₂, 3 mM NaF, 0.5 mM CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulphonate), 3 mg/ml bovine serum albumin (BSA) and 10 µM ³H-thymidine (2 Ci/mmol). In assays with CTP as phosphate donor ATP was substituted with equimolar CTP. Samples of 13 µl were applied on DEAE filters 5, 10 and 15 min after starting the reaction by addition of enzyme extract to the assay mixture to a total volume of 50 µl. The reaction temperature was 37°C. The filters were washed, eluted and the radioactivity determined by scintillation counting as described [3].

One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 nmol dTMP per min.

Protein determination

The protein content was measured by Coomassie brilliant blue as described [18].

RNA isolation

Total RNA was isolated using the guanidine thiocyanate method described by Chomczynski and Sacchi [19]. To improve purification an extra phenol extraction and alcohol precipitation was applied. RNA concentration was estimated from the optical density at 260 nm, and the RNA quality was examined by agarose gel electrophoresis.

Estimation of RNA recovery

³H-uridine was added to the growth medium (5 µCi/ml, 5 Ci/mmol) during PHA-stimulation in two experiments. A total of 5 × 10⁶ labeled cells were harvested on 3 MM filters and non-incorporated ³H-uridine was washed away. From an equal number of cells, RNA was isolated and applied to 3 MM filters. The radioactivity on the filters was determined by scintillation counting. The amount of isotope in RNA was compared with the amount of isotope in the cells. The RNA recovery estimated from these comparisons was in the range of 70–90%.

Reverse transcription

RNA (2.5 µl) was transcribed to cDNA in a 50 µl volume of PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.015% gelatine, 0.1% Tween 20), 7.5 mM MgCl₂, 1 mM of each of the dNTPs, 40 U RNasin, 7 µM random hexamers and 250 units of M-MLV reverse transcriptase RNase H minus. The reaction was terminated after 2 h at 37°C. The extent of reverse transcription was controlled by a parallel reaction where ³H-TTP, instead of TTP was added. Aliquots of the reaction mixture were applied on 3 MM filters. The non-incorporated ³H-TTP was removed from the filter by washing 3 × 10 min in 1 M HCl containing 0.6 mM Na₅P₃O₁₀, 10 min in 0.26 M NaAc/EtOH and finally in EtOH. The radioactivity was measured by scintillation counting (results not shown).

Competitive PCR

The principle in the competitive PCR method, as reported by Gilliland *et al.* [20], is a co-amplification of target cDNA concurrently with the corresponding genomic DNA. Thus, the two templates compete for the same substrates and primers ensuring equal efficiency of amplification. The genomic DNA serves as internal standard. In our experiments we chose exons 1 and 2 with intron 1 from the TK gene as internal standard and exons 1 and 2 as the target cDNA fragment. The fragments were amplified using a pair of primers identical to those reported by Lipson and Baserga [21]. The sizes of the resulting fragments were 138 bp with cDNA as template and 248 bp with genomic DNA as template. The internal standard of 248 bp genomic DNA was prepared by PCR with DNA as a template and using the primers mentioned above. The product was quantified by agarose gel electrophoresis together with different known amounts of DNA. The unknown amount of cDNA was estimated from a set of PCR reactions performed in a dilution series with known amounts of the

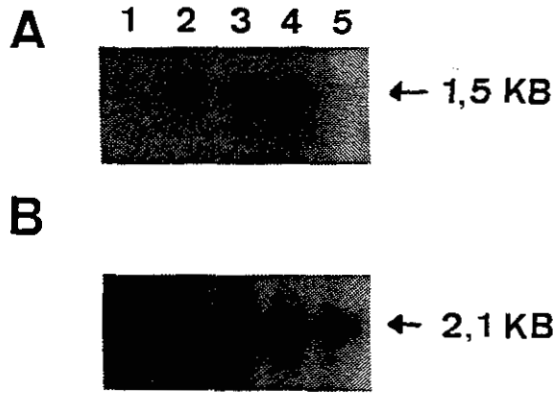


Fig. 1. Northern blot analysis of TK1 mRNA. Total RNA was isolated from lymphocytes from donor 5 with 24 μ g loaded in each lane. The lymphocytes were stimulated to grow with PHA. Non-stimulated (lane 1); 48 h (lane 2); 72 h (lane 3); 96 h (lane 4); 168 h (lane 5). (A) Hybridization with a TK probe detecting a single mRNA species of 1.5 kilobases. (B) After stripping the filter in (A) for TK probe, the filter was rehybridized to a probe for the constitutively expressed β -actin, detecting a mRNA species of 2.1 kb.

genomic DNA. The PCR products were separated by agarose gel electrophoresis. The amount of cDNA (in grams) in the sample was estimated as that amount (in grams) of genomic DNA giving equal intensity of the two amplification products. The number of TK1 cDNA copies was calculated from the amount of cDNA, by division with the molecular weight of the 138 bp cDNA fragment (average molecular weight/base = 308). The number of copies of TK1 cDNA was taken as being representative for the number of copies of TK1 mRNA.

The competitive PCR analyses were performed in a 25 μ l vol. of PCR buffer, 7 pmol of each primer, 200 μ M of each dNTP, 1.5 mM $MgCl_2$ and 0.5 unit of *Thermus aquaticus* DNA polymerase. Heat-denatured cDNA (100°C, 2 min) and internal standard were added to the reaction mixture with a layer of mineral oil to avoid evaporation. The amplification was performed in a Perkin-Elmer/Cetus Thermal Cycler according to the following program: denaturation for 1 min at 95°C, annealing for 1 min at 60°C and polymerization for 1 min at 72°C, for 35 cycles.

Northern blot

Total RNA was prepared as described above. RNA preparations were denatured and electrophoresed through a 1.8% agarose gel containing 2.2 M formaldehyde and transferred to a Hybond N⁺ membrane according to published procedures [22]. The probes used were human TK1 cDNA from plasmid pTK11 [23] and human β -actin cDNA labeled with ³²P-dCTP using Megaprime DNA labeling systems. The TK1 probe was 720 bp and the actin probe was 400 bp. Non-incorporated nucleotides were removed with a SpinBind DNA extraction unit. In a 20 ml hybridization reaction a 100 ng (3.4 μ Ci/ μ g) probe was used. Hybridization was performed according to the guidelines from Amersham Denmark ApS with high stringency washing: 0.1 \times SSPE-buffer (3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA pH 7.7) and 0.1% SDS at 68°C.

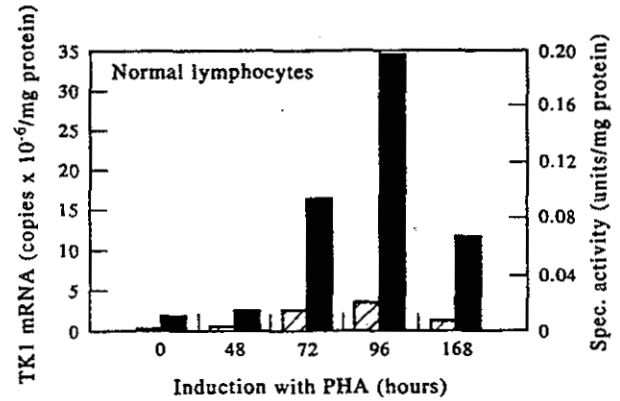


Fig. 2. Cell-cycle-specific variation of TK1 mRNA (copies/mg protein) \square and TK activity (units/mg protein) \blacksquare are illustrated for donor 1. The lymphocytes were stimulated with PHA for the indicated time periods. TK1 mRNA was estimated by competitive PCR and TK activity was measured at standard conditions as described in 'Methods'.

Results

Determination of TK1 mRNA by Northern blotting

Expression of TK1 mRNA during the cell cycle of PHA stimulated lymphocytes was analysed for two donors by Northern blot analysis. As seen in Fig. 1(A) it is clear that TK1 mRNA is not expressed in quiescent lymphocytes, whereas a 1.5 kb band, corresponding to TK1 mRNA, is seen in lymphocytes cultured with PHA. The level of TK1 mRNA increases reaching a maximum after 96 h of culture with PHA, whereafter the level decreases. Hybridization with a probe for the constitutive expressed β -actin shows that equal amounts of RNA were applied to each lane.

TK1 mRNA and TK activity in lymphocytes from healthy donors

With competitive PCR it is possible to measure TK1 mRNA in quiescent lymphocytes. Figure 2 shows the level of TK1 mRNA (copies/mg protein) and the TK activity (units/mg protein) in lymphocytes from donor 1. It is clearly demonstrated that the amounts of TK1 mRNA and TK activity increase concomitantly during incubation of the lymphocytes with PHA, reaching a peak level at 96 h. The same cell-cycle regulated pattern was observed in lymphocytes from the five other donors. Table 1 shows the actual amounts of TK1 mRNA and TK activity in the six donors. In quiescent lymphocytes, the level of TK1 mRNA is very low and in four out of six donors below the limit of detection which in our reactions correspond to 0.006 copies of TK1 mRNA/cell. After PHA stimulation, the level of TK1 mRNA transcripts increases to a maximum level, between

Table 1. The amount of TK1 mRNA (copies/mg protein) and TK activity (units/mg protein) in non-stimulated lymphocytes and lymphocytes incubated with PHA for 96 h

Donor No.	TK1 mRNA (copies $\times 10^6$ /mg protein)		TK activity (units/mg protein)	
	Non-stimulated lymphocytes	PHA-stimulated lymphocytes	Non-stimulated lymphocytes	PHA-stimulated lymphocytes
1	N.D	3.2	0.009	0.195
2	N.D	4.9	0.013	0.120
3	0.215	20.0	0.013	0.292
4	0.061	5.4	0.008	0.503
5	N.D	85.7	0.009	0.285
6	N.D	98.8	0.016	0.760

N.D = Not detectable.

Table 2. The amount of TK1 mRNA (copies/mg protein) and TK activity (units/mg protein) in lymphocytes from five patients with CLL

CLL patient No.	TK1 mRNA (copies $\times 10^6$ /mg protein)	TK activity (units/mg protein)
1	10.3	0.008
2	7.4	0.006
3	22.7	0.013
4	15.2	0.005
5	6.1	0.006

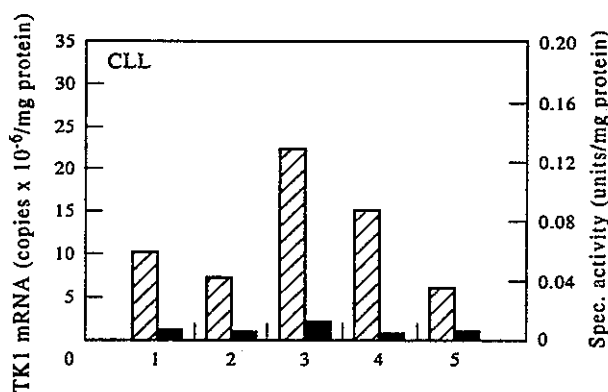


Fig. 3. The amounts of TK1 mRNA (copies/mg protein) \square and TK activity (units/mg protein) \blacksquare in lymphocytes isolated from CLL cells. The numbers on the x-axis refer to the five CLL patients.

50 and 5000-fold higher than in quiescent cells. The broad range in TK1 mRNA level may reflect individual variation between the different donors.

TK1 mRNA and TK activity in CLL cells

Our results show that the TK activity in CLL cells is low and in the same range as in quiescent lymphocytes, between 0.005–0.013 units/mg protein

(Table 2). Surprisingly, despite the low TK activity, lymphocytes from CLL patients express very high levels of TK1 mRNA (Fig. 3). The level of TK1 mRNA/mg protein was between 30 and 300-fold higher than the level in quiescent cells from healthy donors.

Characterization of the TK isoenzyme in CLL cells

Owing to the high TK1 mRNA level it was important to establish to which degree the TK activity was due to TK1 or TK2. The low amounts of TK activity in the crude extracts, however, did not allow any further separation of TK1 and TK2. Therefore, we have distinguished between TK1 and TK2 using the pronounced differences in substrate specificity.

Both isoenzymes utilize adenosine triphosphate (ATP) efficiently as phosphate donor, but TK2 can also use cytidine triphosphate (CTP) efficiently, whereas it is a poor phosphate donor for TK1 [24].

To clarify whether the isoenzyme in CLL cells is similar to TK2 in quiescent cells or to TK1 in dividing cells, we have compared the phosphate donor efficiency of ATP and CTP in quiescent lymphocytes, PHA stimulated lymphocytes and CLL cells. TK activity with CTP as donor is given as a percent of TK activity with ATP as donor.

As seen in Fig. 4, CTP was a poor phosphate donor for the TK enzyme in PHA stimulated lymphocytes, but an efficient phosphate donor for the TK enzyme in quiescent lymphocytes and CLL cells. These results indicate that the TK in CLL cells is the same as in quiescent lymphocytes, namely TK2.

Discussion

The development of the PCR reaction has revolutionized the area of molecular biology. With its high sensitivity, competitive PCR makes it possible to measure gene expression of even a low copy gene,

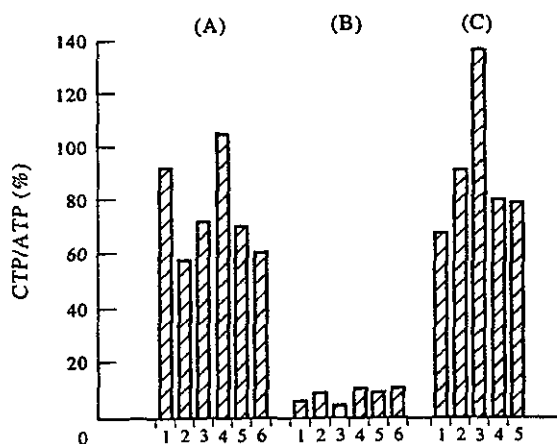


Fig. 4. The ratio between phosphate donor capacity of CTP and ATP. In each experiment the enzyme activity is normalized to 100% with ATP as phosphate donor. (A) Non-stimulated lymphocytes; (B) lymphocytes 96 h after PHA stimulation; (C) CLL cells. The numbers on the x-axis refer to the individual donors and patients.

such as thymidine kinase. We wanted to quantitate TK1 gene expression in lymphocytes from untreated CLL patients, but were restricted by the limited availability of material. The most frequently used method for determination of gene expression is Northern blot analysis, but the results were very weak when carried out on PHA stimulated lymphocytes. To be able to detect TK1 mRNA in the very limited CLL samples, a more sensitive method was required. Using competitive PCR it was possible to quantify the level of TK1 mRNA, even in quiescent lymphocytes. We have estimated a very low, but detectable level, in two donors. The amount of TK1 mRNA in quiescent lymphocytes is very close to the detection limit, which is about 0.006 copies of TK1 mRNA/cell. Below this level, a 248 bp amplification product interferes with the competitive PCR. This is probably as a result of traces of DNA or non-spliced RNA in our RNA preparation. As there is a minor loss during the RNA purification and the cDNA synthesis (see Methods), the actual level of TK1 mRNA is slightly underestimated. However, we presume that the underestimation is in the same range in all samples, since we have used the same protocols for all donors.

As a model system we have used human lymphocytes which are truly G_0 cells. Culturing the quiescent lymphocytes in the presence of PHA stimulates the cells to enter the cell cycle, allowing events in the G_1 and S-phases to be investigated. A clear advantage of using lymphocytes as representatives for normal cells instead of immortal cell lines is the ability of cell lines to grow continuously. This is due

to the occurrence of at least one feature required to turn normal cells into cancer cells.

The expression of TK has been shown to be regulated on multiple levels and the regulation mechanisms differ, depending on the cell system. For example, in cycling HeLa cells, the S-phase increase in TK activity is largely accounted for by an increase in the rate of TK protein translation [7]. In serum-starved cells stimulated to re-enter the cell cycle, the increase in TK activity is accompanied by a corresponding increase in TK1 mRNA. In this system both transcription and post-transcriptional mechanisms account for the induction of TK1 mRNA [4, 25, 26]. In PHA stimulated lymphocytes it is well known that TK activity increases dramatically when the cells enter the cell cycle [1, 27, 28]. However, for this cell system, we have not been able to find any reports regarding the fluctuations in TK1 mRNA level as a characteristic of regulatory mechanisms.

Our results show that both TK1 mRNA and TK activity in PHA stimulated lymphocytes display the same cell-cycle regulated pattern as stimulated serum-starved cells. An increase in TK1 mRNA in lymphocytes at the entry to S-phase is followed by an increase in TK enzymatic activity. The TK1 mRNA level increases about 100-fold when the cells are stimulated. When cells leave S-phase, the TK1 mRNA level and TK enzyme activity decrease.

An important difference between the two cell systems is that lymphocytes are truly G_0 cells, they do not enter S-phase before 48 h after addition of PHA. This is in contrast to stimulated serum-starved cells which reach S-phase after 12 h [25].

In CLL cells we found, surprisingly, that TK1 mRNA expressed per mg protein is 30–300 fold higher than in quiescent lymphocytes. The TK activity level is very low and in the same range as in quiescent lymphocytes. Exploiting the different substrate specificities of TK1 and TK2 using CTP instead of ATP as phosphate donor, it was shown that the TK activity in the CLL cells is due to TK2. This phenomenon of a high TK1 mRNA level with no TK1 enzyme activity has to our knowledge not been described elsewhere.

Our experiments indicate that CLL cells have an abnormal regulation of the S-phase-regulated TK1. TK1 mRNA level is high and is prevented from translation into active enzyme. Owing to its association with the proliferative state of cells, TK activity is regarded as a useful tumor marker with prognostic value for a number of malignant diseases such as human breast cancer [29–31], non-Hodgkins lymphoma [32] and acute lymphatic leukemia [33]. In CLL cells the TK activity is very low and can, therefore, not be used as a tumor marker. On the other

hand, the level of TK1 mRNA is very high and may, therefore, serve as an alternative tumor marker in these cells to predict the diagnosis at an earlier stage.

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PAPER 2

QUANTITATION OF TK1 mRNA IN PATIENTS WITH CHRONIC LYMPHATIC LEUKEMIA

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INTRODUCTION

Thymidine kinase is an enzyme in the pyrimidine salvage pathway that, with ATP as co-substrate, catalyzes the phosphorylation of deoxythymidine to deoxythymidine monophosphate (dTMP) which is subsequently converted to dTTP and utilized for DNA synthesis.

In mammalian cells there are two thymidine kinases (TK), the constitutively expressed TK2, and the S-phase specific TK1 which is only present in dividing cells¹.

Lymphocytes from patients with chronic lymphatic leukemia (CLL) are non-dividing and it is therefore plausible that the low TK activity in these cells almost exclusively is due to TK2. However, a thymidine kinase with similar enzyme kinetic pattern as that observed with TK1 from lymphocytes stimulated to growth by the mitogene phytohemagglutinin has been reported². Since TK1 expression is tightly regulated throughout the cell cycle with transcriptional, translational as well as post-translational regulatory mechanism³, the occurrence of TK1 in non-dividing CLL cells may be due to a change in the control of the cell cycle regulated expression of the TK1 gene.

To investigate the transcriptional expression of TK1 mRNA in CLL cells, we have measured the level of TK1 mRNA with the competitive polymerase chain reaction (competitive PCR), and compared this mRNA level with the TK enzyme activity. Surprisingly, we have found that the ratio of TK1 mRNA/TK activity in lymphocytes from CLL patients was about 60-400 fold higher than in lymphocytes from healthy persons.

METHODS

Lymphocytes from peripheral blood from 6 healthy persons and from 5 patients with untreated CLL were isolated by the Ficoll-Isopaque technique.

Lymphocytes from healthy persons were stimulated to growth by PHA in RPMI 1640 medium supplemented with 10% fetal calf serum, 20 $\mu\text{g}/\text{ml}$ PHA and 20 $\mu\text{g}/\text{ml}$ penicillin/streptomycin at a concentration of 10^6 cells pr ml in 5% CO_2 at 37°C. The lymphocytes were divided in portions of 5×10^6 cells, and in each portion, TK activity was determined by the DE-81 paper method as described⁴ and total protein was determined by the Bradford assay⁵. Total RNA was isolated with the guanidine thiocyanate method⁶, transcribed to cDNA and quantitated by the competitive PCR method⁷. TK1 cDNA, taken as representative for TK1 mRNA, was co-amplified with a dilution series of competitor DNA. Exon 1 and 2 with intron from the TK1 gene served as competitor DNA and exon 1 and 2 of the TK1 gene as the cDNA fragment to be quantitated. The fragments were amplified using a pair of primers identical to those reported by Lipson and Baserga⁸. The sizes of the resulting fragments was 138 bp with cDNA as template and 248 bp with competitor DNA as template. The relative amounts of cDNA versus competitor DNA were measured by scanning of ethidium-bromide stained gels. Because the starting concentration of the competitor DNA was known, the amount of cDNA (in grams) in the sample could be estimated as that amount of competitor DNA where equal intensities of the two amplification products were obtained. The number of TK1 cDNA copies was calculated from the amount of cDNA, by dividing the amount of cDNA with the molecular weight of 1 copy of the 138 bp cDNA fragment.

The amplification was performed in a Perkin-Elmer/Cetus Thermal Cycler according to the following program: denaturation for 1 min at 95°C, annealing for 1 min at 60°C and polymerization for 1 min at 72°C, for 35 cycles.

RESULTS AND DISCUSSION

Table 1 shows the ratio of TK1 mRNA copies and TK activity in non-dividing lymphocytes from 6 healthy persons and in lymphocytes from 5 patients with CLL. As seen, the ratio TK1 mRNA copies/TK activity in CLL cells is 60 to 400 fold higher than in non-dividing lymphocytes. The TK activity in CLL cells is of a magnitude as expected for non-dividing cells, while the expression of TK1 mRNA is very high and in the range of the TK1 mRNA level in PHA stimulated healthy donor lymphocytes. In these experiments the TK1 mRNA level is $3\text{-}98 \times 10^6$ copies/mg protein (results are not shown).

The detection limit in the assay is around 6×10^4 copies of TK1 mRNA/mg protein or 0.006 copies/cell. Below this level, a 248 bp amplification product, interferes with the competitive PCR. This is probably a result of traces of DNA or non-spliced RNA in the RNA preparation. The results indicate that there, as expected, is no TK1 mRNA in non-dividing lymphocytes from healthy persons.

Due to the high TK1 mRNA level in non-dividing CLL cells it was of importance to clarify whether the dominating TK in CLL cells was TK1 or TK2, using the characteristic differences in phosphate donor specificity towards ATP and CTP. Both enzymes can utilize ATP, but only TK2 is capable of utilizing CTP⁹. The relative TK activity with CTP as phosphate donor was expressed as % of activity with ATP as phosphate donor. PHA-stimulated lymphocytes showed a 85-90% decrease in relative

activity, while non-dividing lymphocytes from healthy persons and lymphocytes from CLL patients showed a 7-30% decrease. The conclusion is that the enzyme in CLL cells is the same as in non-dividing lymphocytes from healthy persons, namely TK2.

Table 1. Ratio of TK1 mRNA copies and TK activity.

	TK1 mRNA copies x 10 ⁶ /mg protein	TK activity Units/mg protein	TK1 mRNA copies x 10 ⁶ /TK activity
Non-dividing lymphocytes			
1	< 0.06	0.009	< 6.7
2	< 0.06	0.013	< 4.6
3	0.21	0.013	16.2
4	0.06	0.008	7.5
5	< 0.06	0.009	< 6.7
6	< 0.06	0.016	< 3.8
Lymphocytes from CLL patients			
1	10.3	0.008	1287
2	7.4	0.006	1233
3	22.7	0.013	1746
4	15.2	0.005	3040
5	6.1	0.006	1016

The ratio between TK1 mRNA and TK activity as estimated in non-dividing lymphocytes from 6 donors and in lymphocytes from 5 patients with CLL. The numbers refer to the individual donors and patients. 1 unit is the amount of enzyme that phosphorylate 1 nmol substrate per minute.

The occurrence of a high level of TK1 mRNA without concomitant expression of TK1 enzyme activity may indicate that CLL cells have an abnormal regulation of the cell-cycle regulated TK1. The regulations mechanism are not fully understood, but several investigations have shown that the changes in TK1 mRNA during cell cycle can not fully account for the rise in TK activity. Translational and post-translational modifications may contribute to the regulation of TK1. Chang and Huang¹⁰ have demonstrated that seryl residues of the TK1 polypeptide are phosphorylated in cycling HL-60 cells. An increasing phosphorylation of the polypeptide was followed by an increase in enzyme activity, during the cell cycle. Another post-translational mechanism has been reported by Kauffman and Kelly¹¹. They have shown that amino acid residues near the C-terminal end are

responsible for degradation of thymidine kinase protein in the G₂ and M phase, and that mutations in this part of the gene allow expression in G₀ cells.

It is possible that a post-translational mechanism serve as a secondary back-up system for the regulation of TK. This may explain why we can measure a high TK1 mRNA level but no TK1 activity.

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